Unconventional Secretory Pathways: Protein Folding and Quality Control During FGF2 Membrane Translocation

Dissertation

submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

of the Ruperto Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

Diplom-Biologe Rafael Backhaus

born in Hildesheim

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Zusammenfassung

Fibroblast Growth Factor 2 (FGF2) ist ein proangiogener Wachstumsfaktor, der eine entscheidende Rolle bei der Tumor-induzierten Angiogenese spielt. FGF2 wird über einen bisher nicht bekannten Mechanismus von Säugetierzellen sezerniert, der auch nach Blockierung des ER/Golgi Systems durch Brefeldin A vollständig funktionell bleibt. Da FGF2 auch biomedizinisch ein sehr interessantes Targetprotein darstellt, ist die molekulare Aufklärung des Sekretionsweges von ausserordentlich grosser Bedeutung.

In dieser Arbeit wurde ein experimentelles Modellsystem etabliert, dass mittels Durchflusszytometrie eine exakte Quantifizierung der FGF2 Expressionsrate unter verschiedenen experimentellen Bedingungen erlaubt. Darüber hinaus wurden Testsysteme entwickelt, die den Exportvorgang sowohl mittels konfokaler Laserscanmikroskopie als auch durch biochemische Methoden wie die Zelloberflächenbiotinylierung rekonstituieren.

Eine wesentliche Fragestellung bei der Aufklärung des molekularen Mechanismus der FGF2 Sekretion bestand in der Analyse des Faltungszustandes von FGF2 während des Exportvorganges. Auf der Basis der oben beschriebenen Modellsysteme wurde FGF2 als DHFR Fusionsprotein exprimiert, so dass der Faltungszustand durch einen exogenen Liganden kontrolliert werden konnte. Es konnte gezeigt werden, dass unter Bedingungen, die die Entfaltung des Moleküls nicht erlauben, die FGF2 Sekretionsrate nicht beeinflusst wird. Darüber hinaus konnten mit Hilfe eines sogenannten *Piggyback* Exportsystems Hinweise dafür gewonnen werden, dass eine Interaktion eines zweiten Reportermoleküls mit FGF2 während des Exportvorganges erhalten bleibt. Jedoch war die Effizienz dieses *Piggyback* Transportes gering, so dass diese Ergebnisse schwer interpretierbar blieben. Dennoch sind diese Beobachtungen konsistent mit den Ergebnissen, die mit Hilfe des DHFR Systems erhalten wurden. Weiterhin stimmen die in dieser Arbeit geschilderten Experimente in überein mit neueren Befunden aus unserem Labor, die auf eine Rolle von Heparansulfatproteoglykanen als Exportrezeptoren bei der Sekretion von FGF2 hinweisen. Diese Daten deuten ebenfalls auf einen

Exportvorgang hin, während dessen FGF2 vollständig gefaltet bleibt. Die Summe der Daten hat wichtige Implikationen für den Mechanismus der FGF2 Sekretion, der nach heutigem Kenntnisstand durch eine direkte Translokation über die Plasmamembran erfolgt. Eine Verknüpfung des Exportvorgangs mit dem FGF2 Faltungszustand könnte somit Qualitätskontrolle gewährleisten, die die Sekretion von nicht funktionellen Molekülen ausschliesst.

Summary

Fibroblast growth factor 2, a mediator of tumor-associated angiogenesis, is a mitogenic growth factor involved in various cellular processes. It is released by an unconventional secretory pathway independent of the ER/Golgi system. Due to its strong biomedical relevance it is of great interest to elucidate the molecular machinery involved in non-classical export.

To analyze unconventional secretion, different model systems were established during this study including a FACS-based system which allows for a quantitative analysis of exported material bound to the cell surface and an analysis system employing confocal microscopy to analyze non-classical export qualitatively employing specific antibodies. Additionally, various biochemical analysis methods employing immobilized antibodies, as well as labelling of cell surface proteins using a membrane-impermeable biotinylation reagent to quantify exported FGF2 reporter molecules were established.

In the second part of this thesis, these systems were used to analyze the folding state of FGF2 during unconventional secretion. The first experimental approach, termed DHFR fusion protein system, prevents unfolding during membrane translocation by aminopterin-dependent stabilization of a DHFR domain fused to FGF2. It could be shown that export of FGF2 is not affected under conditions where protein unfolding is prevented, although, based on the same system, mitochondrial import could be blocked. These findings suggest that export of FGF2 does not require unfolding.

The second strategy, termed piggyback export analysis system, monitors the folding state of FGF2 and investigates potential means of quality control associated with unconventional secretion. The system is based on the export of non-covalent cytosolic complexes formed between two interacting domains, one fused to FGF2 and the other to non-exported GFP. To this end, a certain degree of piggyback export, could be detected, however, the efficiency was found to be low. In any case, the results are consistent with those obtained with the DHFR system in that it appears likely that FGF2 remains folded during membrane translocation. These

findings are also supported by recent observations made in our laboratory pointing to a role of heparan sulfate proteoglycans as export receptors requiring FGF2 to be folded during export.

1 Introduction

One of the basic principles of living organisms is to separate the interior from the surrounding environment by a barrier. At the level of cells this barrier is formed by an amphipathic lipid bilayer termed plasma membrane which separates the cytosol from the cell exterior. Additionally, eukaryotic cells possess a highly evolved endomembrane system which allows the formation of specialized compartments known as organelles (Palade, 1975). Organelles create enclosed cavities which provide specialized surroundings for different biological processes. They function in distribution of nutrients and metabolites, storage of substances, assembly and degradation of macromolecules and export of material to the extracellular space. To fulfill these functions transport between organelles and exchange with the cell exterior is essential. The most important processes in this regard are endo- and exocytosis (Besterman and Low, 1983; Bloom and Puszkin, 1981; Sollner, 2003; Stahl and Barbieri, 2002), intracellular transport and secretion. They are mediated by membrane-coated vesicles, which release their content upon fusion with an acceptor membrane (Harter and Wieland, 1996; Rothman, 1994; Rothman and Wieland, 1996; Schmid, 1997). In eukaryotic cells a number of independent organelles functions sequentially to form a network known as the secretory pathway (Lee et al., 2004). It is a specialized transport route which is highly regulated and directly involved in protein biogenesis, modification, sorting, quality control and secretion. The secretory pathway mediates the transport of lipids, proteins and other cargo molecules to intracellular organelles or to the plasma membrane where export occurs. Besides, a number of proteins are known which are exported independently of the secretory pathway by a process termed unconventional secretion (Cleves, 1997; Nickel, 2003; Nickel, 2005).

1.1 Classical Protein Secretion

Classical protein secretion begins with the binding of the signal recognition particle (SRP) to an N-terminal, hydrophobic signal sequence of a nascent polypeptide chain synthesized at a free ribosome in the cytosol (Blobel and Dobberstein, 1975b; Walter

et al., 1984). SRP also binds to the large subunit of the ribosome which causes an arrest in elongation (Walter and Blobel, 1981). This ternary complex is directed to the membrane of the endoplasmatic reticulum (ER) where SRP binds to its receptor (Meyer et al., 1982; Rapoport, 1992a). The SRP receptor is an integral membrane protein consisting of an α- and a smaller β-subunit (Tajima et al., 1986). Once bound to the α-subunit of the SRP receptor the complex is transferred to the translocon, an integral membrane protein complex which forms the protein conducting channel (Rapoport, 1992a). It consists of Sec61α, a protein with 10 membrane spanning α-helices, Sec61β and Sec61γ, which are smaller in size (High et al., 1993; Rapoport, 1992a). When the ribosome is transferred to the translocon SRP is released accompanied by GTP hydrolysis, and elongation continues (Gilmore et al., 1982). The growing polypeptide chain is directly synthesized into the pore formed by the translocon and enters the lumen of the ER in a co-translational manner (Brodsky, 1998; Gilmore, 1993; Walter et al., 1984). The signal peptide is cleaved off by an integral, translocon-associated signal-peptidase (Blobel and Dobberstein, 1975a; Dalbey and Von Heijne, 1992) and the polypeptide chain associates with ER resident chaperones which ensure correct folding (Bukau and Horwich, 1998) and facilitate translocation by functioning as molecular ratchets (Matlack et al., 1999). The translocation from the cytosol to the lumen of the ER is the only step during export where a secretory protein is transported across a membrane. The cytosolic and the luminal face of the membrane, the latter being topologically equivalent to the extracellular space, do not change during further transport steps since they are mediated by membrane-coated vesicles which are generated at a donor compartment and fuse with a downstream acceptor compartment (Lee et al., 2004).

After insertion into the ER the protein can be modified by formation of intramolecular disulfide bonds (Gething and Sambrook, 1992), specific proteolytic cleavage, hydroxylation of proline residues (Lee et al., 2004) as well as N- and O-linked glycosylation. N-linked glycosylation involves the transfer of pre-assembled oligosaccharide precursors to asparagine residues by oligosaccharyl-protein transferases (Sharma et al., 1981). The oligosaccharide precursors are subsequently trimmed by glucosidases which remove glucose and mannose moieties (Hebert et al., 1995). When all modifications are completed and the protein obtained its correct conformation it is packaged into small membrane-coated vesicles with a size of about 50 nm in diameter. These vesicles transport the protein to the Golgi apparatus

eventually passing through the ER-Golgi intermediate compartment (ERGIC) which is formed by fusion of transport vesicles and is involved in sorting processes (Lee et al., 2004).

The Golgi apparatus is organized as a network of cisternal stacks consisting of the cis-, medial- and trans-Golgi (Dunphy and Rothman, 1985; Morre, 1987; Rothman, 1981). Vesicles fuse with an existing cis-Golgi cisterna or a number of vesicles fuse with each other to form a new cis-Golgi stack (Lee et al., 2004). ER-resident proteins transported to the Golgi are retrieved to the ER via retrograde transport (Lee et al., 2004). Additionally, misfolded proteins can be transported back to the ER to undergo ER-associated degradation (ERAD) (McCracken and Brodsky, 1996; Tsuda et al., 2005). Transport through the Golgi is likely to occur according to the cisternal progression model or mediated by transport vesicles. In the cisternal progression model a cis-Golgi stack moves spatially towards the trans-Golgi face thereby undergoing a maturing process in which the enzymatic content and the membrane composition change (Becker et al., 1995). In the vesicular transport model small transport vesicles mediate transport between the Golgi cisternae which are defined compartments with a specific protein and lipid composition (Beckers and Rothman, 1992; Rothman and Orci, 1990). In the Golgi cargo proteins undergo further modifications which have influence on their structure and functional properties. N-linked oligosaccharides transferred in the ER are further trimmed (Zuber et al., 2000), O-linked glycosylation takes place (Ernst and Prill, 2001; Sadeghi and Birnbaumer, 1999) and proteins are modified by sulfatation (Baeuerle and Huttner, 1987; Hille et al., 1984). Once secretory proteins reach the trans-Golgi face they are packaged into specific vesicles to be transported to the plasma membrane. This can occur by bulk flow which mediates incorporation of cargo proteins into vesicle for constitutive secretion (Keller and Simons, 1997; Wieland et al., 1987) or by association with cargo-receptors and adaptor proteins (Pearse and Robinson, 1990; Seeger and Payne, 1992; Tooze and Tooze, 1986). The secretory vesicles are transported to the plasma membrane by movement along the microtubular network (Henley and McNiven, 1996; Hirschberg et al., 1998; Martin-Verdeaux et al., 2003; Wacker et al., 1997) and release their content in a controlled fusion event with the membrane to the extracellular space .

Vesicles mediating the various transport steps are characterized by their protein coat and membrane origin. Known types are clathrin-coated vesicles, COPI and COPII

vesicles (Salama and Schekman, 1995; Schekman and Orci, 1996). Clathrin-coated vesicles are formed at the plasma membrane or the trans-Golgi. They mediate transport to the endosomal/lysosomal compartment. COPI vesicles function in retrograde transport from the Golgi to the ER and mediate intra-Golgi transport between the cisternae (Lippincott-Schwartz, 1993; Lippincott-Schwartz et al., 1989). It is further hypothesized that they might also play a role in transport to the endosomal compartment (Nickel et al., 1998; Rothman and Wieland, 1996). COPII vesicles mediate the anterograde transport of proteins from the ER to the Golgi or the ERGIC (Aridor et al., 1998; Barlowe et al., 1994; Kuehn and Schekman, 1997). All vesicles are formed by polymerization of coat proteins on the cytosolic surface of a donor membrane. Small GTP-binding proteins of the ras-GTPase superfamily function as molecular switches to initiate and control polymerization (ARF for COPI- and clathrin-coated vesicles and Sar1 for COPII vesicles) (Balch, 1990; Boman and Kahn, 1995; Nakano and Muramatsu, 1989; Nickel et al., 2002; Orci et al., 1993; Ostermann et al., 1993; Palmer et al., 1993; Shaywitz et al., 1997). Assembly of the coat results in local membrane deformation which contributes to vesicle shaping and drives the budding process. To select cargo molecules for packaging into vesicles a sorting signal, e.g. in the cytosolic domain of membrane proteins, is required. The sorting signal interacts directly with polymerized coat proteins (Allan et al., 2000; Campbell and Schekman, 1997; Gimeno et al., 1996; Kuehn et al., 1998). Luminal proteins bind to membrane spanning cargo receptors prior to packaging which, in turn, interact with coat proteins. A well characterized example is the binding of the KDEL retrieval sequence of soluble ER resident proteins to the KDEL receptor mediating retrograde transport from the Golgi to the ER (Lewis and Pelham, 1992; Tang et al., 1993). Following vesicle pinch off from the donor membrane the coat is disassembled triggered by hydrolysis of ARF- or Sar1-bound GTP and proteins required for targeting and fusion with the acceptor membrane are exposed (Goldberg, 1999; Reinhard et al., 2003; Tanigawa et al., 1993).

Transport specifity is mediated by a combination of fusion assembly proteins known as SNAREs (Soluble N-ethylmaleimide sensitive factor attachment protein receptor) (Rothman and Warren, 1994; Söllner et al., 1993b), small GTPases of the Rab family (Balch, 1990; Clague, 1999; Novick and Zerial, 1997) and a diverse group of tethering factors (Waters and Pfeffer, 1999). The initial interaction with the acceptor membrane is the assembly of tethering complexes. The mechanistical details of this

process remain elusive but it is likely that loose tethering anchors the vesicle to the target membrane and primes SNAREs for fusion by rearranging SNARE complexes or increasing the probability of SNARE encounters. Tethering factors which mediate the primary interaction can be classified into a group of multi-subunit complexes and a group of coiled-coil proteins (Pfeffer, 2001; Suvorova et al., 2001). Rab proteins mediate docking of the vesicle by interactions with specific coiled-coil Rab effectors on the acceptor membrane. The set of Rab proteins and Rab effectors present on different membranes thereby provide the compartmental identity of an organelle (Christoforidis et al., 1999; Gonzalez and Scheller, 1999; Jahn and Sudhof, 1999; Novick and Zerial, 1997). After docking the interaction of complementary SNAREs present on the vesicle (v-SNARE) and the target membrane (t-SNARE) leads to the formation of the trans-SNARE complex (Rothman and Söllner, 1997; Söllner et al., 1993a; Söllner and Rothman, 1996). This brings the membranes into close proximity and directly drives the fusion process (Nickel et al., 1999; Weber et al., 1998). Upon formation of the trans-SNARE complex the SNARE proteins undergo a conformational change form an unstructured, high-energetic state to an ordered, low-energetic four-helical bundle (Sollner, 2004). The energy provided by this step may be directly used to catalyze the fusion of the two opposing membranes by destabilizing the lipid/water interface and initiating lipid mixing (Jahn et al., 2003). After fusion the SNARE assembly is present as a cis-SNARE complex at the membrane of the target organelle. This complex is disassembled for subsequent rounds of docking and fusion by the combined action of SNAP (soluble N-ethylmaleimide sensitive factor attachment protein) and NSF (N-ethylmaleimide sensitive factor) (Hanson et al., 1997a; Hanson et al., 1997b). The energy required for this process is provided by NSF-mediated ATP-hydrolysis (Jahn et al., 2003). After disassembly t-SNAREs remain in the target membrane and v-SNAREs are recycled to their donor membrane by retrograde transport (Ballensiefen et al., 1998) or maybe by an independent mechanism as proposed by Dietrich et al. based on yeast vacuole studies on homotypic membrane fusion (Dietrich et al., 2005). After this last step the machinery is ready for the next round of vesicle docking and membrane fusion.

The classical secretory pathway involving the ER and the Golgi apparatus can be blocked by certain inhibitors. The fungal metabolite brefeldin A (BFA) inhibits ARF recycling by disrupting the interaction of ARF with its guanine exchange factor (GEF)

which, in turn, impairs COPI vesicles formation (Mossessova et al., 2003), (Robineau et al., 2000). This results in fusion of the cis- and medial-Golgi with the ER and compromises ER/Golgi-dependent protein secretion reversibly (Misumi et al., 1986), (Lippincott-Schwartz et al., 1989). The drug monensin irreversibly blocks transport from the trans-Golgi face leading to Golgi dilatation (Hashieh et al., 1989) which in turn also disrupts the secretory pathway (Tartakoff, 1983).

1.2 Unconventional Protein Secretion

The ER/Golgi-dependent or classical secretory pathway is the main export route for proteins in eukaryotic cells. Soluble secretory proteins typically contain a N-terminal, hydrophobic signal sequence to enter the pathway which transports them through the ER to the Golgi and finally to the extracellular space (see section 1.1).
About 15 years ago proteins were discovered that do not fulfill this requirement but are exported from cells (Muesch et al., 1990). Furthermore, protein secretion was observed in the absence of a functional ER/Golgi system as shown for interleukin 1β (IL-1β) and galectin-1 (Cooper and Barondes, 1990; Hughes, 1999; Nickel, 2003; Nickel, 2005; Rubartelli et al., 1990). These findings were the starting point to unravel the phenomenon of unconventional protein secretion also known as non-classical protein export (Cooper and Barondes, 1990; Florkiewicz et al., 1995; Muesch et al., 1990). Further characterization of unconventionally exported proteins uncovered common features of these different, functionally and structurally unrelated proteins. They do not contain a classical, N-terminal, hydrophobic signal sequence (Rubartelli et al., 1990). They are excluded from the lumen of the ER or the Golgi apparatus. They do not show ER/Golgi-dependent posttranslational modifications, e.g. N-linked glycosylation, despite bearing numerous consensus sites (Hughes, 1999). Their export is not affected by inhibitors like BFA or monensin which compromise the classical secretory pathway (Florkiewicz et al., 1995; Muesch et al., 1990). Various experimental strategies, like measurements of unrelated cytosolic proteins in cellular supernatants, have been applied to exclude unspecific release originating from injured or dead cells (Cleves, 1997; Engling et al., 2002; Hughes, 1999). Furthermore, non-classical export of the proangiogenic growth factor FGF2 was shown to be energy and temperature dependent *in vivo* (Florkiewicz et al., 1995) and

can be influenced by stimulating or inhibiting agents (Cleves, 1997; Hughes, 1999). Additionally, different experimental approaches provided evidence that unconventional secretion of galectins is regulated during cell differentiation (Cooper and Barondes, 1990; Lutomski et al., 1997), release of FGF2 is controlled by NF-κB-mediated signalling (Wakisaka et al., 2002) and that post-translational modifications, e.g. phosphorylation, regulate the release of the non-classically exported homeodomain protein Engrailed (Maizel et al., 2002). Taken together, these findings demonstrate that unconventional secretion is not based on unspecific release by sublethal injury or cell death as proposed formerly (McNeil et al., 1989). It is rather a highly regulated process involving protein-based molecular machineries.

1.2.1 Current Models of Unconventional Export Mechanisms

Although unconventionally secreted proteins share the features described in section 1.2 it is unlikely that they make use of a common unconventional export pathway. Instead, they seem to be released by mechanistically distinct export routes consisting of vesicular and non-vesicular membrane translocation processes (Nickel, 2005). The potential export mechanisms are summarized in the following figure (Fig 1.1).

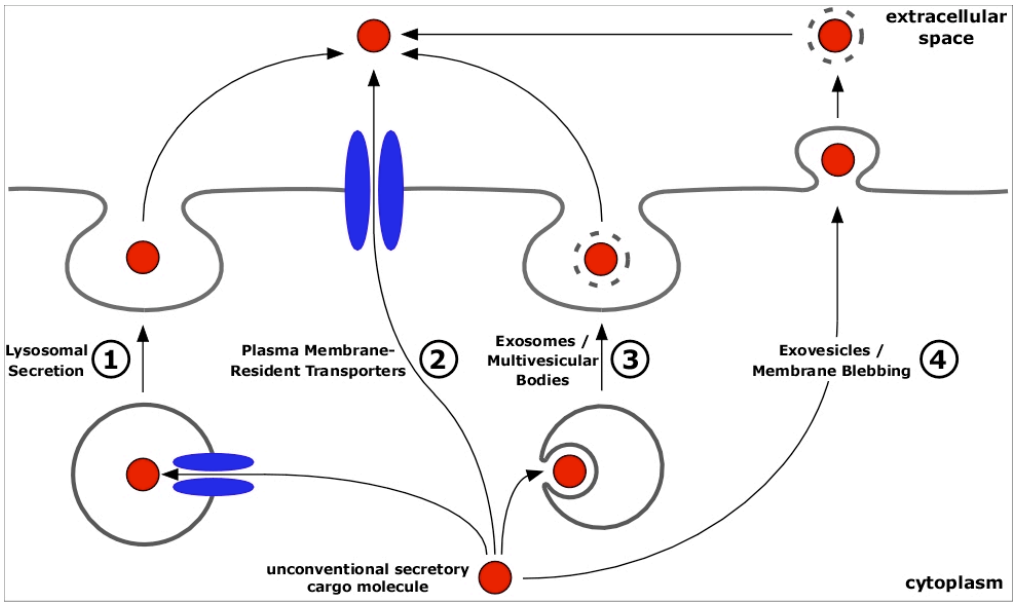

Fig. 1.1 Vesicular and non-vesicular pathways potentially involved in unconventional secretion.
1, export by secretory lysosomes; 2, export mediated by plasma membrane-resident transporters; 3, export through the release of exosomes derived from multivesicular bodies; 4, export mediated by shedding of microvesicles. For details, see main text. (Courtesy of Walter Nickel (Nickel, 2005))

The first model depicted in Fig. 1.1 shows unconventional export mediated by secretory lysosomes. Under certain conditions, lysosomal contents gain access to the exterior of cells when specialized endocytic structures such as secretory lysosomes of cytotoxic T lymphocytes or melanosomes of melanocytes fuse with the plasma membrane (Stinchcombe et al., 2004). An example of a non-classically exported protein using this pathway is IL-1β (Rubartelli et al., 1990). In the second model displayed in Fig. 1.1 a direct translocation of unconventionally secreted proteins by plasma membrane-resident transporters such as adenosine triphosphate-binding cassette (ABC) transporters is proposed (Cleves and Kelly, 1996). Proteins probably exported by this mechanism are FGF-1 (Prudovsky et al., 2002), FGF2 (Schäfer et al., 2004) and the hydrophilic acylated surface protein B (HASPB) of *Leishmania* (Denny et al., 2000; Stegmayer et al., 2005). In the third model, depicted in Fig. 1.1, non-classical export is mediated by the release of exosomes derived from multivesicular bodies. Exosomes are released from cells upon fusion of multivesicular bodies with the plasma membrane (Stoorvogel et al., 2002). The cargo has to be packaged into the exosomal structures prior to the fusion event. An unconventionally secreted protein reported to use this pathway is galectin-3 (Thery et al., 2001). The fourth model presented in Fig 1.2 explains unconventional membrane

translocation by the release of microvesicles in a process termed membrane blebbing (Freyssinet, 2003; Hugel et al., 2005; Martinez et al., 2005). As proposed by Hughes et al. the export of unconventionally secreted members of the galectin family is mediated by this mechanism (Hughes, 1999).

1.3 Non-classically Secreted Proteins

Since the discovery of unconventional secretion (Cooper and Barondes, 1990; Florkiewicz et al., 1995; Muesch et al., 1990) the number of proteins released by non-classical means is increasing steadily. The discovered proteins represent a highly diverse group not sharing structural or functional similarities. In general they have only in common their ER/Golgi-independent export route, but the underlying mechanistical details of membrane translocation differ strongly (Nickel, 2003). A summary of unconventionally secreted cellular, viral and parasitic proteins and the proposed mechanisms of how they exit the cell is presented in the following figure (Fig. 1.2).

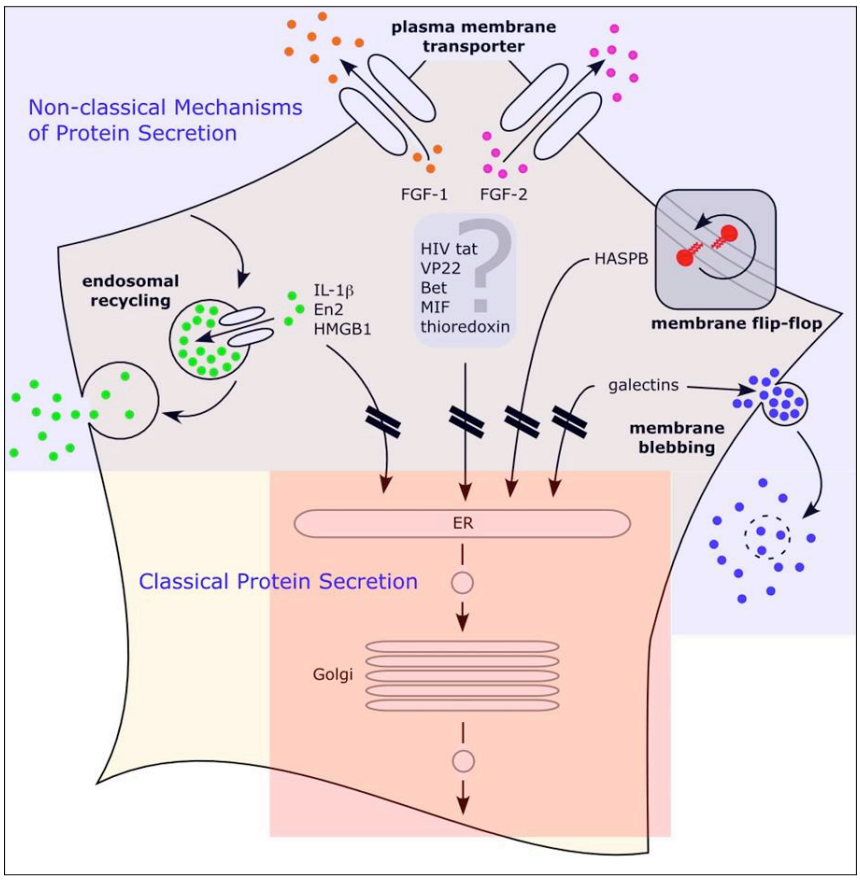

Fig. 1.2 Unconventionally secreted proteins and putative export pathways.

Four different plasma membrane translocation processes involved in non-classical export can be distinguished. They represent the mechanisms described in section 1.2.1. IL-1β, En2 and HMGB1 exit the cell packaged into intracellular vesicle originating form multivesicular endosomes or secretory lysosomes. FGF1 and FGF2 are directly translocated using plasma membrane resident transporters. HASPB of *Leishmania* is also directly translocated across the plasma membrane probably involving a flip-flop mechanism since it is membrane anchored at the inner leaflet due to its dual acylation at the N-terminus. Galectins are exported by membrane blebbing involving the formation of exosomes which are labile structures and release their content to the extracellular space once released. (Courtesy of Walter Nickel (Nickel, 2003))

One group of unconventionally secreted proteins are proangiogenic growth factors such as FGF1 and FGF2. Others are lectins of the extracellular matrix (ECM) such as galectin-1 (Cleves et al., 1996) and galectin-3 (Menon and Hughes, 1999). Additionally, cytokines like IL-1β (Rubartelli et al., 1990), macrophage inhibiting factor (MIF) (Flieger et al., 2003) and thioredoxin (Rubartelli and Sitia, 1991) have been reported to be secreted by unconventional means. Also viral proteins such as Herpes simplex tegument protein VP22 (Elliott and O'Hare, 1997), Human immunodeficiency virus (HIV) Tat protein (Ensoli et al., 1993) and Foamy virus Bet protein (Lecellier et al., 2002) are released by non-classical export. Other proteins sharing unconventional export are HASPB of *Leishmania* (Denny et al., 2000), the

homeodomain-containing transcription factor Engrailed homeoprotein isoform 2 (En2) (Joliot et al., 1998) and high mobility group chromatin-binding protein 1 (HMGB1) (Gardella et al., 2002). Selected proteins from this group are described in more detail in the following sections.

1.3.1 Fibroblast Growth Factors

The protein family of heparin-binding fibroblast growth factors (FGFs) consists of 23 members exhibiting 13-71% amino acid identity (Ornitz and Itoh, 2001). They range in molecular mass form 17 to 34 kDa and are highly conserved among vertebrates in both gene structure and amino acid sequence. Their gene loci are scattered throughout the genome but several genes appear in clusters on different chromosomes. Evolutionary the family was probably generated by gene- and chromosome-duplication accompanied by gene translocation events (Ornitz and Itoh, 2001).

FGFs mediate a variety of functions and are involved in numerous biological processes including angiogenesis, embryonic development, mitogenic activity, cellular chemotaxis, proliferation and differentiation (Bikfalvi et al., 1995; Bikfalvi et al., 1997; Powers et al., 2000; Rogelj et al., 1989). They also function in adult organisms as homeostatic factors, in tissue repair and response to injury (Bikfalvi et al., 1997). Additionally FGFs contribute to pathogenesis of cancer mediating tumor-associated angiogenesis (Liekens et al., 2001) and a subset is involved in neuronal signal transduction in the central and peripheral nervous system (Lou et al., 2005).

As a structural feature FGFs share a central core region of 140 amino acids with 28 highly conserved and six identical amino acids (Ornitz, 2000). Ten of the highly conserved amino acids are thought to mediate the interaction with FGF receptors (FGFRs) (Plotnikov et al., 2000). The central core folds into twelve antiparallel β-strands that form a cylindrical barrel closed by the variable N- and C-terminal regions which is structurally identical to IL-1β (Ago et al., 1991; Zhang et al., 1991). The heparin binding site is formed by basic amino acids residing in the β-strands 10 and 11 and in the loop region between the two β-strands. These regions are believed to be distinct from regions mediating FGFR-binding (Faham et al., 1998).

In addition to receptor binding FGFs interact with heparin, heparan sulfate or herparan sulfate proteoglycans (HSPGs) which are abundant ECM components (Gleizes et al., 1995). These low affinity interactions stabilize FGFs and prevent uncontrolled diffusion and release from the ECM after secretion thereby inducing dimer-formation and the assembly of higher-ordered oligomers (Caldwell et al., 2004; Flaumenhaft et al., 1990; Herr et al., 1997). Furthermore, they mediate clustering and guidance to the actual FGFRs and a concerted mechanism of HSPG- and FGFR-binding is required for the biological activity of FGFs (Pellegrini et al., 2000). FGFRs are transmembrane tyrosine kinase receptors with two to three immunoglobulin (Ig)-like domains which mediate the association with FGFs. Additionally, they contain a heparin binding domain (McKeehan et al., 1998). Four FGFRs are known which result from alternative splicing by which the specifity of FGF-recognizing Ig-like domains is modulated (Johnson et al., 1991; Johnson and Williams, 1993).

Most FGFs contain an N-terminal signal sequence which directs them to the classical secretory pathway (FGFs 3-8, 10, 15, 17-19 and 21-23). FGFs 9, 16 and 20 do not have a classical signal peptide but are nevertheless exported via the ER/Golgi-dependent pathway (Miyakawa et al., 1999; Miyake et al., 1998; Miyamoto et al., 1993). Another group of FGFs (11-14) also lack a signal sequence and remain intracellularly, their function is unknown (Munoz-Sanjuan et al., 2000; Smallwood et al., 1996).

The group of unconventially secreted FGFs is comprised of FGF1 (Burgess et al., 1994; Jackson et al., 1992; Prudovsky et al., 1994), FGF2 (the 18 kDa isoform) (Engleka and Maciag, 1992; Engling et al., 2002; Florkiewicz et al., 1995; Nickel, 2005) and FGF20, the latter being discovered recently and its export mechanism and biological role is currently not known (Hajihosseini and Heath, 2002; Jeffers et al., 2001; Kirikoshi et al., 2000). Non-classically exported FGFs do not contain N-terminal signal sequences and their export occurs independently of the ER/Golgi-pathway. The characteristics of FGF1 and especially FGF2 are discussed in more detail in the following sections since FGF2 is the main focus of the present study.

1.3.1.1 Fibroblast Growth Factor 1

Fibroblast growth factor 1 (FGF1), also known as acidic FGF, is one of the prototype members of the FGF family. It was initially isolated from bovine pituitary extracts in 1974 (Gospodarowicz et al., 1974). FGF1 is known to be unconventionally secreted exhibiting features like the lack of a classical signal sequence and BFA-insensitive export (Tarantini et al., 1995). It was observed that FGF1 release is induced in response to stress conditions such as heat shock (Jackson et al., 1992; Jackson et al., 1995; Tarantini et al., 1998) and serum starvation (Shin et al., 1996). FGF1 forms homodimers in the presence of Cu^{2+} ions mediated by a cysteine residue at position 30 (Engleka and Maciag, 1992; Jackson et al., 1995; Tarantini et al., 1995) which is a prerequisite for secretion. Additionally, dimerized FGF1 binds to S100A13, a member of the S100 family of calcium-binding proteins (Carreira et al., 1998; Landriscina et al., 2001b), and to the p40 extravesicular domain of p65 synaptotagmin 1 (p40) prior to export (Carreira et al., 1998; Landriscina et al., 2001a; Landriscina et al., 2001b). The hetero-oligomeric complex requires the oxidative function of Cu^{2+} ions as an essential post-translational intracellular modifier (Landriscina et al., 2001a). Upon heat shock FGF1 is transported to the plasma membrane as analyzed by real-time confocal microscopy. Based on these observations it was proposed that the assembly of the release complex occurs underneath the plasma membrane. Additionally, this process was shown to be dependent on actin filaments since it can be inhibited by amlexanox, a compound that is known to attenuate actin stress fiber formation (Prudovsky et al., 2002).

Extracellular FGF1 binds to FGFRs 1 to 4 and is internalized by receptor-mediated endocytosis, in a clathrin-dependent and clathrin-independent way. Receptor activation and internalization are required to induce FGF1-dependent proliferation (Wiedlocha and Sorensen, 2004). Externally added FGF1 was found to translocate to early endosomes after 15 min at 37°C. Following association with the respective receptor isoform FGF1 is sorted to the lysosomal compartment for degradation (FGFR 1-3) or to the recycling compartment (FGFR 4) probably dependent on the ubiquitination status of the receptor (Haugsten et al., 2005). FGF1 not destined for degradation is able to cross intracellular membranes to reach the cytosol and the nucleus (Wiedlocha and Sorensen, 2004) mediated by a nuclear localization signal (NLS) which is also crucial for its mitogenic activity (Imamura et al., 1990). FGF1

contains a bipartite NLS localized in the N- and the C-terminal regions of the protein which causes FGF1 translocation from the cytosol to the nucleus (Wesche et al., 2005). FGF1 is thought to stimulate DNA synthesis and is phosphorylated in the nucleus by protein kinase C delta. Phosphorylated FGF1 is exported from the nucleus probably serving as an activity-regulating mechanism (Wiedlocha et al., 2005).

1.3.1.2 Fibroblast Growth Factor 2

Fibroblast growth factor 2 (FGF2) or basic FGF is a mitogenic growth factor which was, like FGF1, first discovered in 1974 by isolation from bovine pituitary extracts (Gospodarowicz et al., 1974). It is a protein of high biomedical relevance with multiple functions as a signalling factor. FGF2 displays pleiotropic effects on different cell types and tissues such as stimulation of proliferation (Rogelj et al., 1989) and migration (Mignatti et al., 1992) or induction of differentiation, e.g. in primary neurons (Williams et al., 1994) and embryogenesis (Poole et al., 2001). It further plays a role in organ biogenesis like cardiogenesis (Sugi et al., 1993) and liver development (Jung et al., 1999) and also acts in wound healing (Bikfalvi et al., 1997). Most importantly, it is a strong mediator of neovascularization and angiogenesis during developmental processes and in association with solid tumor outgrowth (Liekens et al., 2001; Poole et al., 2001). This effect is directly visible in a chicken chorioallantoic membrane assay (CAM) where external FGF2 addition leads to the formation of new capillaries (Wilting et al., 1991). During tumor-associated blood vessel formation FGF2 acts as an initializing factor by stimulating the production and release of vascular endothelial growth factor (VEGF). Released FGF2 and VEGF directly stimulate endothelial cells to proliferate and release extracellular proteases such as urokinase-type plasminogen activator (uPA) that proteolytically activates plasmin which, in turn, degrades components of the ECM. Following ECM-degradation, proliferating endothelial cells can migrate towards the tumor to form new blood vessels (Mignatti and Rifkin, 1996a; Mignatti and Rifkin, 1996b; Rifkin et al., 1990).
FGF2 is found in five isoforms with molecular masses of 18, 22, 22.5, 24 and 34 kDa. The higher molecular weight forms are N-terminally prolonged transcripts of the 18 kDa form which arise by translation-initiation from upstream CUG codons (Arnaud et

al., 1999; Florkiewicz and Sommer, 1989; Prats et al., 1989). The additional N-terminal sequence contains a NLS which causes the higher molecular weight forms to localize predominantly to the nucleus (Renko et al., 1990).

In contrast, the 18 kDa form of FGF2 is a cytosolic protein which is secreted by unconventional means. It does not contain disulfide bonds which are formed in the ER although cysteine residues are present. Mutation of the four intrinsic cysteine residues at position 26, 70, 88 and 93 to serines results in a protein with the same secondary structure and equally mitogenic effects on 3T3 cells as the wild-type FGF2 (Fox et al., 1988). This finding suggests that the formation of disulfide bonds is not necessary for FGF2 functionality (Arakawa et al., 1989).

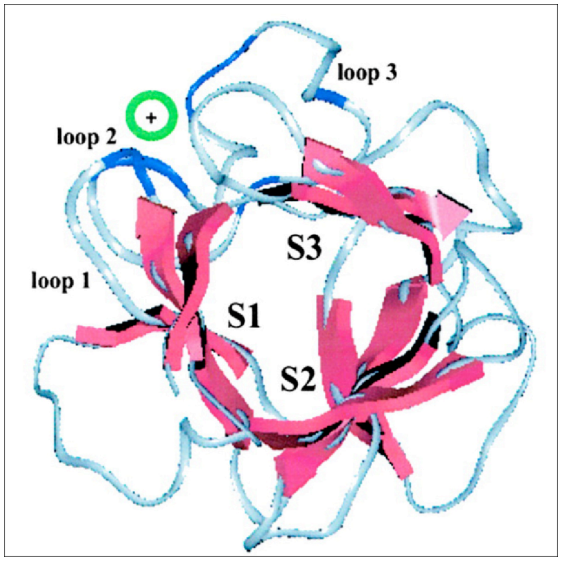

Fig. 1.3 Molecular structure of FGF2 associated with a HSPG side chain.

The HSPG side chain (indicated by the green circle) is arranged to the β-sheets S1, S2, and S3 (in red) of a β-trefoil scaffold of FGF2 in such a way that the pseudoaxis of threefold symmetry is roughly perpendicular to the plane of the paper. The oligosaccharides of HSPGs interact with basic residues (in blue) in the loop regions numbered 1-3. (Picture from Raman et al. (Raman et al., 2003))

Structural characteristics of the FGF2 molecule are the organization of 12 β-strands into a β-trefoil fold. The β-trefoil scaffold is comprised of three antiparallel β-sheets, S1, S2, and S3 that are stacked in a triangular fashion to form a trigonal pyramid-like structure. Each of the antiparallel β-sheets contains four β-strands (Raman et al., 2003). This three-dimensional structure is topologically equivalent to IL-1β (Ago et al., 1991; Eriksson et al., 1991) but only 25% amino acid homology can be observed (Gimenez-Gallego et al., 1985).

FGF2 contains different binding sites for the FGFR and HSPGs. FGFR binding is mediated by a primary and a secondary FGFR binding site which are directly opposed to each other on the molecule. A cluster of hydrophobic residues form the primary receptor binding site (Y24, E96, N101, Y103, L140, and M142) while the secondary receptor binding site is formed by a linear stretch of amino acid residues (K110, Y111, and W114). The primary binding site is conserved among members of the FGF family whereas the secondary binding site varies due to amino acid deletions and mutations (Venkataraman et al., 1999). The binding sites are described as low and high affinity sites depending on their capability to bind to their respective binding site in FGFR. The HSPG-binding site of FGF2 is comprised of three surface exposed loop regions, including one large loop (loop3) between S1 and S3 sheets and two small loops in S1 (loop1) and S3 (loop2) of the β-trefoil scaffold (Raman et al., 2003). It is spatially spread throughout the molecule and only functional upon folding into the correct three-dimensional structure (Seddon et al., 1991).

Extracellular FGF2 binds to heparan sulfates present in the carbohydrate side chains of HSPGs in the ECM. HSPGs are abundant components of the ECM and organized into a core protein backbone, normally a perlecan, glypican or syndecan, and glyosaminoglycan (GAG) side chains. The GAG side chain in HSPGs is an anionic polysaccharide consisting of repetitive disaccharides units (Esko, 1991; Esko and Selleck, 2002). HSPG binding is crucial for FGF2 since it mediates protection against degradation, serves as an FGF2 reservoir for storage of secreted FGF2, and facilitates the formation of the ternary complex of FGF2, HSPG and FGFR. FGF2 bound to HSPGs within the ECM of secreting cells is released upon a stimulus by proteolysis of the HSPG core protein (Rifkin et al., 1990) or GAG side chain cleavage by heparanase (Moscatelli, 1992). It was further proposed that an extracellular FGF-binding protein competes with heparan sulfates for FGF2 causing its release and acting as a carrier molecule (Wu et al., 1991).

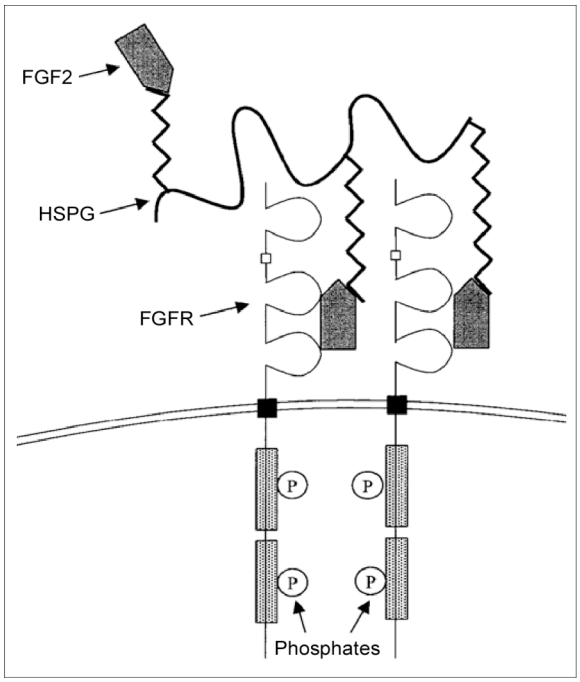

Fig. 1.4 Ternary complex of FGF2, FGFR and HSPG in the ECM.

FGF2 binds to heparan sulfate side chains of HSPG in the ECM. The complex of FGF2 molecules and HSPG binds to the FGFR and induces dimerization. This leads to trans-phosphorylation between the two receptor molecules, which, in turn, activates different signalling pathways of the cell. (Picture from Powers et al. (Powers et al., 2000))

Receptor mediated signalling requires binding of FGF2 to HSPGs and tyrosine kinase FGFRs. Binding of soluble FGF2 to heparan sulfate side chains induces oligomerization and increases the local concentration on the cell surface. As shown by Spivak-Kroizman et al., FGF1 and FGFRs interact with 1:1 stoichiometry (Spivak-Kroizman et al., 1994). The oligomerization of FGFs triggered by binding to heparan sulfates promotes the recruitment of several FGFRs leading to receptor dimerization and activation (Powers et al., 2000). Dimerization of FGFRs induces autophosphorylation of cytosolic tyrosine residues which, in turn, activates phospholipase Cγ (PLCγ), a protein found to be associated with FGFRs (Burgess et al., 1990). PLCγ cleaves phophatidyl-inositol-4,5-bisphosphate to inositol triphosphate (IP_3) and diacylglycerol (DAG) which leads to an increase of the intracellular concentration of Ca^{2+} ions by release from the ER reservoir in response to the stimulus by IP_3. Ca^{2+} ions function as second messengers which induce numerous cellular responses and additionally DAG together with the released Ca^{2+} ions activates phosphokinase C (PKC).

Another signalling pathway which can be activated by FGFR dimerization is Ras/MAP-kinase signalling. It was shown that dimerized FGFRs can phosphorylate a 90 kDa-signalling factor known as SNT-1 or FRS2 (Kouhara et al., 1997; Wang et al., 1996) which links FGF2 induced FGFR dimerization to MAP-kinase signalling.

Regarding the export of FGF2 it was initially supposed that the protein is released by cell damage to induce blood vessel formation in regenerating tissues following injury (McNeil et al., 1989). However, in 1991 it was reported that FGF2 is released from intact cells by a controlled mechanism although it lacks a classical signal sequence (Mignatti and Rifkin, 1991) similar to IL-1β. A milestone was the discovery that FGF2 is selectively exported from COS cells being transiently transfected with the 18 kDa form cDNA (Florkiewicz et al., 1995). Additionally it was discovered that BFA does not inhibit FGF2 secretion and that export is energy-dependent since ATP-depletion abolished FGF2 secretion. First experiments analyzing the kinetics of FGF2 export revealed that it is a relatively slow process since pulse-labelled FGF2 was found extracellularly only after 120 min (Florkiewicz et al., 1995). Taken together these findings showed that the release of FGF2 does not occur in an uncontrolled manner but rather that it is a regulated process dependent on a molecular machinery. Another finding to confirm this hypothesis came again from Florkiewicz et al. who could show that ouabain, a plant-derived alkaloid (cardiac glycoside) from *Strophantus gratus* interferes with FGF2 secretion in a time- and dose-dependent manner (Florkiewicz et al., 1998). Ouabain is a known inhibitor of the Na^+/K^+-ATPase (Lingrel and Kuntzweiler, 1994) and co-purification experiments showed that FGF2 directly interacts with the α-subunit of the Na^+/K^+-ATPase (Florkiewicz et al., 1998). In addition, cells transfected with a rodent α-subunit, insensitive to ouabain, showed no inhibition of FGF2 secretion by ouabain (Dahl et al., 2000). These findings demonstrated the involvement of a proteinaceous machinery and linked FGF2 secretion indirectly to the functionality of the α-subunit of the Na^+/K^+-ATPase without providing evidence for a direct role in FGF2 export. Moreover, Trudel et al. could show that FGF2 is not released into the medium of transfected COS cells but stored extracellularly bound to HSPGs. These findings demonstrated that FGF2 secretion and release are not coupled processes and extracellular FGF2 activity is regulated by controlled release from the ECM giving an explanation why FGF2 is absent from most body fluids and conditioned media (Trudel et al., 2000). Investigating the details of non-classical FGF2 export a study by Gloe et al. provided evidence that FGF2 is

released from shear stress-exposed endothelial cells. They further reported that the release is tightly controlled by cell-matrix interactions mediated by the $\alpha_V\beta_3$ integrin (Gloe et al., 2002). Taverna et al. provided information that three isoforms of FGF2, the 18, 22 and 24 kDa form, can be found in vesicles released from the plasma membrane of serum-starved cells after serum re-addition (Taverna et al., 2003). Linking these two observations it was reported that shear stress induces the shedding of microvesicles (Martinez et al., 2005). Although these observations sound quite reasonable one has to take into consideration that re-addition of serum to previously serum-starved cells is known to induce apoptosis (Hasan et al., 1999). This would lead to unspecific release of cellular content in apoptotic vesicles which could explain the appearance of the 22 and 24 kDa isoform of FGF2 that are not released under normal conditions (Arese et al., 1999; Monzat et al., 1996).
Further analysis of the mechanistical details of FGF2 secretion provided evidence for a microvesicle-independent release. An *in vitro* assay employing plasma membrane-derived inside-out vesicles was exploited to study FGF2 plasma membrane translocation (Schäfer et al., 2004). The lumen of these vesicles is topologically equivalent to the extracellular space and the performed import studies reconstitute the export process. Using this assay it was shown by Schäfer et al. that FGF2 is translocated across the plasma membrane in a temperature- and time-dependent manner (Schäfer et al., 2004). Additionally, treatment with high salt or protease which removes membrane-associated proteins prior to FGF2 addition abolished FGF2 translocation. Moreover, protein translocation occurred selectively as FGF2 and galectin-1 were imported into inside-out vesicles but the unconventionally secreted protein MIF (see section 1.3.4) and classically secreted FGF4 were rejected (Schäfer et al., 2004). These findings gave rise to the proposal of a plasma membrane resident transporter which directly mediates FGF2 secretion.

1.3.2 Galectins

The family of galectins consists of 15 members which are abundant β-galactoside-binding lectins of the ECM (Barondes et al., 1994). All members of the galectin family contain one or two highly conserved carbohydrate recognition domains (CRD) which mediate binding to glycoproteins containing oligosaccharide side chains with terminal

β-galactoside sugar moieties (Hughes, 1999; Perillo et al., 1998) and glycolipids like GM1 (Kopitz et al., 1998; Perillo et al., 1998). Galectins function in different intra- and extracellular processes including cell growth, proliferation, apoptosis, differentiation, migration and adhesion (Liu et al., 2002; Pace et al., 1999; Perillo et al., 1998; Perillo et al., 1995). The best characterized galectins are galectin-1 and galectin-3 which are unconventionally secreted (Hughes, 1999).

Galectin-1 is a low molecular weight protein with a size of 14 kDa containing one CRD. It is mostly found in a homodimeric state with the CRDs located at the opposed ends which results in increased binding properties and crosslinking functions (Lopez-Lucendo et al., 2004). Galectin-1 is synthesized on free ribosomes in the cytosol where it is in an active state due to the reducing environment (Cho and Cummings, 1995). To remain active after secretion into the oxidative extracellular milieu it rapidly binds to β-galactoside-containing ligands. Secretion of galectin-1 is insensitive to BFA (Sato et al., 1993) and it lacks a classical signal sequence (Couraud et al., 1989). Additionally, it is not modified by intramolecular disulfide bond formation although free sulfhydryl groups are present (Hirabayashi and Kasai, 1991). Extracellular galectin-1 binds to counter receptors on the plasma membrane such as laminin (Zhou and Cummings, 1990), T-cell specific CD43 and CD45 (Pace et al., 1999) and fibronectin (Ozeki et al., 1995). Moreover, it is bound by CA125, a tumor-specific cell surface antigen (Seelenmeyer et al., 2003), which points to a role in tumor cell interaction with the ECM (Liu and Rabinovich, 2005).

Galectin-3 is a 30 kDa protein also synthesized on free ribosomes. Its secretion is not affected by BFA and it is does not contain a classical signal sequence (Hughes, 1997). It can form homodimers like galectin-1 and its secretion from breast carcinoma cells is stimulated by the fetal serum protein fetuin (Zhu and Ochieng, 2001). Like galectin-1 it binds to counter receptors localized to the cell surface like laminin (Hughes, 1997; Mehul and Hughes, 1997).

It is assumed that galectin-1 and galectin-3 exit the cell by a process known as membrane blebbing. This process consists of an accumulation of the molecules underneath the plasma membrane and release by shedding of plasma membrane-derived vesicles (Hughes, 1999; Mehul and Hughes, 1997; Sato et al., 1993). Opposed to this model it was recently shown that counter receptor binding, which was thought to occur in the extracellular space after secretion, is essential for the overall release mechanism. Cells devoid of counter receptors do not secrete galectin-

1 and mutated forms of the protein which are deficient in β-galactoside binding are not exported from cells which normally secrete the wt form of the protein (Seelenmeyer et al., 2005). Additionally, Schäfer et al. provided evidence that membrane translocation seems to occur directly from the cytosol to the extracellular space since galectin-1 is imported into the lumen of inside-out vesicles in an *in vitro* assay which mimics secretion, as the lumen of these vesicles is topologically equivalent to the extracellular space (Schäfer et al., 2004).

1.3.3 *Leishmania* HASPB

An example of a parasitic protein exported by unconventional means is the cell surface molecule HASPB of *Leishmania* (Denny et al., 2000). HASPB is expressed only in infectious stages of the parasite's lifecycle and found associated with the outer leaflet of the plasma membrane (Alce et al., 1999; Flinn et al., 1994; Pimenta et al., 1994). The primary structure differs from all unconventionally secreted proteins known as it contains an N-terminal SH4 domain commonly found in src-kinases (Resh, 2004). It is synthesized on free ribosomes in the cytosol, co-translationally myristoylated followed by a palmitoylation step which occurs at the outer leaflet of the Golgi apparatus mediated by a putative Golgi-resident palmitoylacyltransferase (Denny et al., 2000). These acylation modifications occur at amino acid residues of the SH4 domain. Following these modifications the protein is transported to the inner leaflet of the plasma membrane which was shown to be the membrane translocation site of the molecule (Stegmayer et al., 2005). The 18 N-terminal amino acids of HASPB are both required and sufficient to target the protein to the plasma membrane. As shown by mutational analysis a protein lacking this sequence is redistributed to the cytosol (Denny et al., 2000). Also, the myristoylation site is essential for membrane targeting since a myristoylation mutant is also found to be cytosolic (Denny et al., 2000). A palmitoylation mutant is retained at the level of the Golgi suggesting that the outer leaflet of the Golgi membrane is the putative site of palmitoylation in the HASPB biogenesis pathway (Stegmayer et al., 2005). An interesting feature of HASPB is that, upon heterologous expression, it is also exported from mammalian cells, an observation which points to a conserved pathway of non-classical plasma membrane translocation among lower and higher eukaryotes

(Denny et al., 2000). Based on heterologous expression of a HASPB-N18-GFP fusion protein in CHO cells a somatic mutation analysis was performed by Stegmayer et al. resulting in the isolation of a clonal cell line deficient with regard to HASPB secretion. In this mutant, the protein accumulates at the inner leaflet of the plasma membrane suggesting the existence of a plasma membrane-resident transporter which has been compromised by the retroviral mutagenesis (Stegmayer et al., 2005).

1.3.4 Cytokines

Different cytokines were found to exit the cell by non-classical export. The best characterized example is interleukin 1 (IL-1) which was also one of the first proteins discovered to be released by an unconventional mechanism (Rubartelli et al., 1990). It exists in two isoforms IL-1α and IL-1β which are proteolytically processed from two related, but distinct precursors (Dinarello, 1997). Interleukin-1 mediates several biological functions related to inflammatory or infectious immune response and host defense mechanisms (Dinarello, 1985). IL-α is myristoylated, inserted into the plasma membrane and released to the extracellular space by calpain-dependent cleavage independent of the ER/Golgi-pathway (Kobayashi et al., 1990; Stevenson et al., 1993; Watanabe and Kobayashi, 1994). IL-1β is synthesized as a 33 kDa precursor and processed by an interleukin-converting enzyme (ICE) (Black et al., 1988). Only the mature form is then localized to intracellular vesicles which are not related to the ER/Golgi system but rather originate from the endolysosomal compartment (Andrei et al., 1999). IL-1β is not glycosylated despite bearing corresponding consensus sites and its export involves the ABC transporter ABC1 based on inhibition studies using sulfonylurea glyburide (Hamon et al., 1997) and antisense expression inhibition (Zhou et al., 2002). Moreover, studies by MacKenzie et al. suggested that IL-1β is rapidly released from activated monocytes by shedding of microvesicles since bioactive IL-1β was found extracellularly inside microvesicles 2 min after stimulation (MacKenzie et al., 2001).

The unconventional secretion of the pro-inflammatory cytokine macrophage inhibiting factor (MIF) is also dependent on functional ABC transporters (Flieger et al., 2003). The protein mediates pleiotropic functions upon release from monocytes, macrophages and lymphocytes in response to bacterial endo- and exotoxins and

cytokines (Calandra and Bucala, 1997). MIF lacks a classical signal sequence, does not enter the ER and its export from monocytes is not inhibited by BFA (Flieger et al., 2003). Additionally it was reported that MIF can be found in vesicles being released from the plasma membrane of specialized epithelial cells (Eickhoff et al., 2001).
Thioredoxin, another protein exhibiting cytokine functions was found to be exported in an unconventional manner as well. It is an ubiquitous enzyme catalyzing thiol-disulfide exchange reactions (Holmgren, 1989). Certain isoforms act extracellularly as mitogenic cytokines although they lack a classical signal sequence (Rubartelli et al., 1992). Its release is not affected by BFA (Rubartelli and Sitia, 1991; Tanudji et al., 2003). Furthermore, it could be shown that non-classical export of thioredoxin is not dependent on the redox-state of the cell or the protein (Tanudji et al., 2003). The release mechanism remains elusive but secretion is not dependent on ABC transporters and the protein can not be found in intracellular vesicles.

1.3.5 Viral Proteins

Unconventionally secreted proteins of strong biomedical relevance are virus-encoded factors which play a role in the viral replication cycle. The HIV-Tat protein is an auxiliary factor required for replication in addition to structural and enzymatic proteins (Goldstein, 1996). It is an early transactivator protein which is released from intact HIV-infected cells and from cells transfected with HIV-Tat cDNA although it lacks a classical signal sequence (Chang et al., 1997; Ensoli et al., 1993). Furthermore, it contains a region termed the basic transduction domain which is involved in membrane translocation (Becker-Hapak et al., 2001). The basic transduction domain also mediates binding of released Tat to HSPGs in the ECM for storage similar to FGF2 (Chang et al., 1997). Since the release of Tat from cultured cells is temperature sensitive whereas its uptake is not, it was proposed that unconventional secretion occurs in a different way as the protein machinery-independent uptake related to the basic transduction domain (Chang et al., 1997).
Another viral protein exhibiting non-classical export is the Herpes simplex tegument protein VP22. It is expressed as a cytosolic factor lacking a signal sequence and exported by an unknown BFA-insensitive pathway (Elliott and O'Hare, 1997). Additionally its export is sensitive to cytochalasin D which inhibits actin

polymerization. This observation suggests an involvement of the cytoskeleton being consistent with findings that VP22 binds to microfilaments and induces microtubular bundle formation rendering these nocodazole-insensitive (Elliott and O'Hare, 1998).
A third viral protein exiting host cells via unconventional secretion is the Foamy virus protein Bet. It is expressed as a cytosolic protein, secreted by an unknown mechanism and can spread between cultured cells (Giron et al., 1998; Lecellier et al., 2002). Evidence for the secretion via an ER/Golgi-independent pathway came from the finding that Bet lacks a classical signal sequence and its export is not affected by BFA (Lecellier et al., 2002).

1.3.6 Homeodomain Containing Transcription Factors and Chromatin-binding Proteins

Further examples of unconventionally secreted proteins are the transcription factor engrailed homeoprotein isoform 2 (En2) and the high mobility group chromatin-binding protein 1 (HMGB1). These proteins are involved in the process of regulated gene transcription and normally localize to the nucleus.
HMGB1 is secreted in response to inflammation from a limited number of cell types including monocytes and macrophages. Bacterial lipopolysaccharides stimulate monocytes to release HMBG1 which is devoid of a conventional signal peptide. Activated monocytes show a redistribution of HMBG1 from the nucleus to the cytoplasm where it localizes to the endolysosomal compartment. Export occurs by lysosomal exocytosis triggered by an unknown stimulus showing similarities to IL-1β secretion (Gardella et al., 2002).
Analyzing the intracellular distribution of En2 a subpopulation was reported to localize to caveolae-like structures at the plasma membrane. Moreover, a significant portion is present in membrane-bound vesicles as determined by protease protection assays (Joliot et al., 1997). These findings provided first evidence that the protein might be secreted to a certain extent although it does not contain a classical signal sequence. It was further observed that En2 can spread between co-cultured COS cells expressing the chicken orthologue and rat primary neurons. About 5% of the protein are externalized and an 11 amino acid sequence within the homeodomain has been identified which is probably involved in En2 release since deletion of this

sequence abolished export of En2 to the extracellular space (Joliot et al., 1998). Later this sequence was identified as a NES sequence targeting nuclear En2 to the cytoplasm. Furthermore, intercellular transport seems to be regulated by phosphorylation of a serine-rich domain by protein kinase CK2 (Maizel et al., 2002) suggesting that En2 export is a controlled process.

1.4 Quality Control in Unconventional Secretion

If proteins are exported independently of the ER/Golgi-pathway the question arises how quality control is ensured to avoid release of non-functional proteins. During classical secretion quality control measures are applied especially at the level of the ER. Insertion into the ER requires the binding to ER-resident chaperones which facilitate the folding process and misfolded polypeptide chains are removed by dislocation to the cytosol where they are degraded by the proteasome (Rubin and Finley, 1995; Trombetta and Parodi, 2003). An accumulation of misfolded proteins in the ER also induces the unfolded protein response mechanism which leads to transcription of chaperones (Sidrauski and Walter, 1997). Post-translational modifications contribute to functional characteristics of the protein and enzymes like protein disulfide isomerase (PDI) which accomplishes correct formation of disulfide bonds (Freedman et al., 1984; Hillson et al., 1984; Noiva and Lennarz, 1992; Walker and Gilbert, 1997) or oligosaccharyl-protein transferases which mediate N-linked glycosylation by attachment of oligosaccharide alter the protein structure (Abeijon and Hirschberg, 1992; Sharma et al., 1981). The attachment of sugar moieties by N-linked glycosylation serves as another point of quality control. Calnexin or calreticulin, two homologous lectins which require Ca^{2+} for their activity, bind to incorrectly modified glycoproteins or protein segments which expose a terminal glucose which is normally removed during glycosylation (Hebert et al., 1995; Helenius et al., 1997; Tatu and Helenius, 1997). They retain these proteins in the ER and allow another round of glycosylation to correctly complete this modification step. After a protein has achieved its correct conformation and is correctly modified it is recognized by cargo adaptors and packaged into transport vesicles which travel to the Golgi (Helenius and Aebi, 2004; Lee et al., 2004).

For other membrane translocation processes unfolding is a prerequisite. When proteins are imported into mitochondria they have to be unfolded to pass the outer and inner membrane translocation complexes. In the mitochondrial matrix the translocated polypeptide chain is immediately bound by mitochondrial chaperones which ensure correct folding (Schatz and Dobberstein, 1996). Taken together these elaborate mechanisms demonstrate the importance of quality control for membrane translocation processes and secretion. It is therefore rather unlikely that quality control measures do not apply for unconventional secretion.

Non-classical export starts with synthesis of polypeptide chains on free ribosomes in the cytosol. The proteins do not enter the ER or the Golgi and are therefore excluded from the post-translational modifications which can serve as quality control measures like in the case of N-linked glycosylation in combination with the calnexin/calreticulin chaperone system. It could be possible that cytosolic chaperones mediate correct folding although no evidence contributing to this hypothesis was found so far. If a direct plasma membrane translocation step is involved, as proposed for FGF1, FGF2 and HASPB, unconventional secretion would be mechanistically similar to ER-mediated membrane translocation with the difference that quality control measures can not be applied anymore once a protein is released to the extracellular space. Release of non-functional proteins could be a consequence. As shown by Schäfer et al. in an *in vitro* plasma membrane transport assay FGF2 seems to be translocated by a plasma membrane resident transporter (Schäfer et al., 2004). One could imagine that the transporter only recognizes correctly folded molecules as a substrate. A proposed feature of non-classically exported FGF2 could be that the targeting motif which mediates secretion is formed by amino acid residues spread throughout the molecule and not by linear export sequence (Nickel, 2005). Comparable to the HSPG-binding site (see section 1.3.1.2) (Raman et al., 2003) this motif would be inseparably linked to a correct three-dimensional conformation of the molecule. Quality control could be mediated by rejection of misfolded molecules. This scenario is similar to protein import into peroxisomes, a process by which fully folded proteins are translocated into the peroxisomal lumen without the existence of a chaperone system (Titorenko et al., 2002).

Evidence contributing to the plasma membrane-resident transporter quality control hypothesis comes from protein import into chloroplasts and bacterial transporters involved in protein secretion. These system also mediate transport of fully folded

cargo molecules (Robinson and Bolhuis, 2004). In the case of the bacterial twin-arginine translocation (Tat) system it was observed that the transporter even mediates export of oligomeric, co-factor containing complexes and can sense the folding state of the cargo as a quality control measure (DeLisa et al., 2003)

1.5 Use of Dihydrofolate Reductase in Protein Folding Analysis

Dihydrofolate Reductase (DHFR) is an enzyme of the nucleotide synthesis pathway involved in thymidylate synthesis (Hartman, 1993). It catalyzes the NADPH-dependent regeneration of tetrahydrofolate from dihydrofolate. Tetrahydrofolate is the carrier of the activated methylene group that is added to dUMP to form dTMP. DHFR can be inhibited by methotrexate or aminopterin which bind competitively to the active center with a $K_i < 10^{-9}$ M. Methotrexate and aminopterin are analogues of folate and inhibit the reaction cycle that generates thymidylate for DNA synthesis. These Inhibitors are used in cancer treatment as antimetabolites because actively proliferating cells need large amounts of nucleotides for DNA replication. They are known since the middle of last century (Hertz et al., 1956; Hunter and Carroll, 1949).
DHFR can be used in experimental systems to investigate protein folding because the protein is highly stabilized in its folded conformation upon binding of i.e. methotrexate or aminopterin. Chaperones or membrane translocation machineries like pore-complexes are not able to unfold DHFR in the presence of aminopterin or methotrexate. Under these conditions, DHFR is even resistant to protease digestion (Salvador et al., 2000). Therefore, it is possible to generate fusion constructs of cargo proteins linked to DHFR whose export or membrane translocation is dependent on unfolding. Another important feature is that the system can be used for *in vivo* studies since aminopterin or methotrexate can pass cellular membranes probably involving a folate transporter and bind to intracellular DHFR. The system is well known and different studies were performed to analyze import into organelles or transport machineries in general. Examples are mitochondrial protein import (Eilers and Schatz, 1986; Rassow et al., 1989; Verner and Schatz, 1987; Wienhues et al., 1991), chloroplast membrane translocation (Endo et al., 1994; Guera et al., 1993), substrate specificity of the GroEL system (Braig et al., 1993; Mayhew et al., 1996), protein import into lysosomes (Salvador et al., 2000), glycosomal protein import in

trypanosomes (Häusler et al., 1996), uptake of bacterial toxins by cells (Haug et al., 2003) or bacterial protein export (Arkowitz et al., 1993). Even questions addressing classical secretion processes were investigated using the DHFR system (Kida et al., 2005).

1.6 Aim of the Present Study

The aim of the present study was to analyze the folding state of FGF2 during unconventional secretion. For this purpose two experimental systems were applied. The first analyzes whether FGF2 has to be unfolded for non-classical export. The second investigates whether correct folding is maintained during unconventional export of FGF2 or whether the conformation of the cargo molecule is changed. Moreover, the second approach investigates the existence of quality control measures for unconventional export processes. To experimentally address these questions cell lines were generated using a retroviral transduction system, which express different FGF2 fusion-proteins in a doxicycline-dependent manner (Engling et al., 2002).

For the first experimental approach an *in* vivo system which reconstitutes non-classical FGF2 export in CHO cells was established. An advantage of CHO cells is that they do not possess FGFRs which excludes induction of differentiation processes mediated by exported FGF2. To generate reporter cell lines a construct consisting of FGF2 fused to the fluorescent reporter molecule GFP and DHFR (FGF2-GFP-DHFR) was stably integrated into the genome of the target cell line $CHO_{MCAT\text{-}TAM2}$. The GFP tag facilitates the detection of exported material which rebinds to HSPGs on the cell surface by flow cytometry and confocal microscopy using its intrinsic fluorescence activity or by specific antibody staining (Engling et al., 2002). Furthermore, it allows the analysis of the intracellular distribution of the fusion protein accessible by confocal microscopy. The addition of the DHFR domain integrates a unit which mediates control of the folding state in an aminopterin-dependent manner (see section 1.5) (Eilers and Schatz, 1986). As a control a mitochondrial targeting sequence was fused to GFP and DHFR (MTS-GFP-DHFR) to verify the functionality of the aminopterin-dependent translocation arrest by inhibition of unfolding of the reporter construct during mitochondrial import. To investigate

whether FGF2 needs to be unfolded for export, flow cytometry and confocal microscopy analyses were performed in the absence and presence of aminopterin. Specific antibodies directed against the N- and extreme C-terminus of the molecule were used to analyze whether translocation of the reporter construct occurs completely.

The second experimental approach makes use of a so called piggyback export analysis system. It consists of FGF2 fused to the F_c-part of the mouse immunoglobulin 2α (IgG2α). A second reporter construct was generated which is comprised of GFP fused to bacterial Protein A. IgG2α and Protein A are known to form a stable complex if both molecules are folded correctly. The two reporter constructs were introduced into CHO cells using a bicistronic expression vector which allows expression of two distinct proteins from one mRNA in a doxicycline-dependent manner. The system was used to investigate whether FGF2 maintains its folding state during membrane translocation. As a read-out the extracellular appearance of the complex consisting of FGF2-IgG2α and GFP-Protein A was monitored. Additional cell lines were generated which contain conventionally exported FGF4 with a signal sequence (FGF4-S), unconventionally secreted FGF1 instead of FGF2 or no FGF moiety at all to be used as controls. The system addresses the question whether the machinery mediating FGF2 plasma membrane translocation is capable of exporting oligomeric complexes like the bacterial Tat-system and whether, therefore, putative means of quality control could apply during the export process (see section 1.4) (DeLisa et al., 2003).

2 Material and Methods

2.1 Material

2.1.1 Chemicals

Agar	Becton Dickinson, Le Pont de Claix, France
Agarose electrophoresis grade	Invitrogen, Paisley, UK
Aminopterin	Sigma-Aldrich Chemie GmbH, Steinheim
Ammonium chloride	Carl Roth GmbH, Karlsruhe
Ampicillin sodium salt	Gerbu Biotechnik GmbH, Gaiberg
APS (Ammonium peroxo disulfate)	Carl Roth GmbH, Karlsruhe
αMEM	Biochrom AG, Berlin
Bromphenol Blue Na-salt	Serva Electrophoresis GmbH, Heidelberg
BSA (Bovine serum albumine, Albumin fraction V)	Carl Roth GmbH, Karlsruhe
β-Mercaptoethanol	Merck, Darmstadt
Calcium chloride dihydrate	Applichem, Darmstadt
Cell dissociation buffer (CDB)	Invitrogen, Paisley, UK
Chloroquine	Sigma-Aldrich Chemie GmbH, Steinheim
CL-4B Sepharose (Beads)	Amersham Biosciences AB, Uppsala, Sweden
Clear Nail Protector	Wet'n Wild USA, North Arlington, USA
Complete Mini (Protease Inhibitor Cocktail Tablets)	Roche Diagnostics, Mannheim
Deoxycholic acid sodium salt	Sigma-Aldrich Chemie GmbH, Steinheim
DMEM	Biochrom AG, Berlin
DMSO (Dimethyl sulfoxide)	J.T. Baker, Deventer, USA
dNTP-Mix	Peqlab, Erlangen
Doxicycline	Clontech, Palo Alto, USA
ECL Western Blotting Detection Reagent	Amersham Biosciences AB, Uppsala, Sweden
EDTA (Ethylene diamine tetraacetic acid)	Merck, Darmstadt
Ethanol pro analysi	Riedel-de Haën, Seelze
EZ-Link Sulfo-NHS-SS-Biotin	Pierce, Perbio Sciences, Bonn
FCS (Fetal Calf Serum)	PAA Laboratories GmbH, Linz, Austria
Fluoromount G	Southern Biotechnologies Inc., Birmingham, USA
Glacial acidic acid	Carl Roth GmbH, Karlsruhe
Glycerol	Carl Roth GmbH, Karlsruhe
Glycine	Applichem, Darmstadt

Hepes	Carl Roth GmbH, Karlsruhe
Isopropanol	Merck, Darmstadt
Kanamycin sulfate	Gerbu Biotechnik GmbH, Gaiberg
L-Glutamine	Biochrom AG, Berlin
Magnesium chloride hexahydrate	Applichem, Darmstadt
Methanol pro analysi	Merck, Darmstadt
Milk Powder	Carl Roth GmbH, Karlsruhe
Nonidet P40 (NP-40)	Roche, Mannheim
Paraformaldehyde	Electron Microscope Sciences, Hatfield, UK
Penicillin/Streptomycin for cell culture	Biochrom AG, Berlin
Ponceau S	Serva Electrophoresis GmbH, Heidelberg
Potassium dihydrogen carbonate	Carl Roth GmbH, Karlsruhe
Potassium hydroxide	J.T.Baker, Deventer, USA
Protein A-Sepharose (Beads)	Amersham Biosciences AB, Uppsala, Sweden
PVDF Membrane Immobilon FL	Millipore Corporation, Bedford
PVDF Membrane Immobilon P	Millipore Corporation, Bedford
Rotiphorese Gel 30 (37.5:1)	Carl Roth GmbH, Karlsruhe
Sodium chloride	J.T. Baker, Deventer, USA
Sodium dodecyl sulfate	Serva Electrophoreis GmbH, Heidelberg
Sodium hydrogen carbonate	J.T. Baker, Deventer, USA
Sodium hydroxide	J.T. Baker, Deventer, USA
Superdex 200 Beads	Amersham Biosciences AB, Uppsala, Sweden
Temed (*N*,*N*,*N*',*N*'-tetramethylethylenediamine)	Bio-Rad, München
Trichloroacetic acid	Carl Roth GmbH, Karlsruhe
Tris	Carl Roth GmbH, Karlsruhe
Trition X-100	Roche, Mannheim
Trypsin	Sigma-Aldrich Chemie GmbH, Steinheim
Trypsin / EDTA for cell culture	Biochrom AG, Berlin
Tryptone	Becton Dickinson, Le Pont de Claix, France
Tween 20 (Polyoxyethylenesorbitan monolaurate)	Carl Roth GmbH, Karlsruhe
UltraLink immobilized streptavidin (Beads)	Pierce, Perbio Sciences, Bonn
Whatman MM	Whatman AG, Würzburg
Xylencyanol FF	Serva Electrophoresis GmbH, Heidelberg
Yeast Extract	Becton Dickinson, Le Pont de Claix, France

2.1.2 Technical Devices

Agaros Gel Electrophoresis	
Anthos 2001 Microplate Photometer	Anthos, Hombrechtikon, Switzerland
Bacterial Incubator Infors HT ITE	Infors AG, Einsbach
Bacterial Shaker Centromat R	Braun, Melsungen
Centrifuge 5415 R	Eppendorf, Hamburg
Centrifuge 5417 R	Eppendorf, Hamburg
Centrifuge Avanti J-25	Beckman Coulter, Krefeld
Centrifuge Megafuge 1.0 R	Kendro, Langenselbold
Centrifuge Optima TLX Ultracentrifuge	Beckman Coulter, Krefeld
Centrifuge Rotor Sorvall SS-34	Kendro, Langenselbold
Centrifuge Sorvall Evolution RC	Kendro, Langenselbold
Centrifuge Sorvall RC 6	Kendro, Langenselbold
Ultracentrifuge Rotor TLA-45	Beckman Coulter, Krefeld
FACSAria	Becton Dickinson, Heidelberg
FACSVantage	Becton Dickinson, Heidelberg
FACSCalibur	Becton Dickinson, Heidelberg
Gel Doc 2000	Bio-Rad, München
Incubator Heraeus CO_2-Auto-Zero	Kendro, Langenselbold
LKB Ultraspec III	Amersham Biosciences, Freiburg
Microscope Axiovert 40 C	Zeiss, Göttingen
Microscope LSM 510 Meta Confocal	Zeiss, Göttingen
Mini Trans-Blot Cell	Bio-Rad, München
Mini-PROTEAN 3 Electrophoresis System	Bio-Rad, München
Nanodrop ND-1000 Spectrophotometer	Peqlab, Erlangen
Odyssey Infrared Imaging System	LI-COR Biosciences, Bad Homburg
PCR Primus Advanced 25 and 96	Peqlab, Erlangen
pH-Meter 766 Calimatic	Knick, Egelsbach
Power Pack 200 and 300	Bio-Rad, München
Roto-Shake Genie	Scientific Industries, Bohemia, USA
SMART System	Amersham Biosciences, Freiburg
Sonifier Cell Disruptor B 30	Heinemann, Schwäbisch Gmünd
Sonorex Super RK 103 h	Bandelin, Berlin
Super RX Medical X ray film	Fujifilm, Düsseldorf
Thermomixer compact and comfort	Eppendorf, Hamburg
Tricorn 5/150 Column	Amersham Biosciences, Freiburg
Wide Mini-Sub Cell GT	Bio-Rad, München

2.1.3 Plasmids

173.174.IgG2apBSII	Ingrid Haas, MPI, Freiburg
peGFP-1	Clontech, Mountain View, USA
pET15b-FGF2	Matthias Wuttke, AG Nickel, BZH, Heidelberg
pET24-FGF1	Matthias Wuttke, AG Nickel, BZH, Heidelberg
pET24-FGF4-S	Matthias Wuttke, AG Nickel, BZH, Heidelberg
pFB-hrGFP	Stratagene, La Jolla, USA
pGEM-T	Promega, Madison, USA
pGEM-T-TAP-Tag	Christoph Zehe, AG Nickel, BZH, Heidelberg
pQE16	Qiagen, Hilden
pRevTRE2	Clontech, Mountain View, USA
pRevTRE2-FGF2-GFP	Sabine Wegehingel, AG Nickel, BZH, Heidelberg
pRTi	Jörg Mölleken, AG Wieland, BZH, Heidelberg
pVPack Eco	Stratagene, La Jolla, USA
pVPack GP	Stratagene, La Jolla, USA
pYES-mtGFP1	Benedikt Westermann, LMU, München

2.1.4 Primers and Oligonucleotides

Primers and oligonucleotides were purchased from Thermo Electron Company. All sequences are listed in 5’ to 3’ direction. Reverse primers and oligonucleotides labelled with ‘rev’ in the title were ordered as reverse complement sequences.

<u>PCR primers for DHFR constructs</u>

DHFR-Cystein-BsrGI-for: 44 bases, T_m: 66°C
Overhang – BsrGI – 2 bases for in frame – sequence DHFR (mutation: ser to cys)
gactgg-tgtaca-ag-atg gtt cga cca ttg aac t**gc** atc gtc gcc

FGF2-GFP-DHFR-REV: 37 bases, T_m:60°C
Sequence DHFR – ClaI – overhang
ccagatctcatcaccatcaccatcactaa-atcgat-gg

mtpre HindIII for: 27 bases, T_m: 59°C
HindIII – sequence MTS
ccaagctt-atggcctccactcgtgtcc

mtpre AgeI rev: 24 bases, T_m: 61°C
sequence MTS (without stop codon) - AgeI
cgcgcctactcttcc-gcaccggtc

PCR primers for piggyback constructs

L-FGF1-for: 39 bases, T_m: 54°C
PmeI – Kozak sequence – sequence FGF1
gtttaaac-cgccacc-atggctgaaggggaaatcaccacc

L-FGF1-rev: 30 bases, T_m: 56°C
sequence FGF1 (without stop codon) – HindIII
ctccccctgccagtctcttctgat-aagctt

L-FGF2-for: 35 bases, T_m: 53°C
PmeI – Kozak sequence – sequence FGF2
gtttaaac-cgccacc-atggcagccgggagcatcac

L-FGF2-rev: 39 bases, T_m: 56°C
sequence FGF2 (without stop codon) – HindIII
gctatacttttttcttccaatgtctgctaagagc-aagctt

L-FGF4-for: 33 bases, T_m: 56°C
PmeI – Kozak sequence – sequence FGF4-S
gtttaaac-cgccacc-atgtcggggcccgggacg

L-FGF4-rev: 26 bases, T_m: 55°C
sequence FGF4-S (without stop codon) – HindIII
cccacttcctccccaggctg-aagctt

L-GFP-for: 43 bases, T_m: 55°C

BamHI – FseI – Kozak sequence - sequence eGFP

ggatcc-ggccggcc-cgccacc-atggtgagcaagggcgaggagc

L-GFP-rev: 30 bases, T_m: 56°C

sequence eGFP – SalI

ctcggcatggacgagctgtacaag-gtcgac

L-IgG2a-for: 41 bases, T_m: 59°C

HindIII – 2 base for in frame - Kozak sequence – artificial ATG – sequence IgG2α

aagctt-gc-cgccacc-atg-gatcaggagcccagagggcccac

L-IgG2a-rev: 31 bases, T_m: 54°C

sequence IgG2a (without stop codon) – NotI

gcttctcccggactccaggtaaa-gcggccgc

L-ProtA-for: 28 bases, T_m: 53°C

SalI – sequence ProtA

gtcgac-aaaaccgcggctcttgcgcaac

L-ProtA-rev: 29 bases, T_m: 56°C

sequence ProtA (without stop codon) – HindIII

cgaattccgcggggaagtcaacc-aagctt

<u>Oligonucleotides for piggyback constructs:</u>

L-LuxA-NES-for: 62 bases, T_m: 71°C

NotI open – 1 base for in frame – SGG-Linker – NES sequence– PacI – ClaI open

5'- ggccgc-t-agcggcggc-ctgcagaagaagctggaggagctggagctggactaa-ttaattaa-at -3'

L-LuxA-NES-rev: 60 bases, T_m: 71°C

NotI open – 1 base for in frame – SGG-Linker – NES sequence– PacI – ClaI open

3'- cg-a-tcgccgccg-gacgtcttcttcgacctcctcgacctcgacctgatt-aattaatt-tagc-5'

L-LuxB-NES-for: 60 bases, T_m: 75°C

HindIII open – SGG-Linker – NES sequence– SbfI – NotI open

5'- agctt-agcggcggc-ctgcagaagaagctggaggagctggagctggactaa-cctgcagg-gc-3'

L-LuxB-NES-rev: 60 bases, T_m: 76°C

HindIII open – SGG-Linker – NES sequence– SbfI – NotI open

3'- a-tcgccgccg-gacgtcttcttcgacctcctcgacctcgacctgatt-ggacgtcc-cgccgg-5'

2.1.5 DNA Modifying Enzymes

AmpliTaq Polymerase	Perkin Elmer (Roche), Branchburg, USA
BamHI	New England Biolabs, Frankfurt
BsrGI	New England Biolabs, Frankfurt
Calf Intestinal Phosphatase (CIP)	New England Biolabs, Frankfurt
ClaI	New England Biolabs, Frankfurt
FseI	New England Biolabs, Frankfurt
HindIII	New England Biolabs, Frankfurt
NotI	New England Biolabs, Frankfurt
PacI	New England Biolabs, Frankfurt
PmeI	New England Biolabs, Frankfurt
SalI	New England Biolabs, Frankfurt
SbfI	New England Biolabs, Frankfurt

2.1.6 Bacteria and Bacterial Media

For transformation and plasmid amplification competent DH5α cells (Invitrogen) or XL1-Blue supercompetent cells (Stratagene) were used. They were grown in LB medium (Luria Bertani medium) or on LB agar plates supplied with ampicillin or kanamycin in a final concentration of 100 µg/ml to select for successfully transformed cells carrying plasmids with a resistance gene.

Bacteria: subcloning efficiency DH5α competent cells,

Genotype: F^- φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1

XL1-Blue supercompetent cells,

Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)]

LB medium:	0.5 - 1.0%	NaCl (w/v)
	1%	Tryptone (w/v)
	0.5%	Yeast Extract (w/v)
		ddH_2O

LB agar plates:	0.5 - 1.0%	NaCl (w/v)
	1%	Tryptone (w/v)
	0.5%	Yeast Extract (w/v)
	1.5%	Agar (w/v)
		ddH_2O

2.1.7 Eukaryotic Cell Lines

HEK293T cells (Human Embryonic Kidney cells) were used as a host cell line for the production of retroviral particles carrying the various reporter constructs.
CHO cells (Chinese Hamster Ovary cells) were used as target cells for retroviral transduction. They were stably transduced with cDNA constructs and subsequently used for the expression of reporter molecules to function as an eukaryotic *in vivo* system to investigate proteins in a living cell environment. All reporter cell lines used for later analyses were generated using CHO cells.

Eukaryotic cell lines: CHO cells (ECACC; Ref. No. 85050302)
HEK293T cells (ATCC, Ref. No. CRL-11268)

2.1.8 Eukaryotic Cell Culture Media

αModification of the Minimal Essential Medium (αMEM)

The αModification of the Minimal Essential Medium (Biochrom AG) was used to cultivate CHO cells. Dry medium was dissolved in 5 l ddH_2O, and 10 g of sodium hydrogencarbonate were added to adjust the pH to 7.4, which was checked continuously. The prepared medium was sterile filtered into autoclaved bottles and stored at 4°C. Before addition to cells the medium was supplemented with 10% (v/v) fetal calf serum (FCS) and 100 µg/ml Streptomycin / Penicillin. If the medium was stored longer than six weeks, 2 mM glutamine were added.

Dulbecco's Modified Eagle Medium (DMEM)

Dulbecco's Modified Eagle Medium (Biochrom AG) was used to cultivate HEK cells. Dry medium was dissolved in 5 l ddH_2O and 10 g of sodium hydrogencarbonate were added to adjust the pH to 7.4, which was checked continuously. The prepared medium was sterile filtered into autoclaved bottles and stored at 4°C. If the medium was stored longer than six weeks, 2 mM glutamine were added. Before addition to cells the medium was supplemented with 10% (v/v) fetal calf serum and 100 µg/ml Streptomycin / Penicillin.

2.1.9 Antibodies

Primary antibodies:

To detect GFP containing reporter constructs, affinity-purified anti-GFP antibodies (Pineda Antibodies, acidic elution) were used (Engling et al., 2002). They were applied in a 1:200 dilution for Western blot and *FACS* analysis and in a 1:50 dilution for confocal microscopy. When performing immunoprecipitation or co-immunoprecipitation experiments 10 µl of affinity-purified anti-GFP antibodies (Pineda Antibodies, basic elution, (Engling et al., 2002) or affinity-purified anti FGF2 antibodies (Pineda Antibodies, acidic elution) were used per reaction. To analyze

DHFR containing reporter constructs, monoclonal anti DHFR antibodies (Becton Dickinson) were applied in a 1:250 dilution for Western blot analysis. To probe for reporter proteins containing a His_6-tag, Penta-His antibodies (Qiagen) were used. They were applied in a 1:200 dilution in Western blot, *FACS* and confocal microscopy analysis. To detect mouse IgG2α containing reporter constructs, goat anti mouse IgG Allophycocyanin-coupled antibodies (Molecular Probes) were used in a 1:500 dilution for *FACS* analysis. For Western blot analysis goat anti mouse IgG Alexa 680-coupled antibodies (Molecular Probes) were applied in a 1:1000 dilution.

Secondary antibodies:

Secondary antibodies for Western blot analysis were goat anti rabbit IgG HRP-coupled antibodies (Bio-Rad), goat anti mouse IgG HRP-coupled antibodies (Bio-Rad), monoclonal mouse anti rabbit IgG clone RG-16 HRP-coupled antibodies (Sigma-Aldrich), goat anti rabbit IgG Alexa 680-coupled antibodies (Molecular Probes) and goat anti mouse IgG Alexa 680-coupled antibodies (Molecular Probes). HRP-coupled antibodies, except RG-16, were applied in a 1:5000 dilution and Alexa 680-coupled antibodies in a 1:10000 dilution. RG-16 HRP-coupled antibodies were diluted 1:3000. Secondary antibodies for *FACS* analysis were goat anti rabbit IgG and goat anti mouse IgG antibodies, both conjugated with Allophycocyanin (Molecular Probes). They were used in a 1:750 dilution. Secondary antibodies for confocal microscopy were goat anti rabbit or goat anti mouse IgG Alexa 546-coupled antibodies (Molecular Probes). They were applied in a 1:1000 dilution.

2.2 Molecular Biological Methods

2.2.1 Bacterial Transformation

To transform DH5α cells, 1 µl of plasmid DNA (1-10 ng DNA) or 5 µl of a ligation reaction were added to 30 µl bacteria and incubated on ice for 30 min followed by a heat shock of 20 s at 37°C and an additional incubation period of 2 min on ice. After that 1 ml LB medium without antibiotics was added followed by incubation at 37°C for 1 h under constant shaking (300 rpm).

To transform XL1-Blue cells, 1 µl of plasmid DNA (1-10 ng DNA) or 5 µl of a ligation reaction were added to 50 µl bacteria and incubated on ice for 30 min followed by a heat shock of 45 s at 42°C and an additional incubation period of 2 min on ice. After that 1 ml LB medium without antibiotics was added followed by incubation at 37°C for 1 h under constant shaking (300 rpm).
Bacteria were then spread on LB plates supplemented with 100 µg/ml ampicillin or kanamycin and incubated at 37 °C for 12 to 16 h or used to inoculate liquid cultures of LB medium supplemented with the respective antibiotic at 37°C for 12 to 16h under constant skaking (180 rpm).

2.2.2 Selection and Amplification of Plasmids

If bacteria were grown on agar plate in correct density, they form colonies each originating from a single bacterium. To obtain genetically identical plasmids, bacteria from one colony were transferred to 5-10 ml LB medium using a 20 µl pipet tip. The liquid cultures which contain the respective antibiotic to select for bacteria carrying the plasmid with the resistance gene were incubated at 37°C for 12 to 16h under constant shaking (180 rpm).

2.2.3 Plasmid Preparation

Plasmids were prepared from overnight LB medium cultures of transformed bacteria by the application of Qiagen or Macherey & Nagel Plasmid DNA purification kits. The kit used is dependent on the volume of the overnight culture.

Culture Volume	Qiagen Kit	Macherey & Nagel Kit
5 - 10 ml	QIAprep Spin Miniprep Kit	Nucleospin Plasmid
20 - 50 ml	QIAGEN Plasmid Midi Kit	Nucleobond-PC 100
More than 150 ml	QIAGEN Plasmid Maxi Kit	Nucleobond-PC 500

Purification was performed following the manufacturer's manual employing alkaline lysis and binding of DNA to silica membranes or anion-exchange resins, respectively. Elution of the DNA was performed using appropriate volumes of ddH_2O.

2.2.4 Determination of DNA Concentration

The concentration of a DNA solution was determined photometrically by measuring the absorption at 260 nm wavelength. The measurement was either performed in a photometer with a diluted DNA solution using a quarz cuvette with a thickness of 10 mm or by directly measurement 1 µl of the DNA solution in a Nanodrop photometer. The concentration of double stranded DNA was calculated based on the fact that an optical density (OD) of 1 corresponds to a concentration of 50 µg/ml.
To determine contamination the OD at 280 nm was measured additionally. The ratio OD_{260} / OD_{280} represents the grade of purity since pure DNA shows a value between 1.8 and 2.0. Values above 2.0 show contamination with RNA, values below 1.8 contamination with protein.

2.2.5 Agarose Gel Electrophoresis

To separate mixtures of DNA molecules by size, agarose gel electrophoresis was used. Separation was achieved by subjecting the negatively charged DNA molecules after loading on a gel matrix with a defined pore size to an electric field where they migrate to the anode. The migration speed depends on the size of the DNA molecules and is limited by the pore size of the gel which was controlled by the amount of agarose used.
Agarose gels were prepared by heating 1% agarose (w/v) in TAE buffer. After the agarose was dissolved, ethidiumbromide in a final concentration of 0.5 µg/ml was added. The gel was poured into an agarose gel casting chamber and a plastic comb was inserted which forms the loading wells. After hardening the gel can be stored at 4°C until use up to two weeks.
To perform electrophoresis the gel was transferred into an agarose gel running chamber (Wide Mini-Sub Cell GT, Bio-Rad) and TAE was added until the gel is completely covered with liquid. Samples containing DNA sample buffer in a 1:5 dilution were loaded on the gel and electrophoresis is performed at 100 V until sufficient separation was reached as visualized by the migration behaviour of the blue bromphenol marker front. Agarose gels were documented using the Gel Doc 2000 imaging system (Bio-Rad).

TAE buffer (50x):	2 M	Tris
	1 M	Glacial acidic acid
	50 mM	EDTA, pH 8
		ddH_2O
DNA sample buffer (5x):	0.25%	Bromphenol blue (w/v)
	0.25%	Xylencyanol FF (w/v)
	30%	Glycerol (w/v)
		ddH_2O

2.2.6 DNA Marker

As a size standard two premixed DNA ladders were used, the 1 kB DNA ladder and the 100 bp DNA ladder (New England Biolabs). They contain DNA fragments of defined sizes ranging from 100 to 1500 bp (100 bp ladder) to analyze smaller DNA fragments or from 500 to 10.000 bp (1 kb ladder) to analyze large inserts and vectors. The markers were applied by loading 10 µl of a stock solution containing 0.05 µg/µl DNA in DNA sample buffer. Since each band of the marker contains a defined amount of DNA, the marker can be used to approximate the mass of DNA of an unknown sample by comparing band intensities visually.

2.2.7 Polymerase Chain Reaction

To amplify a gene or DNA fragment the polymerase chain reaction (PCR) was used (Lawyer et al., 1989; Saiki et al., 1988). During PCR a DNA template defined by a forward and reverse primer is amplified and can be used for further cloning to generate desired reporter constructs. PCRs were performed with the enzyme AmpliTaq polymerase (Perkin Elmer) which generates adenosine overhangs at the 3’-end. This is important when using the pGEM-T vector system for further cloning since the vector contains thymidine overhangs at its 3’-end for simplified ligation of PCR products. The following reaction mix was used for PCRs.

50 ng	template DNA
25 pmol	forward primer
25 pmol	reverse primer
2.5 units	Taq polymerase
10 mM	dNTPs
25 mM	$MgCl_2$
1x	PCR buffer II
	ddH_2O

The reaction was performed employing a Primus Advance Thermocycler (PeqLab). The following program was used to amplify template DNA.

Denaturation	2 min, 95°C		
Amplification	45 s, 94°C	Denaturation	30 cycles
	1 min, $T<T_m$ of primers	Hybridization	
	1 min, 72°C	Elongation	
Elongation	10 min, 72°C		
Store	∞, 4°C		

The annealing temperature was chosen depending on the melting temperatures (T_m) of the primers used. 5°C were subtracted from the lowest T_m of all primers used ($T = T_m - 5°C$). The melting temperature for each individual primer was calculated according to the following equation.

$$T_m = 81.5 + 16.6 \times \log\left[Na^+\right] + 41 \times \%\,GC - \frac{675}{N}$$

$[Na^+]$ = 0.05 M; %GC = GC content of annealing sequence; N = number of annealing basepairs

When problematic primers were used which result in very low yields or lead to no amplification at all, up to 10% DMSO (dimethyl sulfoxide) were added to the reaction mix. DMSO reduces secondary structures like loops or hairpins and the primers can anneal more easily at the template. A disadvantage of DMSO is, that mutations and mispairing of bases occur more frequently. When using DMSO, it is of great importance, to sequence obtained PCR products, to verify the correct sequence before using them for further cloning.

2.2.8 PCR Purification

To purify PCR products and remove primers and reaction mix components, the samples were processed using a PCR purification kit (QiaQuick PCR purification kit, Qiagen). DNA is bound to a silica membrane under high salt conditions and eluted after washing with an appropriate volume of ddH_2O.

2.2.9 Gel Extraction of DNA Fragments

To purify desired DNA fragments after restriction digests, the reaction mix was separated on a 1% agarose gel. The bands were transiently visualized with a UV lamp (366 nm) and cut out of the gel with a sharp blade. To purify the DNA from the agarose gel the samples were processed using a gel extraction kit (Qiagen). The agarose was melted in a specific buffer and DNA is bound to a silica membrane under high salt conditions. Following a washing step Elution was performed in an appropriate volume of ddH_2O.

2.2.10 Restriction Digests

Restriction enzymes were purchased from New England Biolab. Restriction digests were performed according to the manufacturer's manual. An optimized buffer system consisting of buffer 1 to 4 of which one is optimal for a specific enzyme was used. Additionally, unique buffers for certain enzymes are available. In the case of a double digest a buffer was chosen which provides the highest cleavage efficiency for both enzymes or the digest was performed sequentially with a DNA purification step in between. Depending on the enzyme and the quality of the DNA 1 to 5 units/µg DNA were used in the restriction digest incubated 2 to 4 h at 37°C.

2.2.11 DNA Dephosphorylation

To dephosphorylate linearized vectors at the 5'-end after restriction digests in order to prevent self-ligation *Calf Intestinal Phosphatase* (CIP, New England Biolabs) was added to the reaction mix in a concentration of 1 u/µg DNA for 30 min at 37°C. The enzyme was heat inactivated by incubation at 70°C for 10 min.

2.2.12 Ligation of DNA Fragments

In order to ligate PCR products or other DNA fragments to each other or into linearized vectors a ligation kit was used (Ver. 2.1, Takara Bio Inc.). The ligation partners were digested with the same restriction enzymes to provide compatible ends. Following the manual, 50 ng of vector (or the longer DNA fragment) were used. The amount of insert (or the shorter DNA fragment) was calculated according to the following equation.

$$\frac{\text{amount vector}\,[\text{ng}] \times \text{number of basepairs insert}\,[\text{bp}]}{\text{number of basepairs vector}\,[\text{bp}]} = \text{amount insert}\,[\text{ng}]$$

The DNA solutions and 5 µl of Takara Solution 1 which contains the T4 DNA ligase and an optimized buffer in a 2 fold concentration were mixed and ddH_2O was added to a total volume of 10 µl. The reaction was incubated for 2 h at room temperature or for at least 16 h at 4°C. After the incubation period the enzyme was heat inactivated by incubation at 70°C for 10 min.

2.2.13 DNA Sequencing

Cloned inserts or cDNA constructs in different plasmids were sequenced in order to rule out mutations and to verify the correct sequence. Therefore, plasmid samples and primers were sent to commercial sequencing companies (Seqlab, Göttingen or GATC, Konstanz). Obtained sequences were analyzed using the Lasergene software suite (Lasergene, DNAStar) or the 'align 2 sequences' function on the BLAST project in the world wide web (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

2.2.14 Cloning Strategy: cDNA Constructs of the DHFR Approach

To generate the cDNA constructs FGF2-GFP-DHFR and MTS-GFP-DHFR the plasmid pQE16 (Qiagen) was used as a template to clone the DHFR moiety. pQE16 contains the open reading frame (ORF) of murine DHFR with a mutation of wildtype cysteine at position 7 to serine by exchange of two basepairs at the respective position. To reverse the mutation a forward primer was designed which contains the nucleotide sequence coding for a cysteine residue at position 7 (see section 2.1.4, primer: DHFR-Cystein-*BsrGI*-for). The construct MTS-GFP-DHFR was generated according to the cloning strategy depicted in Fig. 2.1.

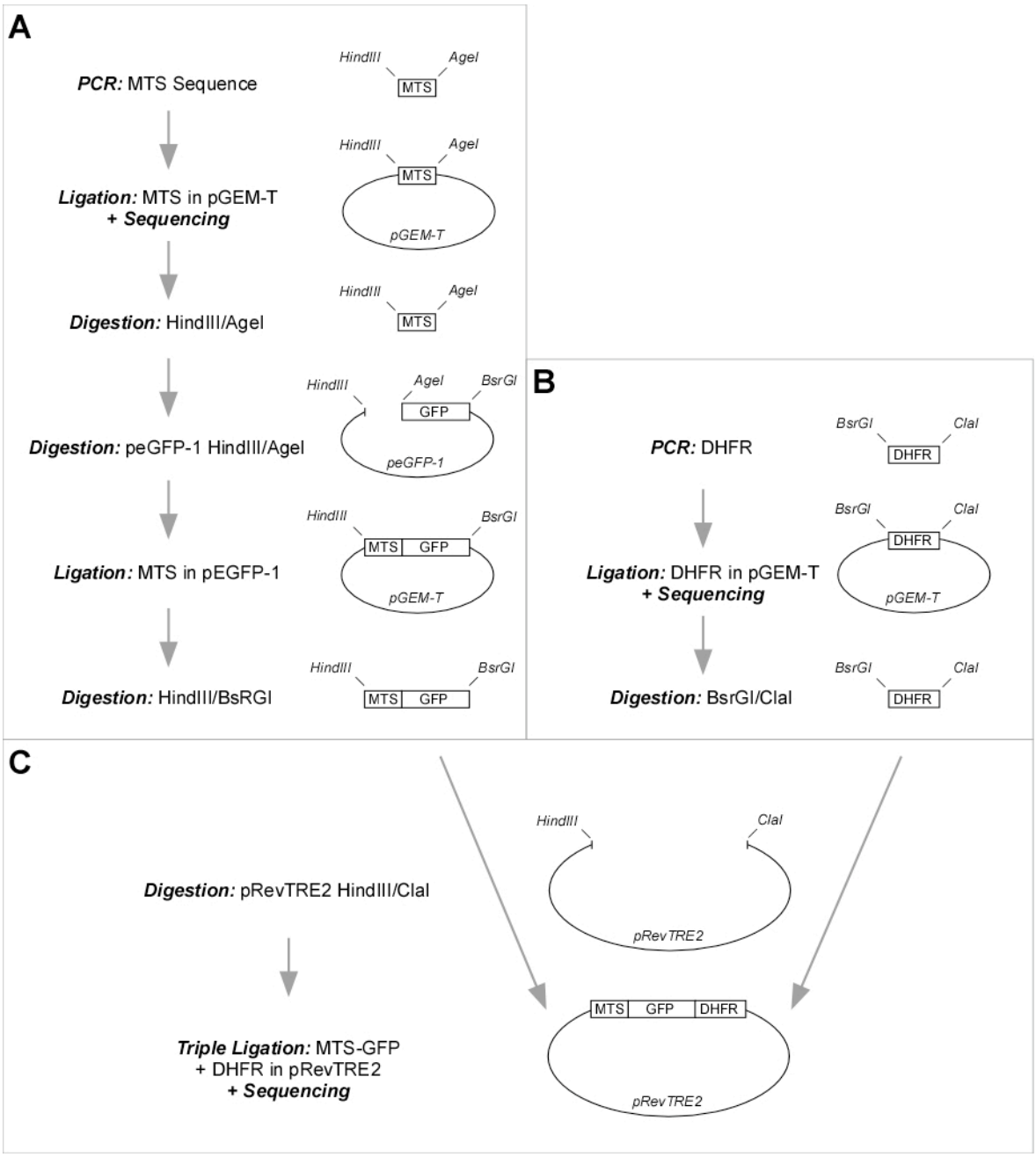

Fig. 2.1 Schematic overview: cloning strategy DHFR constructs.

MTS = mitochondrial targeting sequence from the S9 subunit of the F_0-ATPase (*Neurospora crassa*), GFP = enhanced green fluorescent protein (*Aequoria victoria*), DHFR = dihydrofolate reductase (*Mus musculus*)

As a first step a PCR was performed using the mitochondrial targeting sequence (MTS) of the S9 subunit of the mitochondrial F_0 ATPase in the plasmid pYES-mtGFP-1 as a template. The PCR product was purified and subcloned into the pGEM-T vector. After bacterial transformation and amplification of positive clones, the insert was sequenced and finally excised using the restriction enzymes *HindIII* and *AgeI*. The restriction sites for this digestion step were artificially introduced by the primers

used in the PCR (see section 2.1.4, primers: mtpre *HindIII* for, mtpre *AgeI* rev). In parallel the vector peGFP-1 (Clontech) which contains enhanced *green fluorescent protein* (GFP) was linearized using the same enzymes. After purification of the vector and the MTS insert a ligation was performed generating the construct MTS-GFP which is flanked 5' by a *HindIII* and 3' by a *BsrGI* restriction site. Sequencing was performed to verify correct ligation and to rule out frame shifts. Digestion with *HindIII* and *BsrGI* excised the insert MTS-GFP which was used for later cloning steps (Fig. 2.1 A). The ORF of DHFR was amplified by PCR using the pQE16 vector (Qiagen) as a template. As described above, the serine at position 7 was mutated to a cysteine to restore wildtype conditions. The PCR product was subcloned into the pGEM-T vector and sequenced. Following amplification and purification of correct clones a digestion with the restriction enzymes *BsrGI* and *ClaI* was performed to generate the DHFR insert (Fig.2.1 B). The obtained inserts, MTS-GFP and DHFR, were ligated into the retroviral transduction vector pRevTRE2 in a triple ligation reaction. The vector was linearized with *HindIII* and *ClaI* to provide sticky ends which guarantee ligation of the inserts in the right sequence and orientation due to the used restriction sites. After amplification and purification, the insert was checked by digestion using *HindIII* and *ClaI*. The digestion reactions were analyzed by agarose gel electrophoresis followed by sequencing of positive clones to verify successful insertion (Fig. 2.1 C).

To generate the construct FGF2-GFP-DHFR a similar cloning strategy was applied. Due to the availability in our laboratory it was not necessary to generate the insert FGF2-GFP. The plasmid pRevTRE2-FGF2-GFP was digested with *NotI* and *BsrGI* to obtain the insert FGF2-GFP. The vector pRevTRE2 was digested using *NotI* and *ClaI*. Then the inserts FGF2-GFP and DHFR, which was digested with *BsrGI* and *ClaI* as described above (Fig. 2.1 B), were ligated into pRevTRE2. After amplification and purification the construct was analyzed by digestion with *NotI* and *ClaI*. Digestion reactions were analyzed by agarose gel electrophoresis followed by sequencing of positive clones.

Both constructs, MTS-GFP-DHFR and FGF2-GFP-DHFR in pRevTRE2, were used for retroviral transduction of $CHO_{MCAT\text{-}TAM2}$ cells (see section 2.3.4) to generate cell lines expressing the reporter molecules.

2.2.15 Cloning Strategy: cDNA Constructs of the Piggyback Approach

To generate the cDNA constructs used for the piggyback approach a three step cloning strategy was applied. Each construct consists of two ORFs enconding distinct proteins expressed from a bicistronic expression vector termed pRTi. pRTi is a derivative of the retroviral transduction vector pRevTRE2, which was modified by insertion of an internal ribosome entry site (IRES) into the existing multiple cloning site (MCS). The result is a bicistronic expression vector with two MCS separated by the IRES element. To be able to use the restriction enzymes required for the cloning strategy the MCS upstream of the IRES was further extended with additional restriction sites.

The strategy to generate the cDNA constructs consists of three steps. In a first step the construct inserted upstream of the IRES element was generated. Second, the construct inserted downstream of the IRES element was produced. In the final step the two inserts were ligated sequentially into the pRTi vector. A schematic overview is presented in the following figure (Fig. 2.2).

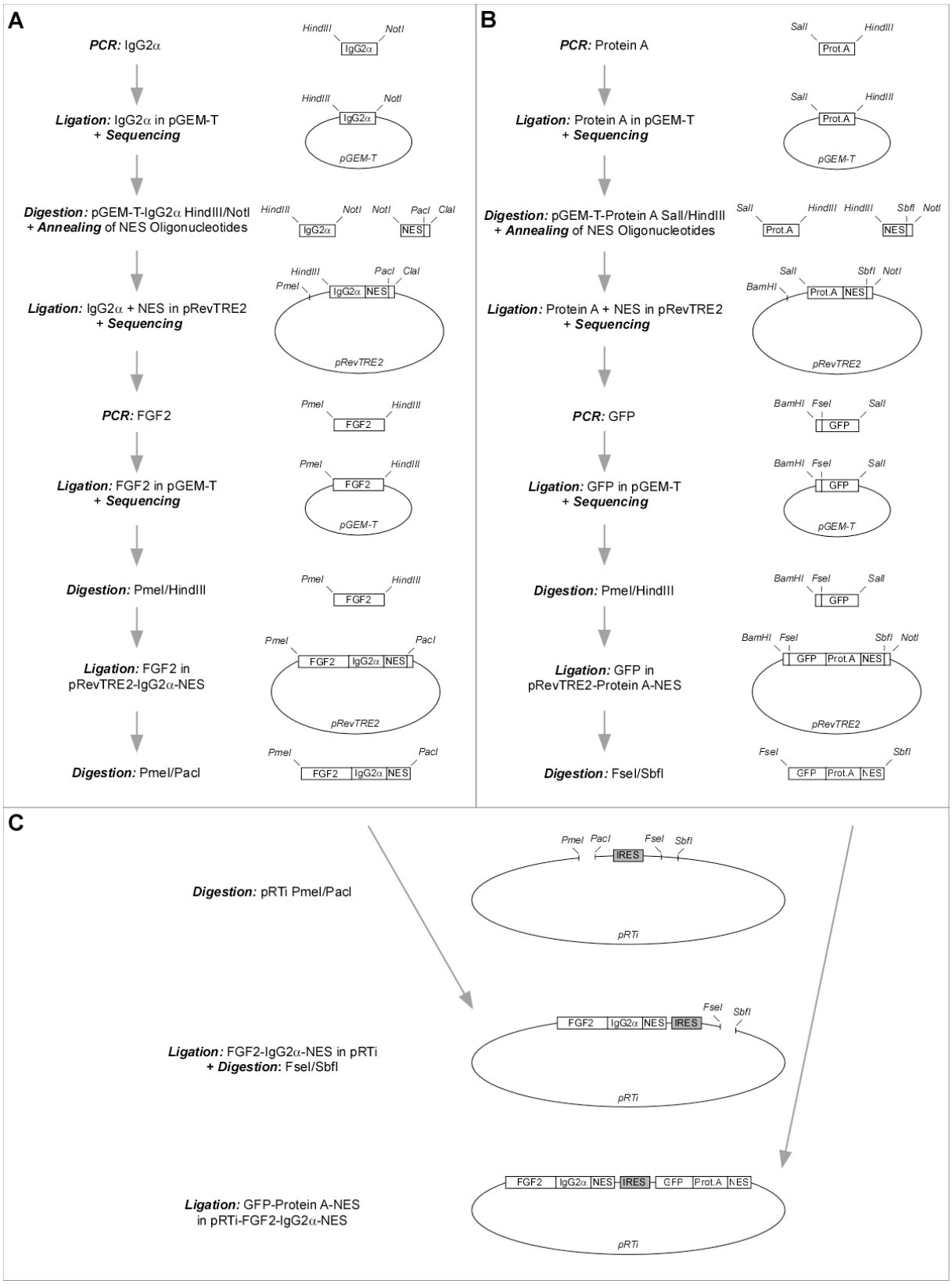

Fig. 2.2 Schematic overview: cloning strategy piggyback constructs.

IgG2α = F_c domain of immunoglobulin 2α (*Mus musculus*), Protein A = Protein A (*Staphylococcus aureus*), NES = nuclear export signal from mitogen activated protein kinase kinase 1 (*Rattus norvegicus*), FGF2 = fibroblast growth factor 2 (*Homo sapiens*), GFP = green fluorescent protein (*Aequria victoria*), IRES = internal ribosome entry site

To generate the construct inserted upstream of the IRES element, namely FGF2-IgG2α-NES, a PCR was performed to amplify the ORF of the mouse immunoglobulin 2α (IgG2α) from the plasmid 173.174.IgG2apBSII. Two artificial restriction sites, *HindIII* and *NotI*, were introduced by the primers used (see section 2.1.4, L-IgG2a-for and L-IgG2a-rev). The product was purified and ligated into the pGEM-T vector. After bacterial transformation, selection of clones, multiplication in bacteria and plasmid purification the insert was sequenced to verify correct amplification during PCR. Then a digestion was performed using the restriction enzymes *HindIII* and *NotI* and the excised insert was purified applying gel extraction. In parallel the synthetic oligonucleotides coding for a nuclear export signal (NES) were annealed (see section 2.1.4, L-LuxA-NES-for, L-LuxA-NES-rev). They were designed to have a *NotI* restriction site at the 5'-end, as well as a *PacI* followed by a *ClaI* restriction site at the 3'-end. After annealing the overhangs form sticky ends which allow direct ligation without digestion. The IgG2α insert and the NES oligonucleotide were ligated into the pRevTRE2 vector which was digested using *HindIII* and *ClaI*. After bacterial transformation, selection of clones, amplification in bacteria and plasmid purification the insert was sequenced to verify correct ligation and exclude frame shifts. In a next step a PCR was performed amplifying the ORF of human FGF2 from the plasmid pET15b-FGF2 and introducing artificial *PmeI* and *HindIII* restriction sites (for primers see section 2.1.4, L-FGF2-for and L-FGF2-rev). The PCR product was purified by gel extraction and ligated into pGEM-T. After transformation of bacteria clones were selected and propagated. The plasmids were purified and sequenced to check for correctly amplified inserts. In the next step the FGF2 insert was excised using *PmeI* and *HindIII* and ligated into pRevTRE2-IgG2α-NES. The plasmid was digested using the same enzymes due to the existence of a *PmeI* site upstream of the *HindIII* site in the MCS which was used before to insert IgG2α-NES. Therefore, it was then possible to link the two inserts into one continuous ORF. Following ligation, bacteria were transformed, clones were selected and propagated, plasmids were purified and the insert was sequenced. The correct insert was excised using *PmeI* and *PacI* and used for the next cloning step. A detailed overview of the procedure is depicted in Fig. 2.2 A.

To generate the construct GFP-Protein A-NES which was inserted downstream of the IRES element the same cloning strategy was applied. In a first step, the ORF of protein A from the plasmid pGEM-T-TAP-Tag was amplified via PCR and artificial

SalI and *HindIII* restriction sites were introduced (for primers see section 2.1.4, L-ProtA-for and L-ProtA-rev). The PCR product was subcloned into pGEM-T, sequenced and excised using *SalI* and *HindIII*. The Protein A insert was ligated into pRevTRE2 digested with *SalI* and *NotI*. Thereby, it was linked in a triple ligation with NES oligonucleotides which were designed with a *HindIII* restriction site at the 5'-end and a *SbfI* followed by a *NotI* restriction site at the 3'-end (for oligonucleotides ee section 2.1.4, L-LuxB-for and L-LuxB-rev). After ligation a correctly ligated clone was chosen and verified by sequencing. To insert the GFP moiety, a PCR was performed using the plasmid peGFP-1 as a template to generate the GFP insert which was subcloned into pGEM-T and sequenced. The primers used were designed to introduce a *BamHI* followed by a *FseI* restriction site at the 5'-end and a *SalI* restriction site at the 3'-end of the ORF (for primers see section 2.1.4, L-GFP-for and L-GFP-rev). After digesting the previously generated Plasmid pRevTRE2-Protein A-NES with *BamHI* and *SalI* the GFP insert was ligated into the linearized vector to form the construct GFP-Protein A-NES. It was subsequently excised using *FseI* and *SbfI* and used for following cloning steps (Fig. 2.2 B).

In the final step the pRTi vector was digested with *PmeI* and *PacI* which are located in the MCS upstream of the IRES element and the insert FGF2-IgG2α-NES which was excised using the same enzymes was ligated into the linerarized vector. The ligation products were used to transform bacteria and clones were selected. After verifying successfully ligated clones, was propagated and a digestion was performed using *FseI* and *SbfI*. These enzymes cut in the MCS downstream of the IRES element and were also used to excise the insert GFP-Protein A-NES. Subsequently, the insert was ligated into the vector and bacteria were transformed again and clones were chosen. After verification of correct ligation one final clone was chosen which contains FGF2-Ig2α-NES upstream and GFP-Protein A-NES downstream of the IRES element (Fig. 2.2 C).

To generate the cDNA constructs consisting of FGF1 or FGF4 with a signal sequence (FGF4-S) the same cloning strategy was applied using FGF1 in the plasmid pET24-FGF1 or FGF4-S in the plasmid pET24-FGF4-S as templates for the PCR instead of FGF2 (Fig. 2.2 A). To produce the construct IgG2α without FGF moiety the insert IgG2α-NES was excised directly from pRevTRE2 using *PmeI* and *PacI*. The *PmeI* restriction site is present in the MCS upstream of the insert and the *PacI* restriction site at the 3'-end of the insert introduced via PCR (see Fig. 2.2 A). All

constructs were used for retroviral transduction of $CHO_{MCAT-TAM2}$ cells (see section 2.3.4) to generate cell lines expressing the reporter molecules.

2.3 Eukaryotic Cell Culture Techniques

2.3.1 Maintaining Cell Lines

Adherent cell lines were grown on culture dishes in their respective culture medium at 37°C with 5% CO_2. The cells were splitted dependent on confluency every 3 to 5 days by washing with PBS and addition of 0.125% trypsin/EDTA in PBS (v/v). After 1 min incubation the trypsin solution was removed and the cells were resuspended in the appropriate volume of medium used for the culture dish. Cells were then seeded in the desired dilution on new culture dishes prepared with fresh medium.

PBS (Phosphate buffer saline):	140 mM	NaCl
	2.7 mM	KCl
	10 mM	Na_2HPO_4
	1.8 mM	KH_2PO_4
		ddH_2O
Trypsin / EDTA:	0.125%	Trypsin (w/v)
	0.5 mM	EDTA
		PBS

2.3.2 Freezing of Eukaryotic Cells

To prepare frozen stocks for long term storage cells grown to about 100% confluency were washed once with PBS and trypsinized. Then the cells were resuspended in normal growth medium, transferred to a 15 ml tube and collected by centrifugation (200 g, 5 min, 4°C). The pellet was carefully resuspended in 2 ml freeze medium and transferred to 2 ml cryo-vials (Greiner). An alternative procedure is to resuspend the cells directly in freeze medium after trypsinization. The cryo-vials were frozen at

-80°C in special cryo boxes which ensure a temperature decrease of 1°C per minute. For long term storage the deep frozen cryo-vials were transferred to liquid nitrogen cell storage tanks.

Freeze Medium:	20%	FCS (v/v)
	10%	DMSO (v/v)
	100 µg/ml	Streptomycin / Penicillin
		αMEM or DMEM

2.3.3 Thawing of Eukaryotic Cells

To defreeze cells cryo-vials were removed from liquid nitrogen and immediately thawed in a water bath at 37°C. The content was transferred to 20 ml fresh, prewarmed culture medium in a 50 ml tube and cells were sedimented by centrifugation (200 g, 5 min, 4°C). To remove DMSO the medium was discarded and the cell pellet was resupended in fresh culture medium. The cells were then seeded on culture dishes of the same size they were taken from to prepare the frozen stocks and incubated at 37°C with 5% CO_2.

2.3.4 Retroviral Transduction

To stably integrate reporter genes into the genome of target cells a MBS Mammalian Transfection Kit (Stratagene) was used following the instructions of the manufacturer's manual. The procedure takes 5 days and consists of preparation of plasmids coding for virus components and the reporter gene, production of retroviral particles using HEK293T host cells, harvesting of retroviral particles and infection of target cells. The target cell line $CHO_{MCAT\text{-}TAM2}$ (Engling et al., 2002) expresses the murine cationic amino acid transporter MCAT-1 (Albritton et al., 1989; Davey et al., 1997) on the cell surface which is recognized by the virus and mediates docking and uptake. Additionally, the doxicycline-sensitive transactivator rtTA2-M2 (Urlinger et al., 2000) is constitutively expressed and allows protein expression in a doxicycline-dependent manner. The cDNA reporter constructs were cloned into the retroviral transduction vector pRevTRE2 or its derivative the vector pRTi which allows the

simultaneous expression of two proteins from a single, bicistronic mRNA. Both vectors contain a doxicycline-responsive element (TRE) which mediates doxicycline-dependent protein expression. As a transduction control hrGFP in the vector pFB was used which is constitutively expressed after successful infection. The plasmid containing the reporter construct was mixed with two other plasmids, pVPack GP and pVPack eco, encoding the viral gag-pol elements (GP) and the viral envelope protein (Eco).

On Day 1 DNA was precipitated using ethanol and stored at 4°C. Virus producing HEK293T cells were seeded on freshly prepared culture dishes to be used for transfection the next day. On day 2 HEK293T cells were transfected with the three plasmids prepared the day before following the manual of the MBS mammalian transfection kit (Stratagene) and incubated for 72 h at 37°C to produce retroviral particles. On day 3 $CHO_{MCAT\text{-}TAM2}$ cells were seeded on culture dishes in the desired dilution to be used for transduction 48 h later. On day 5 virus particle-containing medium was harvested from transfected HEK293T cells and passed through a sterile 45 µm filter. Subsequently, this medium was transferred to target cells and transduction occurs by virus mediated gene transfer leading to stable integration of the cDNA reporter constructs into the target cell genome. Normal growth medium was added and cells were incubated for two days with retroviral particles. Cells were further analyzed using flow cytometry and transduction efficiency was measured by counting GFP positive cells transduced with pFB-hrGFP.

Vectors:	pVPack GP	Stratagene
	pVPack Eco	Stratagene
	pFB-hrGFP	Clontech, derived from Moloney Murine Leukemia Virus (MMLV)
	pRevTRE2	Clontech, derived from MMLV, contains tet-response element (TRE)
	pRTi	modified pRevTRE2 vector kindly provided by Jörg Mölleken and further modified during this study

2.3.5 Addition of Doxicycline

Doxicycline (Clontech) was added to the culture medium of different reporter cell lines to induce protein expression by the tetracycline/doxicycline-responsive element. A stock solution of 1 mg/ml in PBS was diluted 1:1000 in culture medium to achieve a final concentration of 1 µg/ml. The incubation period varied dependent on the experimental conditions from 16 to 24 h.

2.3.6 Addition of Aminopterin

Aminopterin (Sigma-Aldrich Chemie GmbH), a substrate analogon of DHFR, was used in experiments analyzing different DHFR fusion constructs. It was added in a concentration of 50 µM to the culture medium by dilution of a 50 mM stock solution 1:1000. Addition was performed simultaneously with doxicycline incubation because the aminopterin-responsive reporter constructs are expressed in a doxicycline-dependent manner. The time period of aminopterin incubation varied from 16 to 24 h.

2.4 Biochemical Methods

2.4.1 Preparation of Cell Lysates

Cells grown on culture dishes were detached using PBS/EDTA and sedimented by centrifugation (200 g, 4°C, 5 min). The pellet was washed once by careful resuspension in PBS followed by centrifugation. After that cells were resuspended in an appropriate volume of SDS sample buffer and processed for SDS-PAGE analysis.
If defined amounts of protein were needed the pellet was subjected to detergent-based lysis followed by determination of total protein concentration. Therefore, lysis was performed by resuspension of the pellet in PBS supplemented with 1% Triton X-100 (w/v) followed by sonication in a water bath for 3 min. To solubilize membrane proteins samples were incubated at room temperature for 15 min combined with vortex-mixing every 3 min. Insoluble material was sedimented by centrifugation

(16.000g, 4°C, 10 min). The supernatant was used to determine total protein concentration and for SDS-PAGE analysis.

PBS/EDTA:	0.5 mM	EDTA
		PBS

2.4.2 Preparation of Cell Free Supernatants

To prepare cell free supernatants without detergent cells were grown to a confluency of about 80%. The cell monolayer was washed three times with PBS followed by detachment of cells using PBS/EDTA. Detached cells were carefully resuspended and collected by centrifugation (200 g, 4°C, 5 min). The pellet was resuspended in PBS containing protease inhibitors (Protease Inhibitor Cocktail Tablets, Complete Mini, Roche, 1 tablet / 10 ml) and subjected to two freeze-thaw-cycles in liquid nitrogen. The cells were further disrupted by sonication using a sonication tip for initially 15 s and then 10 s (Pulse 50%) in an ice water bath. Membranes were removed by a two step centrifugation procedure first sedimenting insoluble cell fragments (200 g, 4°C, 10 min) and second performing an ultracentrifugation (100.000 g, 4°C, 1h). The supernatant was used for further analysis.

2.4.3 Determination of Protein Concentration

The concentration of a protein solution was determined employing the BCA Protein Assay Kit (Pierce). It uses a combination of the biuret reaction (reduction of Cu^{2+}-cations to Cu^{1+}-cations by proteins in an alkaline medium) and the colorimetric detection of the Cu^{1+}-cations by a bicinchoninic acid-containing color reagent.
10 µl of protein sample were added to 490 µl ddH_2O (dilution 1:50). In parallel a dilution series of a 0.5 µg/µl BSA stock solution in ddH_2O was prepared to be used as a standard in later evaluation. Applied amounts were 0, 1.25, 2.5, 3.75, 5, 7.5 and 10 µg BSA in 500 µl ddH_2O. 500 µl of a mixture of kit reagent A and B (ratio 50:1) were added to each sample followed by an incubation at 60°C for 30 min. After another incubation at room temperature for 10 min 150 µl of each reaction were transferred into a 96 well plate. The read out was performed using an Anthos 2001 Microplate

Photometer. Protein concentrations of the unknown samples were calculated by multiplication of the obtained values with a factor of 50 since the samples were diluted 1:50 at the beginning of the procedure.

2.4.4 Sample Preparation for SDS Polyacrylamide Gel Electrophoresis

SDS sample buffer (4x) was added to samples in a ratio of 1:4 followed by an incubation at 95°C for 5 min. Before loading the samples onto the gel a centrifugation step was performed (16,000 g, 4°C, 1 min) to collect all liquid at the bottom of the reaction tube. In the case of cell lysates directly prepared in SDS sample buffer, insoluble DNA aggregates were sedimented (16.000g, 4°C, 10 min) and the supernatant was applied to the gel.

SDS sample buffer (4x):	200 mM	Tris-HCl, pH 6.8
	25%	Glycerol (w/v)
	2%	SDS (w/v)
	0.2%	Bromphenol blue (w/v)
	0.7 M	β-Mercaptoethanol
		ddH_2O

2.4.5 SDS Polyacrylamide Gel Electrophoresis

To separate proteins which were denatured by sodium dodecyl sulfate (SDS) according to their molecular mass SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (Laemmli, 1970) using the Mini PROTEAN III Electrophoresis System (Bio-Rad).

Gels with dimensions of 80 x 73 mm and a thickness of 0.75 mm were casted between to glass plates by pouring freshly prepared separating gel solution containing 10% or 13% acrylamide into the gel cassette fixed in a casting frame. Unpolymerized separating gel solution was overlayed with isopropanol to achieve an even surface. After polymerization isopropanol was poured off and remains were removed using filter paper (Whatman 3MM, Whatman AG). Then unpolymerized stacking gel solution was poured into the gel cassette and a plastic comb was

inserted from the top which forms the loading wells in the stacking gel. After polymerization gels can be stored at 4°C wrapped in wet paper towels up to 3 weeks. To perform electrophoresis the gel was placed into the electrode assembly device inside a clamping frame in the tank of the Mini PROTEAN III system. Electrophoresis running buffer was added to the inner and outer chamber of the tank and the plastic comb was carefully removed. Samples were loaded into the wells of the stacking gel using extralong loading pipet tips. Electrophoretic separation was performed at 200 V until the bromphenol blue front of the SDS sample buffer reached the end of the separating gel.

Separating Gel Solution:	10% Gel	13% Gel	
	2 ml	1.68 ml	ddH_2O
	1.25 ml	1.25 ml	1.5 M Tris-HCl, pH 8.8
	50 µl	50 µl	10% SDS (w/v)
	1.66 ml	2 ml	Acrylamide/Bis 30%(w/v)
	25 µl	25 µl	10% APS (w/v)
	2.5 µl	2.5 µl	Temed

Stacking Gel Solution:	4.8% Gel	
	1,53 ml	ddH_2O
	0,625 ml	0.5 M Tris-HCl, pH 6.8
	25 µl	10% SDS (w/v)
	335 µl	Acrylamide/Bis 30% (w/v)
	12.5 µl	10 % APS (w/v)
	2.5 µl	Temed

Electrophoresis running buffer:	25 mM	Tris-HCl, pH 8.3
	192 mM	Glycine
	0.1%	SDS (w/v)
		ddH_2O

2.4.6 SDS-PAGE Protein Molecular Weight Standards

As protein molecular weight standards either peqGOLD Protein-Marker I (Peqlab) or Odyssey Protein Molecular Weight Marker (LICOR) were used. peqGold marker, ranging from 14 to 116 kDa, as used when analyzing gels by Western blot using the ECL detection system. Odyssey marker, ranging from 10 to 250 kDa, was applied when performing the analysis using an *Odyssey infrared imaging system* since the marker proteins are prestained with Coomassie which is visualized by this system. Markers were applied by loading 1 to 5 µl of the premixed solutions.

2.4.7 Western Blot Transfer

To transfer proteins separated by SDS-PAGE to a polyvinylidene fluoride (PVDF) membrane for further analysis (Towbin et al., 1979) a wet blot transfer device was used (Mini Trans-Blot Cell, Bio-Rad). A PVDF membrane and two pieces of filter paper (Whatman 3MM, Whatman AG) were cut to the size of the separating gel. The PVDF membrane was activated by incubation in 100% methanol for 1 min followed by rinsing with ddH_2O. Filter paper, two sponges and a sandwich blotting cassette were equilibrated in blotting buffer. All parts were assembled as depicted in Fig. 2.3.

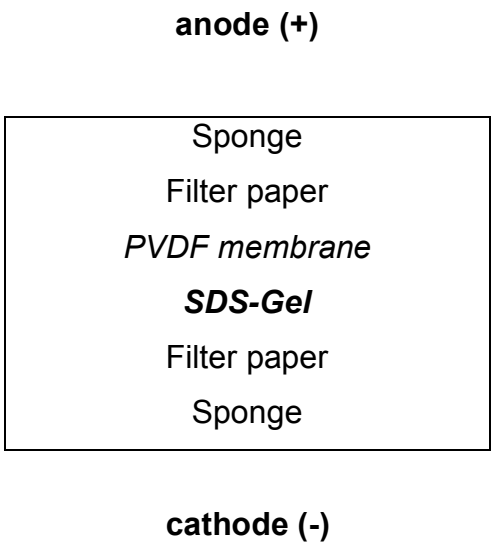

Fig. 2.3 Schematic overview: Assembly of a Western blot wet blot sandwich cassette.

The assembled blotting cassette was inserted into the transfer tank, an ice block for cooling was added and the tank was filled with blotting buffer. Protein transfer was performed at 100 V for 1 h with constant stirring.

Blotting buffer:	192 mM	Glycine
	25 mM	Tris, pH 8.4
	20%	MetOH (v/v)
		ddH_2O

2.4.7.1 Reversible Ponceau Staining of Proteins

To verify successful protein transfer PVDF membranes were reversibly stained with Ponceau S (Serva Electrophoresis GmbH). The PVDF membrane was incubated in Ponceau solution for 1 min. Subsequently, excessive Ponceau solution was washed away using ddH_2O until the proteins bands became clearly visible. Marker bands were labelled using a ball pen and the membrane was completely destained by incubating in PBS-T on a shaker. Ponceau staining was not performed if the LICOR detection system was used because this would increase background signals.

PBS-T:	0.05%	Tween 20 (w/v)
		PBS

Ponceau Solution:	0.25%	Ponceau S (w/v)
	3%	Trichloroacetic acid (v/v)
		ddH_2O

2.4.7.2 Immunochemical Protein Detection Using the ECL System

Western blotting was performed as described above using Immobilon-P PVDF membrane (Millipore Corporation). The membrane was incubated in blocking buffer for 1 h at room temperature or at 4°C overnight on a shaker. Following blocking, the membrane was rinsed with PBS-T and incubated with the primary antibody in the desired dilution for 1 h at room temperature on a shaker. Three 5 min washing steps

with PBS-T were performed and the membrane was incubated with secondary goat anti rabbit IgG or goat anti mouse IgG antibodies coupled to HRP in a 1:5000 dilution. If samples derived from an immunoprecipitation experiment (IP) were analyzed monoclonal anti-rabbit Immunoglobulin clone RG-16 HRP-coupled antibodies in a 1:5000 dilution were used to detect only native antibodies excluding those derived from the IP procedure. After three times 5 min washing with PBS-T min on a shaker, visualization was performed using the enhanced chemiluminescence-system (ECL, Amersham Pharmacia). The membrane was incubated with the ECL solution for 1 min at room temperature and chemiluminescence was detected using medical x-ray films (Super RX Medical X ray film, Fuji).

Blocking buffer:	5%	Milk powder (w/v)
		PBS-T
Primary antibody buffer:	3%	BSA (w/v)
	0.02%	Sodium azide (w/v)
		PBS-T
Secondary antibody buffer:	3%	Milk powder (w/v)
		PBS-T

2.4.7.3 Immunochemical Protein Detection Using the LICOR System

Western blotting was performed as described using Immobilon-FL PVDF membrane (Millipore Corporation) optimized for fluorescence detection. The membrane was incubated in blocking buffer for 1 h at room temperature on a shaker. Following blocking, the membrane was rinsed two times 5 min with PBS-T and incubated with the primary antibody in the desired dilution for 1 h at room temperature on a shaker. Four times 5 min washing with PBS-T was performed followed by incubation with secondary goat anti rabbit IgG or goat anti mouse IgG antibodies coupled to the fluorophor Alexa 680 diluted 1:10000 for 30 min at room temperature under constant shaking in the dark. Finally the membrane was washed four times for 5 min with PBS-T on a shaker and once with PBS without Tween 20. Visualization was performed using the *Odyssey infrared imaging system*.

Blocking buffer:	5%	Milk powder (w/v)
		PBS
Primary antibody buffer:	3%	BSA (w/v)
	0.02%	Sodium azide (w/v)
	0.1%	Tween 20 (w/v)
		PBS
Secondary antibody buffer:	3%	Milk powder (w/v)
	0.01%	SDS (w/v)
	0.1%	Tween 20 (w/v)
		PBS

2.4.8 Protease Protection Assay

To analyze the stability of the DHFR domain present in the reporter constructs FGF2-GFP-DHFR and MTS-GFP-DHFR a protease protection assay was performed. Cells expressing the reporter constructs were lysed by addition of a detergent-containing lysis buffer and scraped off the culture dishes using a rubber policeman. In order to complete lysis the cell suspension was incubated at 4°C for 30 min combined with mixing by pipetting every 5 min. Finally, insoluble material was removed by centrifugation (16.000g, 4°C, 10 min).

The supernatants were incubated for 15 min at room temperature in the absence or presence of 50 µM aminopterin added from a stock solution (2.5 mM aminopterin in lysis buffer) by diluting 1:50. Subsequently, trypsin in a final concentration of 200 µg/ml was added by direct dilution of a stock solution (10 mg/ml) in a ratio of 1:50 followed by an incubation for 30 min at 4°C. After the trypsin treatment protease inhibitors (Complete Mini, Roche) were added to stop the reaction and samples were mixed with SDS sample buffer in a 3:1 ratio for analysis by SDS-PAGE and Western blot.

Lysis buffer:	100 mM	Hepes-KOH, pH 7.4
	2 mM	$CaCl_2$
	0.2%	Triton X-100 (w/v)
		ddH_2O

Trypsin stock solution:	10 mg/ml	trypsin (w/v)
		Lysis buffer

2.4.9 Immunoprecipitation of Proteins

To immunoprecipitate GFP containing reporter molecules a mixture of Protein A-Sepahrose beads, CL-4B beads (Amersham Pharmacia) and 20% ethanol (1:1:2) was prepared and 40 µl slurry, corresponding to 20 µl pure beads, was used per sample. The beads were washed three times with IP-Buffer 1. Sedimentation was performed at 3000 g, 1 min, 4°C. To couple affinity-purified GFP antibodies to the beads they were incubated with 10 µl anti-GFP antibodies (basic elution) in 190 µl IP-Buffer 1 per 20 µl packed beads overnight at 4°C using end-over-end rotation. Following the coupling procedure, unspecific binding sites were quenched by two times washing with IP-Buffer 2 containing 1% BSA and a final washing step using IP-Buffer 1. After sedimentation and removal of the buffer the sample consisting of 1 ml culture medium obtained from the respective reporter cell line grown on 6 well-plates and 500 µl PBS Ca^{2+}/Mg^{2+} obtained from a washing step (see 2.5.12 Biotinylation) were added to the beads followed by an 2 to 4 h incubation at room temperature using end-over-end rotation. After this incubation the beads were washed three times with IP-Buffer 0. Bound material was eluted by addition of SDS sample buffer and incubation at 95°C for 5 min. Subsequently, samples were subjected to SDS-PAGE and Western blot analysis

IP-Buffer 0:	25 mM	Tris-HCl, pH 7.4
	150 mM	NaCl
	1 mM	EDTA
		ddH_2O

IP-Buffer 1:	25 mM	Tris-HCl, pH 7.4
	150 mM	NaCl
	1 mM	EDTA
	0.5%	NP-40 (w/v)
		ddH_2O

IP-Buffer 2:	25 mM	Tris-HCl, pH 7.4
	150 mM	NaCl
	1 mM	EDTA
	0.5%	NP-40 (w/v)
	1%	BSA
		ddH_2O

2.4.10 Co-Immunoprecipitation of Proteins

To co-precipitate proteins bound to GFP containing reporter molecules cell free supernatants were prepared as described in section 2.5.2. The samples were subjected to immunoprecipitation as described in section 2.5.9. Additionally, anti-FGF2 antibodies were coupled to Protein A-sepharose beads and NP-40-free buffers were used to perform washing steps following antibody coupling to the beads prior to incubation with the samples. In general, use of detergent was avoided when handling the samples in order not to disrupt protein-protein interactions.

2.4.11 Gel Filtration Analysis

To perform a gel filtration analysis cell free supernatants were prepared as described (see section 2.5.2). A column of 150 mm length and a diameter of 5 mm with a bed volume of 3 ml (Tricorn 5/150, Amersham) was packed with Superdex 200 size exclusion beads (Amersham) using a peristaltic pump thereby avoiding air bubbles. Superdex 200 beads retain proteins from 10 to 600 kDa on the column. The packed column was mounted in a SMART FPLC system (Amersham) and washed with 10 ml filtered and degased ddH_2O and subsequently with 10 ml IP-Buffer 0 (see section 2.5.9). 50 µl of each sample were injected into the tube system of the SMART and proteins were retained according to their size on the column when moving at a flow rate of 100 µl/min. The absorption at 260 nm wavelength which corresponding to protein molecules was measured simultaneously and plotted against the eluted volume. Fractions of 50 µl were collected starting from 0.9 ml to 3 ml after sample injection resulting in 42 fractions. 50 µl of SDS sample buffer was added and every second fraction from 10 to 28 was analyzed by SDS-PAGE and Western blot.

2.4.12 Biotinylation of Cell Surface Proteins

To analyze exported, cell surface bound material a biotinylation assay was performed as described (Seelenmeyer et al., 2005). A membrane impermeable biotinylation reagent was added to cells and covalently binds to all free ε-amino groups of lysine residues present in surface proteins. Following lysis, biotinylated proteins were purified by incubation with streptavidin beads and the amounts of biotinylated and non-biotinylated proteins were compared to determine the ratio of exported to non-exported reporter molecules.

Cells were grown on 6 well-plates in the presence of doxicycline to a confluency of about 80%. Following two times washing with 500 µl cold PBS Ca^{2+}/Mg^{2+} they were incubated with 0.5 mg/ml biotinylation reagent (EZ-link Sulfo-NHS-SS-biotin, Pierce) in incubation buffer for 30 min at 4°C. To quench unbound biotinylation reagent cells were washed once with quenching buffer followed by an incubation with 500 µl quenching buffer for 20 min at 4°C. Two additional washing steps using PBS were preformed and 200 µl lysis buffer was added. Cells were incubated 10 min at 37°C with lysis buffer and subsequently scraped off the 6 well-plates using a rubber policeman. The cell solution was homogenized by pipetting and transferred to an eppendorf tube on ice. To complete lysis the samples were subjected to sonication in a water bath and incubated for 15 min at room temperature mixed by vortexing every 5 min. To remove insoluble material a sedimentation was performed (16,000 g, 10 min, 4°C) and 10 µl of the supernatant was saved to be used as input sample for later analysis. The rest was added to 40 µl packed streptavidin beads equilibrated with lysis buffer and incubated for 1 h at room temperature under constant end-over-end rotation to allow binding of biotinylated proteins to streptavidin moiety. After the incubation period, the beads were washed two times with washing buffer 1 and another two times with washing buffer 2. Sedimentation in between the washing steps was performed by centrifugation (3.000 g, 4°C, 1 min). After the last washing step the supernatant was carefully discarded and bound material was eluted by incubation in SDS sample buffer for 5 min at 95°C. Subsequently, samples were analyzed by SDS-PAGE and Western blot.

PBS Ca^{2+}/Mg^{2+}:	1 mM	$MgCl_2$
	0.1 mM	$CaCl_2$
		PBS
Incubation buffer:	150 mM	$MgCl_2$
	10 mM	Triethanolamine, pH 9
	2 mM	$CaCl_2$
		ddH_2O
Quenching buffer :	100 mM	Glycine
		PBS Ca^{2+}/Mg^{2+}
Lysis buffer:	62.5 mM	EDTA
	50 mM	Tris-HCl, pH 7.5
	0.4%	Deoxycholate (w/v)
		Protease Inhibitor tablet (1 per 10 ml)
		ddH_2O
Washing buffer 1:	62.5 mM	EDTA
	50 mM	Tris-HCl, pH 7.5
	0.4%	Deoxycholate (w/v)
	1%	NP-40
	0.5 M	NaCl
		ddH_2O
Washing buffer 2:	62.5 mM	EDTA
	50 mM	Tris-HCl, pH 7.5
	0.4%	Deoxycholate (w/v)
	0.1%	NP-40
	0.5 M	NaCl
		ddH_2O

2.5 Flow Cytometry

2.5.1 Sample Preparation for *FACS* Analysis Detaching Cells From Culture Dishes

To analyze GFP fluorescence and exported reporter molecules by specific antibody cell surface staining cells were processed according to the following protocol and analyzed using flow cytometry.
Cells were grown on 6 well-plates to a confluency of about 70% in the absence or presence of doxicycline. After removal of the growth medium they were washed with 500 µl PBS and 500 µl *Cell Dissociation Buffer* (CDB, Invitrogen) or PBS/EDTA were added. Cells were incubated for 10 min at 37°C and detached by resuspension using a pipet. After transfer to an eppendorf tube on ice, a centrifugation step was applied (200 g, 4°C, 5 min) and the supernatant was discarded. The pellet was carefully resuspended in 300 µl αMEM supplemented with primary antibodies in the desired dilution. After an incubation period of 1 h at 4°C under constant end-over-end rotation, cells were sedimented and the pellet was washed once with αMEM without antibodies. Then, secondary antibodies coupled to the fluorophor allophycocyanin were added in a 1:750 dilution in αMEM and cells were incubated for 30 min as described above. To remove secondary antibodies cells were washed once with αMEM without antibodies and the pellet was resuspended in 500 µl sorting medium containing propidium iodide in a concentration of 1 µg/ml to stain dead cells. Samples were subsequently analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

Primary antibodies:	αMEM
	Affinity-purified rabbit anti-GFP anibodies (acidic elution) 1:200
	monoclonal mouse anti-His-tag antibodies 1:200
Secondary antibodies:	αMEM
	goat anti rabbit IgG APC-coupled antibodies 1:750
	goat anti mouse IgG APC-coupled antibodies 1:750

Sorting Medium:	5%	CDB (v/v)
	0.2%	FCS (v/v)
		αMEM without FCS

2.5.2 Plate Labelling Technique

To prepare samples for *FACS* analysis using the plate labelling technique cells were grown on 12 well-plates to a confluency of about 70% in the absence or presence of doxicycline. Following washing with 500 µl PBS the primary antibody in the desired dilution in 300 µl αMEM was added and the plates were incubated for 1 h at 4°C under constant shaking. Subsequently, cells were washed three times with 500 µl PBS and secondary antibodies were added in the desired dilution in 300 µl αMEM. Samples were incubated for 30 min at 4°C under constant shaking in the dark followed by three times washing with 500 µl PBS. Cells were detached by addition of 200 µl PBS/EDTA followed by an incubation period of 10 min at 37°C. Using a pipet samples were resuspended and transferred to an eppendorf tube which was prepared with 500 µl αMEM without FCS additionally containing propidium iodide in a concentration of 1 µg/ml. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

Primary antibodies:	αMEM
	Affinity-purified rabbit anti-GFP anibodies (acidic elution) 1:200
	Goat anti mouse IgG Allophycocyanin-coupled antibodies 1:500 (no secondary antibodies needed)
Secondary antibodies:	αMEM
	Goat anti rabbit IgG Allophycocyanin-coupled antibodies 1:750

2.5.3 FACS Sort

FACS-based sorting was performed in collaboration with Dr. Blanche Schwappach from the *Center of Molecular Biology Heidelberg* (ZMBH). Cells induced by addition of doxicycline for 12 h or grown after a sort in the absence of doxicycline for 7 days were detached from culture dishes after washing with PBS using sterile CDB (Invitrogen). After sedimentation at 200 g, 4°C, 5 min CDB was removed and the pellet was carefully resuspended in sorting medium. The cells were filtered using cell strainer caps (Becton Dickinson) into 5 ml round bottom *FACS* tubes (Becton Dickinson) and propidium iodide in a final concentration of 1 µg/ml was added. Subsequently, cells were sorted using a FACSVantage or FACSAria sorting device (Becton Dickinson) as pools of 50.000 or 100.000 cells in 6 well-plates or as single cells to generate clonal cell lines in 96 well-plates.

2.6 Confocal Microscopy

2.6.1 Sample Preparation for Confocal Microscopy

Cells were grown on coverslips placed in 24 well-plates. Following two times washing with PBS on ice 200 µl 3% PFA in PBS per well was added and the cells were fixed without permeabilization for 20 min. After removal of PFA cells were washed four times with PBS. Coverslips were mounted on microscopic slides using Fluoromount G (Southern Biotechnology Associates). After hardening at room temperature overnight in the dark the specimens were sealed at the edge of the coverslip employing clear nail polish (Wet'n Wild USA) and analyzed using a LSM 510 Meta confocal microscope (Zeiss).

PFA in PBS:	3%	PFA (w/v)
		PBS

2.6.2 Immunostaining of Cell Surface Proteins for Confocal Microscopy

Samples were prepared as described in section 2.7.1. Following fixation with PFA and two times washing with PBS, the samples were quenched by incubation with 250 µl quenching buffer for 10 min at room temperature. Unspecific antibody binding sites were blocked by incubation in blocking buffer for 10 min at room temperature. Primary antibodies were added in the desired dilution in 250 µl blocking buffer for 1 h at room temperature. Following three times washing with PBS, unspecific binding sites were blocked again by incubation in 250 µl blocking buffer for 10 min at room temperature. Secondary antibodies were added in 250 µl blocking buffer and the specimens were incubated for 30 min at room temperature. After four times washing the samples were mounted as described in 2.7.1 and analyzed using a LSM 510 Meta confocal microscope (Zeiss).

Quenching buffer:	50 mM	NH_4Cl
		PBS
Blocking buffer:	1%	BSA (w/v)
		PBS

Primary antibodies: Affinity-purified rabbit anti-GFP antibodies (acidic elution) 1:50
Monoclonal mouse anti-His-tag antibodies 1:200
Blocking buffer

Secondary antibodies: Goat anti rabbit or goat anti mouse IgG antibodies Alexa 546-coupled 1:1000
Blocking buffer

3 Results

Fibroblast Growth Factor 2 (FGF2) belongs to the protein family of heparin binding growth factors (Powers et al., 2000). It is a strong mediator of angiogenesis (Moscatelli et al., 1986) and neovascularization (Wilting et al., 1991) and is often associated with tumor growth (Zetter, 1998) and developmental processes (Bikfalvi et al., 1997). FGF2 is unconventionally secreted by an unknown machinery that functions independently of the classical ER/Golgi pathway (Florkiewicz et al., 1995; Nickel, 2005).

The purpose of the current study was to analyze the folding state FGF2 during unconventional secretion. To investigate the conformation of FGF2 during membrane translocation two independent experimental systems were applied. First a *dihydrofolate reductase fusion protein system* and second a *molecular piggyback export analysis system*.

3.1 Dihydrofolate Reductase Fusion Protein System to Study Protein Folding During Membrane Translocation Events

The dihydrofolate reductase fusion protein system is based on the fact that a substrate analogon, aminopterin, can bind to the enzyme dihydrofolate reductase (DHFR) in a very tight manner (Salvador et al., 2000). DHFR can be inhibited via binding of aminopterin to its active center. Of great importance in this concern is, that upon binding of aminopterin the conformation of the enzyme is highly stabilized so that it can not be unfolded during membrane translocation processes or by chaperone activity. Thus, if a fusion protein consisting of a molecule which is translocated across a membrane and DHFR needs to be unfolded, the process can be blocked by the addition of aminopterin. This system was widely used in analyzing mitochondrial protein import, glycosomal protein import in trypanosomes or protein import into peroxisomes (Eilers and Schatz, 1986; Wienhues et al., 1991).

For the current study a fusion protein consisting of FGF2, enhanced green fluorescent protein (GFP) and DHFR was generated and the export of the construct was analyzed in an *in vivo* system based on CHO cells with regard to aminopterin-

influence on membrane translocation applying different experimental approaches. Of great importance for export analysis is that, following secretion, FGF2 binds to heparan sulfates of proteoglycans in the ECM on the cell surface (Moscatelli, 1987). This allows the detection of exported material by externally added antibodies.
The detection of cell surface-bound material is the basis for the analysis by flow cytometry. In a *FACS*-based assay reporter molecules on the cell surface are immunolabelled with antibodies that, in turn, are detected by secondary antibodies coupled to a fluorescent dye. The *FACS* system detects the fluorescence of the dye and simultaneously the intrinsic fluorescence of the GFP moiety on a single cell basis. This allows a quantitative analysis of expression levels (GFP fluorescence) and exported reporter protein (cell surface staining). Another analysis technique used to investigate FGF2 export is confocal microscopy. It uses the same basic principle of antibody labelling of exported, surface bound material and intrinsic GFP fluorescence. It allows a qualitative and spatial analysis of the reporter construct with regard to intracellular localization and cell surface distribution. Furthermore, biochemical analysis methods were applied. Cell lysates prepared from cells which express the reporter construct were analyzed for doxicycline-dependent protein expression by SDS-PAGE and Western blot using specific antibodies. Moreover, a protease protection assay was performed to analyze binding of aminopterin to the reporter construct and the influence of aminopterin on its stability.

3.1.1 Generation of Cell Lines

To generate reporter cell lines which express the reporter proteins the respective cDNA constructs were cloned into the pRevTRE2 vector (Clontech, see Material & Methods, section 2.2.14). The vector allows stable integration of the constructs into the genome of target cells and reporter protein expression in a doxicycline-dependent manner due to the doxicycline/transactivator-responsive element. As a prerequisite the cells have to express the doxicycline-sensitive transactivator rtTA2-M2 (Urlinger et al., 2000) to make use of the doxicycline-dependent protein expression system.

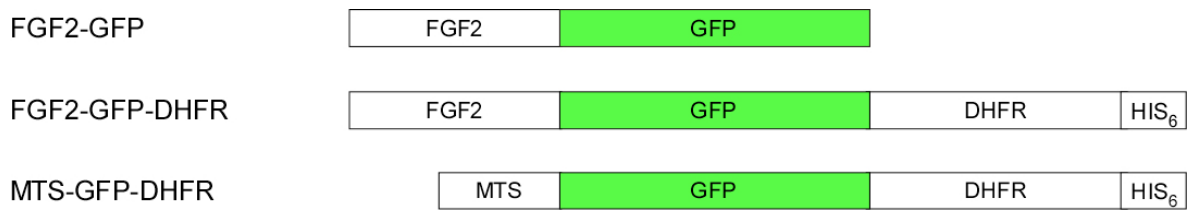

Fig. 3.1 Schematic overview: cDNA constructs.

FGF2 = *fibroblast growth factor* 2, GFP = *green fluorescent protein*, DHFR = *dihydrofolate reductase*, His_6 = hexa-histidin-tag, orientation = left to right corresponds to 5' to 3'

The construct FGF2-GFP was already existent and a clonal cell line was available in our laboratory (Engling et al., 2002). The calculated molecular mass of this construct is 44 kDa. FGF2-GFP-DHFR corresponds to a construct in which FGF2 is fused to GFP and a DHFR domain (Eilers and Schatz, 1986) with a C-terminal His_6-tag. The calculated molecular mass is 67 kDa. MTS-GFP-DHFR entitles a construct where the mitochondrial targeting sequence (MTS) of the S9 subunit of the F_0 ATPase (Salvador et al., 2000) is fused to GFP and a DHFR domain with a C-terminal His_6-tag. The calculated molecular mass of this construct is 57 kDa.

The reporter cell lines were generated using $CHO_{MCAT\text{-}TAM2}$ cells (Engling et al., 2002) which constitutively express the murine cationic transporter MCAT-1 (Albritton et al., 1989; Davey et al., 1997) and the doxicycline-sensitive transactivator rtTA2-M2 (Urlinger et al., 2000). Retroviral particles carrying the reporter constructs were produced and used for gene transfer (see Material & Methods, section 2.3.4). After viral transduction reporter molecule expression can be induced by addition of doxicycline to the culture medium.

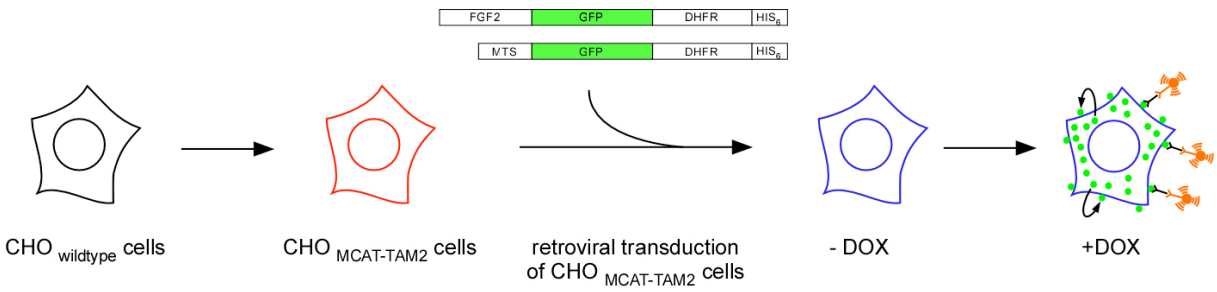

Fig. 3.2 Schematic overview: Retroviral transduction procedure to generate reporter cell lines.

CHO = Chinese Hamster ovary (cells), FGF2 = Fibroblast Growth Factor 2, GFP = green fluorescent protein, DHFR = dihydrofolate reductase, His_6 = Hexa-Histidine-tag, DOX = Doxicycline.

The virally transduced cells were subjected to a *FACS*-based sorting procedure to generate clonal cell lines (see Material & Methods, section 2.5.3). Three days after retroviral transduction, 1 µg/ml doxicycline was added to the culture medium for 12 h. Subsequently, cells were detached from culture dishes using a protease free buffer system and processed for *FACS* analysis. Dead cells were excluded by staining with propidium iodide, which intercalates into the DNA after membrane damage. 50,000 cells from each cell line were isolated by *FACS* sorting based on GFP fluorescence using a FACSVantage sorting device (Becton Dickinson). The obtained pools of cells were incubated for 7 days in the absence of doxicycline followed by the isolation of 50,000 cells from each population that did not display any GFP fluorescence at this point. Each population was now cultured for another 7 days including 12 h in the presence of 1 µg/ml doxicycline at the end of this period. Single cells were obtained by *FACS* sorting based on GFP fluorescence. Exemplarily the results of the sorting procedure are displayed for the cell line CHO FGF2-GFP-DHFR in Fig. 3.3.

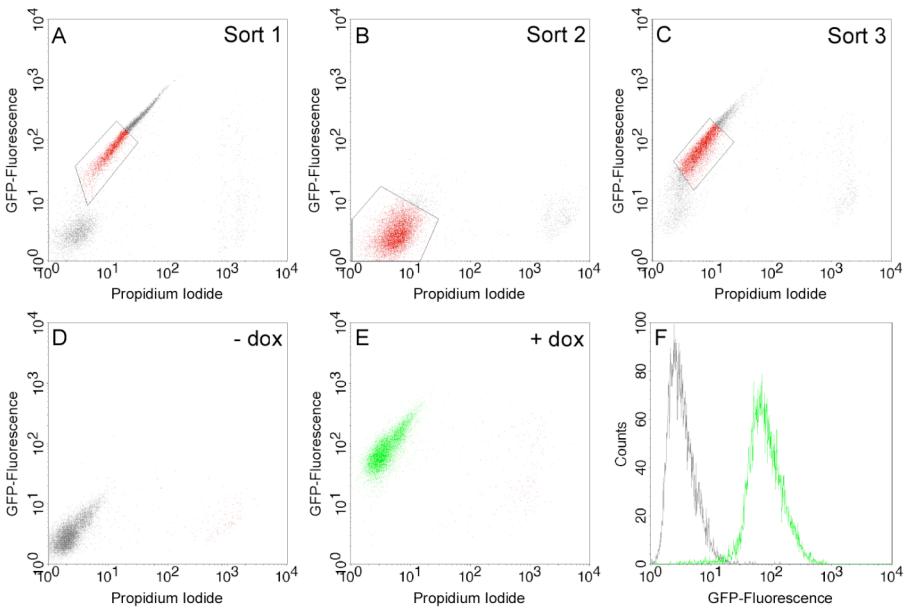

Fig. 3.3 *FACS* sorting based on GFP fluorescence to generate the cell line FGF2-GFP-DHFR.

Cells were detached from culture dishes using a protease free buffer system and processed for *FACS* analysis to measure GFP fluorescence. Sort 1 displays cells 3 days after viral transduction incubated in the presence of 1 µg/ml doxicycline for 12 h (panel A). 50,000 cells were sorted within the sorting gate (red population). Sort 2 shows cells grown for 7 days in the absence of doxicycline after sort 1 (panel B). Again 50,000 cells were sorted within the sorting gate. Sort 3 shows cells 7 days after sort 2 incubated in the presence of 1 µg/ml doxicycline for 12 h. Single clones were sorted within the sorting gate and propagated to generate clonal cell lines. Panel D and E show the clonal cell line FGF2-GFP-DHFR obtained from sort 3 grown in the absence or presence of 1 µg/ml doxicycline for 18 h. Panel F displays a histogram overlay of the obtained data from D and E verifying the shift in GFP fluorescence dependent on the addition of doxicycline (grey curve: - doxicycline, green curve: + doxicycline).

The obtained single clones were tested and propagated to generate clonal cell lines. The cell lines were termed $CHO_{FGF2\text{-}GFP\text{-}DHFR}$ and $CHO_{MTS\text{-}GFP\text{-}DHFR}$ in order to reflect the reporter molecules expressed.

To detect reporter molecules on the cell surface in forthcoming experiments appropriate antibodies can be used (Fig. 3.2, see scheme cell plus doxicycline). All three constructs can be detected applying affinity-purified polyclonal anti-GFP antibodies (Seelenmeyer et al., 2003) due to the presence of the GFP moiety in the fusion proteins (Engling et al., 2002). The two constructs designed with the DHFR domain can furthermore be analyzed employing commercially available monoclonal anti-DHFR antibodies (Becton Dickinson). Furthermore the His_6-tag at the C-terminus of the DHFR domain allows the use of commercially available anti-His_6-tag antibodies (Qiagen) to detect specifically the DHFR fusion constructs and within them the extreme C-terminus which is of great importance for later localization studies (see sections 3.1.8 to 3.1.10).

3.1.2 Biochemical Analysis of Doxicycline Dependent Protein Expression

To analyze the doxicycline-dependent protein expression, cells transduced with the different reporter constructs, were grown in the absence or presence of 1 µg/ml doxicycline for 18 h. Cell lysates obtained from these cultures were analyzed by SDS-PAGE and Western blot applying primary affinity-purified anti-GFP antibodies and secondary HRP-coupled antibodies. Visualization was performed using the chemiluminescence-based ECL system and x-ray film detection.

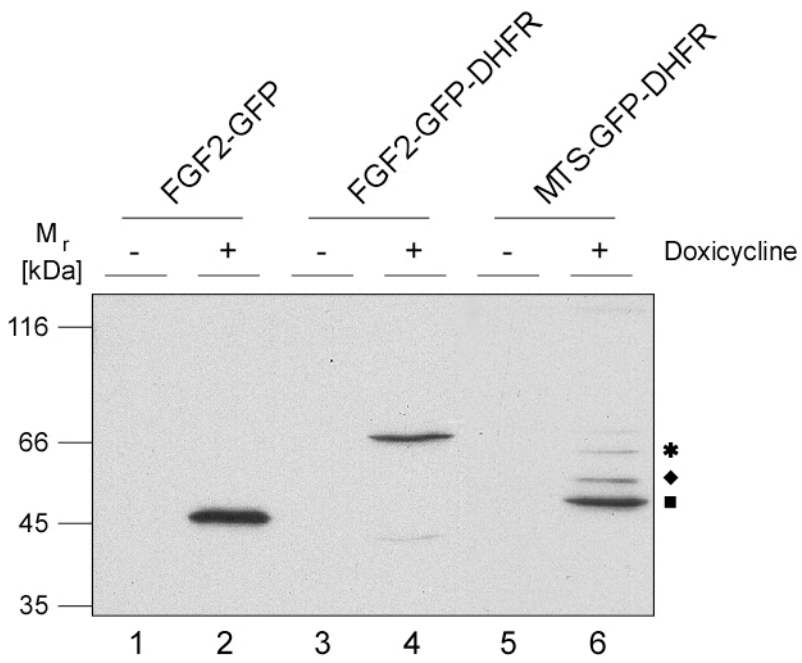

Fig. 3.4 Western Blot analysis of doxicycline-dependent protein expression.

Reporter cell lines were grown in the absence or presence of 1 µg/ml doxicycline for 18 h. Cell lysates were separated on 10% SDS gels followed by Western blot transfer. Detection was performed applying affinity-purified anti-GFP antibodies. When analyzing FGF2-GFP material derived from 10,000 cells was loaded. In the case of FGF2-GFP-DHFR and MTS-GFP-DHFR, material derived from 20,000 cells was analyzed. Visualization was performed using the ECL system and x-ray film detection. Three species of MTS-GFP-DHFR are detectable: a precursor (∗), an intermediate form (◆) and the fully processed form (■).

All reporter cell lines show doxicycline-dependent protein expression when compared to non-induced cells (Fig. 3.4, compare lanes 1, 3 and 5 to lanes 2, 4 and 6). The fusion proteins show a migration behaviour corresponding to their calculated molecular masses when compared to a protein standard. FGF2-GFP migrates at about 45 kDa (Fig 3.4, lane 2), FGF2-GFP-DHFR at about 67 kDa (Fig 3.2,lane 4) and MTS-GFP-DHFR around 60 kDa (Fig. 3.4, lane 6).

Expression of MTS-GFP-DHFR results in production of three isoforms. The slow migrating precursor form (∗) displaying a size of 57 kDa, the intermediate form (◆) and the fully processed form (■), which are both smaller in size (Fig. 3.4, lane 6). They correspond to the mitochondrial import steps and are the result of two membrane translocation events into the mitochondrial matrix during which the MTS is cleaved twice. This result is a first hint that the MTS construct is imported into mitochondria.

3.1.3 Characterization of the Reporter Cell Lines by Confocal Microscopy

Cells were grown on cover slips in the absence or presence of 1 µg/ml doxicycline for 18 h and processed without permeabilization for confocal microscopy. Immunostaining was performed applying primary affinity-purified anti-GFP antibodies and Alexa 546-coupled secondary antibodies. All samples were analyzed using a Zeiss LSM 510 confocal microscope.

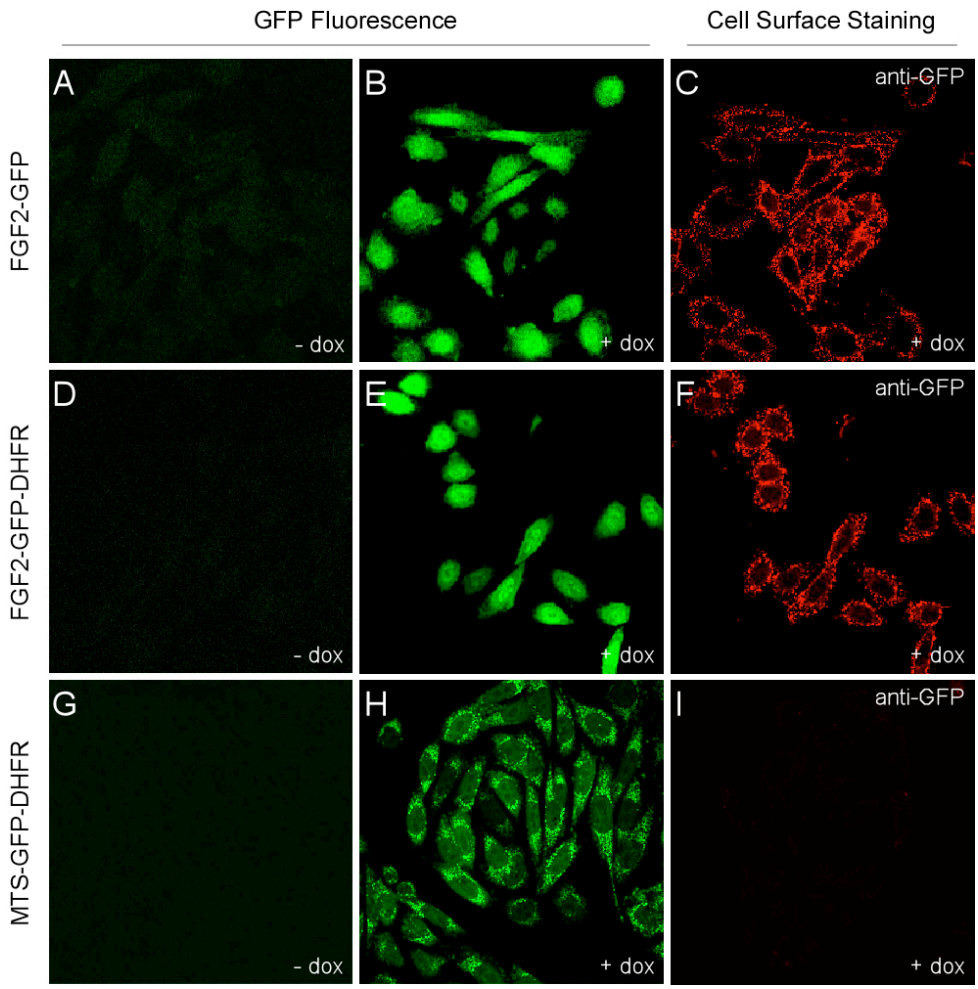

Fig. 3.5 Characterization of generated cell lines by confocal microscopy.

Generated cells were cultivated on cover slips in the absence or presence of 1µg/ml doxicycline for 18h. Samples were prepared without permeabilization and immunostained applying affinity-purified anti-GFP antibodies. The microscopic analysis was performed using a Zeiss LSM 510 confocal microscope. Panels A, B, D, E, G and H show total GFP fluorescence, panels C, F and I cell surface staining.

All cell lines exhibit doxicycline-dependent protein expression analyzed by total GFP fluorescence (Fig. 3.5 A and B, D and E, G and H). Moreover, the reporter constructs FGF2-GFP and FGF2-GFP-DHFR show cytosolic and nuclear localization upon expression (Fig. 3.5 panels B and E). MTS-GFP-DHFR localizes to dot-like structures throughout the cytoplasm and is excluded from the nucleus and the plasma membrane. This staining pattern is consistent with mitochondrial localization (Fig. 3.5 panel H). Combined with the biochemical analysis performed previously, it is safe to conclude that the MTS construct is indeed imported into mitochondria (see section 3.1.2 and 3.1.6).

FGF2-GFP and FGF2-GFP-DHFR can be detected on the cell surface applying externally added anti-GFP antibodies (Fig. 3.5 panel C and F), which demonstrates that both are translocated across the plasma membrane. MTS-GFP-DHFR is not detectable on the cell surface using anti-GFP antibodies (Fig. 3.5 panel I).

3.1.4 Analysis of Doxicycline Dependent Protein Expression and Secretion by Flow Cytometry

Cells transduced with the reporter constructs were grown in 6-well culture dishes in the absence or presence of doxicycline, respectively 1 µg/ml for 18h. After detaching the cells from the culture dishes using a protease-free buffer system, immunostaining was performed using either anti-GFP or monoclonal anti-His_6-tag primary antibodies and allophycocyanin-coupled secondary antibodies. Additionally, propidium iodide staining was performed to exclude dead cells from the subsequent evaluation. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

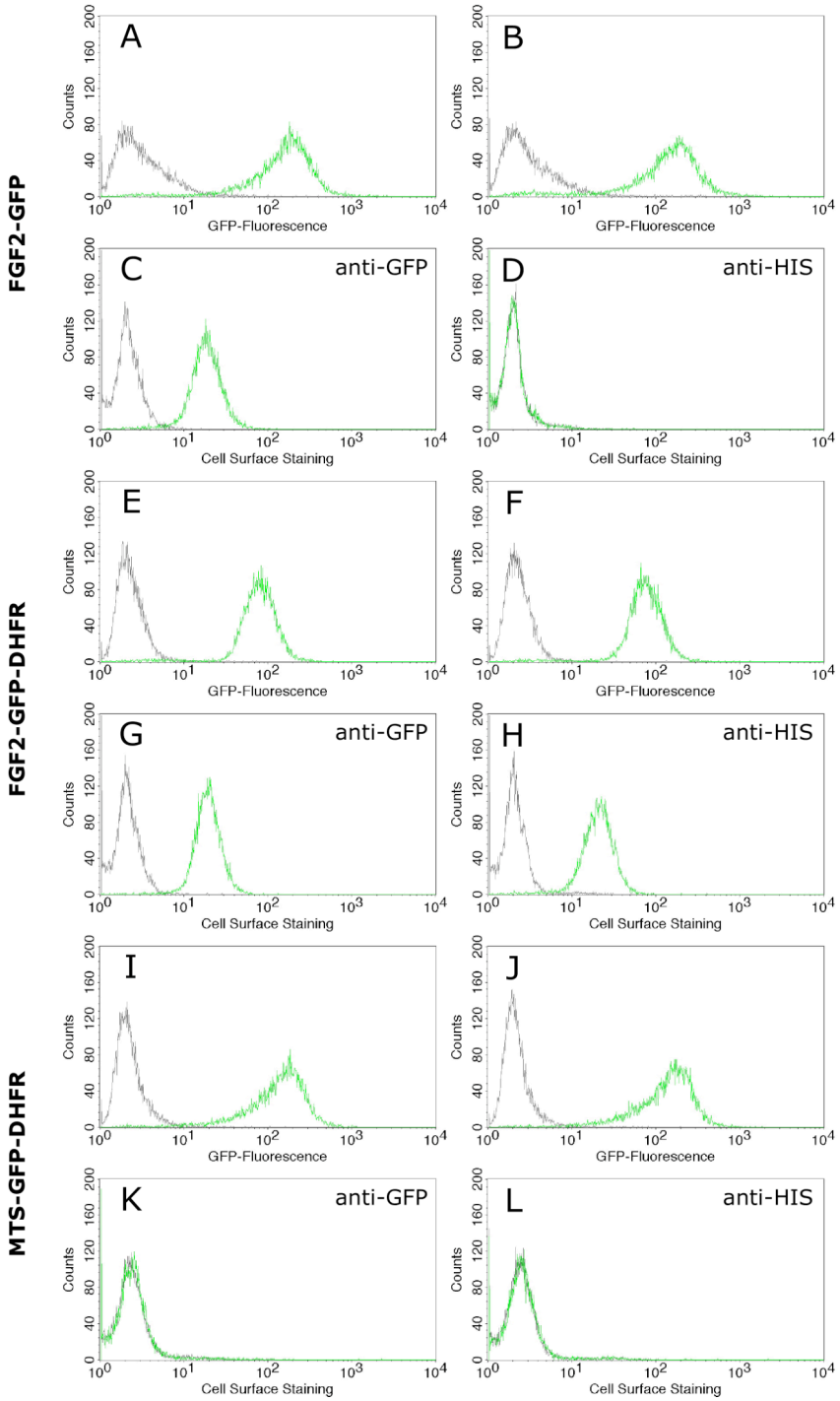

Fig. 3.6 Quantitative analysis of doxicycline-dependent protein expression and secretion based on flow cytometry.

FGF2-GFP cells (A, B), FGF2-GFP-DHFR cells (C, D) and MTS-GFP-DHFR cells (E, F) were grown in the absence (grey curves) and presence (green curves) of 1 µg/ml doxicycline for 18 h. Cells were detached from culture plates employing a protease-free buffer and immunostained using either affinity-purified anti-GFP (A, C, E) or monoclonal anti-His_6-tag antibodies (B, D, F). Living cells were analyzed simultaneously for GFP fluorescence and cell surface staining using a FACSCalibur flow cytometer (Becton Dickinson).

All cell lines show expression of the reporter constructs dependent on the addition of doxicycline as shown by the diagrams representing GFP fluorescence (Fig. 3.6, panels A to F, GFP fluorescence). The grey curves represent cells grown in the absence of doxicycline, the green curves show cells grown in the presence of doxicycline. A clear shift in the presence of doxicycline can be observed.
The proteins FGF2-GFP and FGF2-GFP-DHFR are detectable on the cell surface using GFP antibodies when protein expression is induced (Fig. 3.6, panels A and C, cell surface staining, green curves). FGF2-GFP-DHFR is also detectable on the cell surface employing anti-His_6-tag antibodies (Fig. 3.6, panel D, cell surface staining, green curve). As expected FGF2-GFP, which lacks the C-terminal His_6-Tag can not be detected on the cell surface using anti His_6-tag antibodies (Fig. 3.6, panel B, cell surface staining). Importantly, MTS-GFP-DHFR is not detectable on the cell surface neither with anti-GFP nor with anti-His_6-tag antibodies (Fig. 3.6, panels E and F, cell surface staining)

In summary, employing different methods, it could be shown that all cell lines express the reporter constructs exclusively in the presence of doxicycline. The reporter proteins FGF2-GFP and FGF2-GFP-DHFR are translocated across the plasma membrane and can be detected on the cell surface employing anti-GFP or anti-His_6-tag antibodies. MTS-GFP-DHFR is shown to be imported into mitochondria confirmed by confocal microscopy and Western blot analysis.

3.1.5 Influence of Aminopterin on the Mitochondrial Import of the Reporter Construct MTS-GFP-DHFR

To test if the import of the MTS construct into mitochondria is influenced by aminopterin cells were grown on coverslips in the presence of 1 µg/ml doxicycline for 18 h. Aminopterin was added as indicated, respectively 50 µM for 18 h. Samples were fixed using paraformaldehyde and analyzed by confocal microscopy. Visualization was performed using a Zeiss LSM 510 confocal microscope.

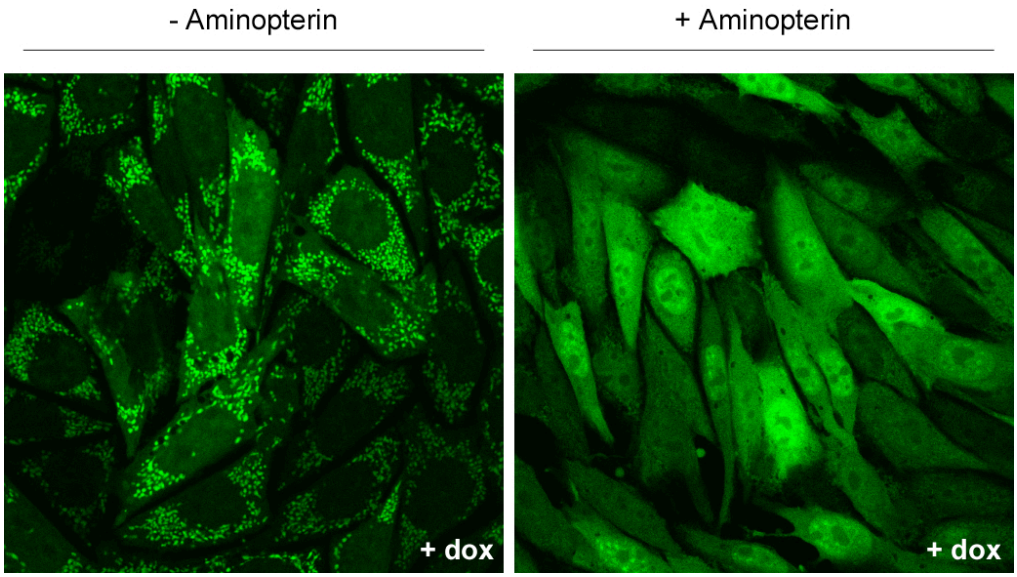

Fig. 3.7 Influence of aminopterin on the mitochondrial import of MTS-GFP-DHFR.

MTS-GFP-DHFR cells were grown on coverslips for 18 h in the presence of 1 µg/ml doxicycline and 50 µM aminopterin as indicated. Samples were prepared for microscopic analysis and GFP fluorescence was analyzed using a Zeiss LSM 510 confocal microscope.

Without aminopterin the reporter construct is not found in the cytoplasm, at the plasma membrane or in the nucleus. It localizes to mitochondria confirmed by the staining pattern, which was shown to represent mitochondrial localization (see section 3.1.3)

Upon addition of aminopterin the localization pattern changes dramatically. The protein exhibits cytosolic and nuclear localization. No distinct mitochondrial staining is detectable anymore. Aminopterin blocks the mitochondrial import of the fusion construct and, therefore, the expressed protein is distributed throughout the cytoplasm. It can also be found in the nucleus due to the GFP moiety since GFP is known for its nuclear localization to a certain extent by diffusion along the concentration gradient in order to establish an equilibrium.

3.1.6 Biochemical Analysis of the Influence of Aminopterin on MTS-GFP-DHFR Import Into Mitochondria

MTS-DHFR-GFP cells were cultivated in media supplied either with 1 µg/ml doxicycline or 1 µg/ml doxicycline and 50 µM aminopterin for 18 h. Cell lysates were prepared and analyzed by SDS-PAGE and Western blot using anti-GFP antibodies

and HRP-coupled secondary antibodies. Visualization was performed using the chemiluminescence-based ECL system and x-ray film detection.

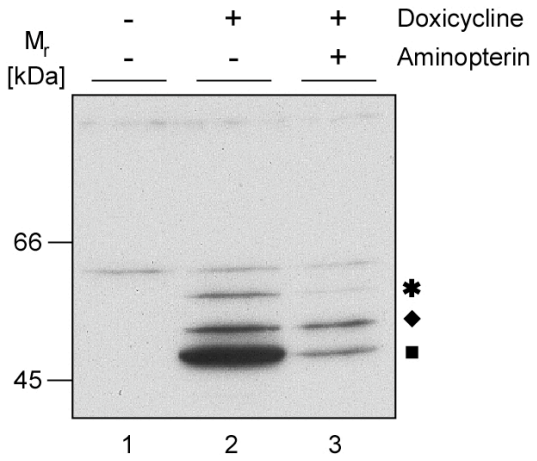

Fig. 3.8 Biochemical Analysis of MTS-GFP-DHFR import into mitochondria in the absence or presence of aminopterin.

MTS-GFP-DHFR cells were grown in the absence or presence of 1 µg/ml doxicycline for 18h. Where indicated 50 µM aminopterin was added during the protein induction period. Obtained cell lysates were analyzed by SDS-PAGE and Western blotting employing affinity-purified anti-GFP antibodies. Material of about 20,000 cells was loaded in each lane. Visualization was performed using the ECL system and x-ray film detection. The previously described isoforms of MTS-GFP-DHFR are detectable; the precursor (✱), the intermediate form (◆) and the fully processed form (■).

The protein MTS-GFP-DHFR is expressed in a doxicycline-dependent manner (Fig. 3.8, lane 2). The three isoforms observed previously are detectable (see section 3.1.2), the slow migrating precursor form (✱), the intermediate form (◆) and the fully processed form (■) which migrates fastest (Fig. 3.8, lane 3). The visible band migrating slower than the precursor form corresponds to a cross reaction signal since it is detected even in the absence of doxicycline. In the absence of aminopterin an accumulation of the processed form can be observed.

Upon addition of aminopterin the distribution and detectable amounts of the isoforms change dramatically (Fig. 3.8, compare lanes 2 and 3). The amount of the processed form is strongly reduced. Regarding the intermediate form the signal remains the same and when analyzing the precursor form one can also see a strong reduction. Taken together the whole amount of protein found in the cells is largely reduced.

The observed reduction of the overall protein amount occurs because fusion proteins binding to the mitochondrial import complexes and blocking these, since they can not be translocated when bound by aminopterin, are probably actively degraded by the cell. This is done to clear the import complexes in order to keep them in a functional

state. Therefore, the amount of the precursor form bound to the cytosolic face of the mitochondria is strongly reduced and also the amount of imported protein detectable as fully processed form is diminished since the import is blocked. The transient intermediate form is not reduced since the small fraction of protein passing through the intermembrane region even in the presence of aminopterin is rapidly translocated to the mitochondrial matrix.

3.1.7 Protease Protection Assay to Analyze the Stability of the DHFR Domain in the Absence or presence of Aminopterin

To test if aminopterin binds to the DHFR domain and if it is able to stabilize the conformation of the molecule a protease protection assay was performed. FGF2-GFP and FGF2-GFP-DHFR cells expressing their reporter molecules were lysed in a detergent-containing buffer followed by centrifugation to remove insoluble material. The Lysates were subjected to trypsin protease treatment (200 mg trypsin/ml) in the absence or presence of 50 µM aminopterin for 30 min at 4°C. Samples were analyzed employing SDS-PAGE and Western blot. Fusion proteins were detected using primary monoclonal anti-DHFR antibodies (Becton Dickinson) and HRP-coupled secondary antibodies. Signals were visualized using the chemiluminescence-based ECL system and x-ray film detection.

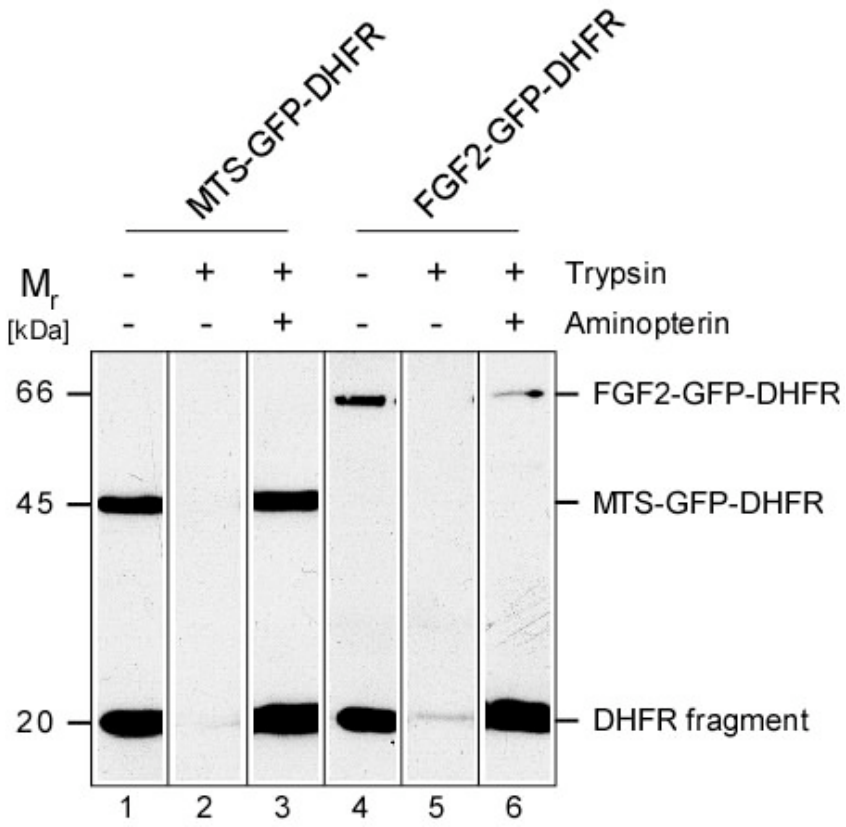

Fig. 3.9 Protease protection assay analyzing the stabilizing effect of aminopterin on the DHFR domain.

MTS-GFP-DHFR and FGF2-GFP-DHFR cells expressing the reporter constructs were lysed in a detergent-containing buffer. The lysates were cleared by centrifugation and subjected to protease treatment (200 µg trypsin/ml) in the presence or absence of aminopterin (50 µM) for 30 min at 4°C. The samples were subjected to SDS-PAGE and Western blot transfer. Detection was performed applying monoclonal anti-DHFR antibodies. Lanes 1-3: MTS-GFP-DHFR; Lanes 4-6: FGF-2-GFP-DHFR. As can be seen in lanes 1 (MTS-GFP-DHFR) and 4 (FGF-2-GFP-DHFR), post lysis degradation results in the appearance of the DHFR fragment without adding exogenous protease.

In the absence of trypsin and aminopterin the fusion proteins are detectable and show the expected migration behaviour (Fig. 3.9 lanes 1 and 4). Also a fragment with a size of 20 kDa corresponding to the DHFR domain alone is detectable, which results from degradation occurring during sample preparation. Addition of trypsin in the absence of aminopterin results in complete proteolysis of the reporter molecules (Fig. 3.9 lanes 2 and 5). By contrast, trypsin treatment in the presence of aminopterin shows stabilization of both the complete fusion proteins and the DHFR fragments (Fig. 3.9 lanes 4 and 6). The stabilization of the fusion proteins and protection against proteolysis demonstrates tight binding of aminopterin to DHFR. Only in the presence of the drug protection is achieved giving a proof for binding and stabilization of the conformation by the substrate analogon.

3.1.8 Rationale For the Analysis of the Aminopterin Effect on FGF2-GFP-DHFR Export

As depicted in Fig. 3.10 the use of affinity-purified anti-GFP antibodies to analyze the effect of aminopterin on protein secretion in the *in vivo* assays is not sufficient to obtain correct results.

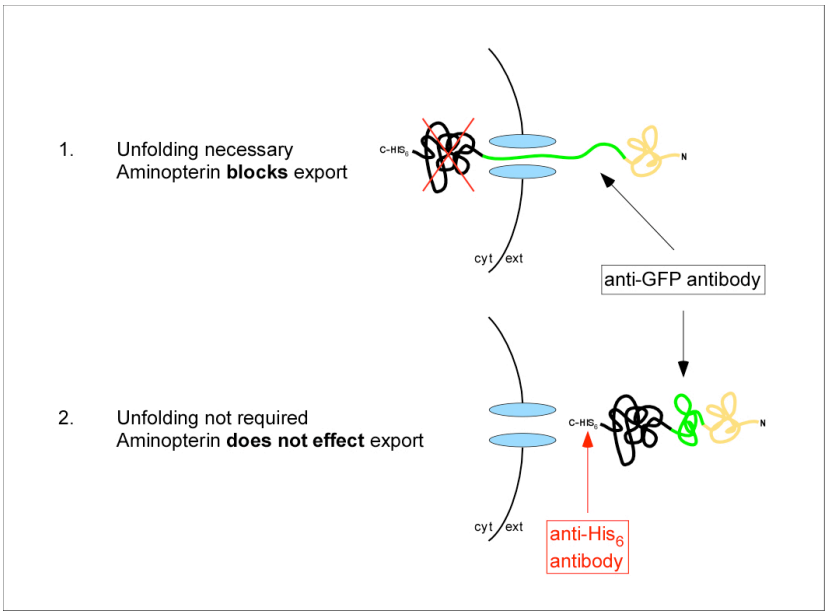

Fig. 3.10 Schematic overview: Rationale for the analysis of FGF2-GFP-DHFR in the presence of aminopterin.

Anti-GFP antibodies can also detect fusion proteins, which might be stuck in a putative membrane pore or protein conducting channel upon aminopterin binding to the DHFR domain (Fig. 3.10, 1.). A part of the GFP molecule could reach out into the extracellular space and function as an antibody epitope since the used antibodies are polyclonal and detect multiple epitopes in the sequence of the GFP moiety. Misleading observations would be made when investigating the possible inhibition of protein export mediated by aminopterin binding by employing polyclonal anti-GFP antibodies only.

In contrast, the use of anti-His_6-tag antibodies allows the detection of completely secreted molecules since the translocation signal is located in the N-terminal FGF2 moiety of the fusion construct and the C-terminal His_6-tag is only available as an antibody epitope when secretion of the molecule is complete (Fig 3.10, 2.). It is

therefore necessary to have the possibility to detect the extreme C-terminus of the reporter constructs what can be achieved by the use of anti-His_6-tag antibodies.

3.1.9 Effect of Aminopterin on FGF2-GFP-DHFR Export As Analyzed by Confocal Microscopy

Cells expressing the reporter construct FGF2-GFP-DHFR were cultured on cover slips in the absence or presence of 50 µM aminopterin for 18 h. The specimens were fixed without permeabilization and cell surface localization was probed using primary anti-GFP or anti-His_6-tag antibodies and secondary Alexa 546-coupled antibodies. Samples were analyzed using a Zeiss LSM 510 confocal microscope.

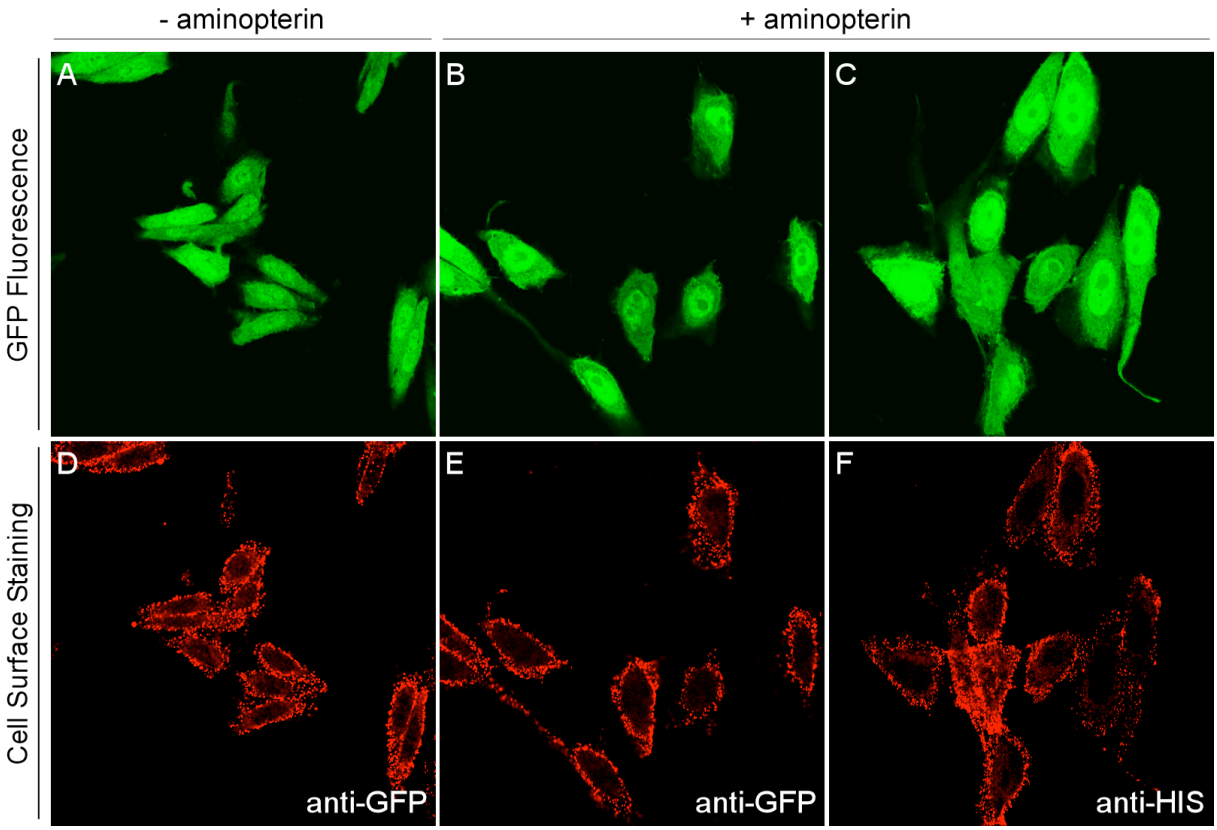

Fig. 3.11 Microscopic analysis of FGF2-GFP-DHFR export in the absence or presence of aminopterin.

FGF-2-GFP-DHFR cells were grown on cover slips in the presence of 1 µg/ml doxicycline for 18 h and in the absence (panel A and D) or presence of 50 µM aminopterin (panels B, C, E and F). Cells were fixed without permeabilization using paraformaldehyde followed by immunostaining applying either affinity-purified anti-GFP antibodies (panels D and E) or monoclonal anti-His_6-tag antibodies (panel F). Total GFP fluorescence (panels A to C) as well as cell surface staining (panels D to F) were analyzed using a Zeiss LSM 510 confocal microscope.

Analysis of the GFP fluorescence shows that protein expression is functional in the absence or presence of aminopterin (Fig. 3.11, panels A, B and C).
In the absence of aminopterin the expressed reporter molecules are translocated across the plasma membrane and can be detected on the cell surface using anti-GFP antibodies (Fig. 3.11, panel D). In the presence of the drug the protein is also exported and recognized on the cell surface by both anti-GFP and anti-His_6-tag antibodies (Fig. 3.11, panels E and F). The addition of aminopterin does not influence FGF2-GFP-DHFR export or inhibit its secretion as shown by the detection of the GFP moiety and also the extreme C-terminus of the fusion protein outside of the cells.

3.1.10 Effect of Aminopterin on FGF2-GFP-DHFR Export As Analyzed by Flow Cytometry

FGF2-GFP-DHFR cells were grown in the absence or presence of 1 µg/ml doxicycline and 50 µM aminopterin for 18 h. After preparation for *FACS* analysis by washing and detaching the cells using a protease-free buffer system, exported reporter proteins on the cell surface were immunolabelled employing anti-GFP or anti- His_6-tag antibodies. Primary antibodies were detected using allophycocyanin-coupled secondary antibodies. Dead cells were identified by propidium iodide staining and are thereby excluded from later evaluation. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

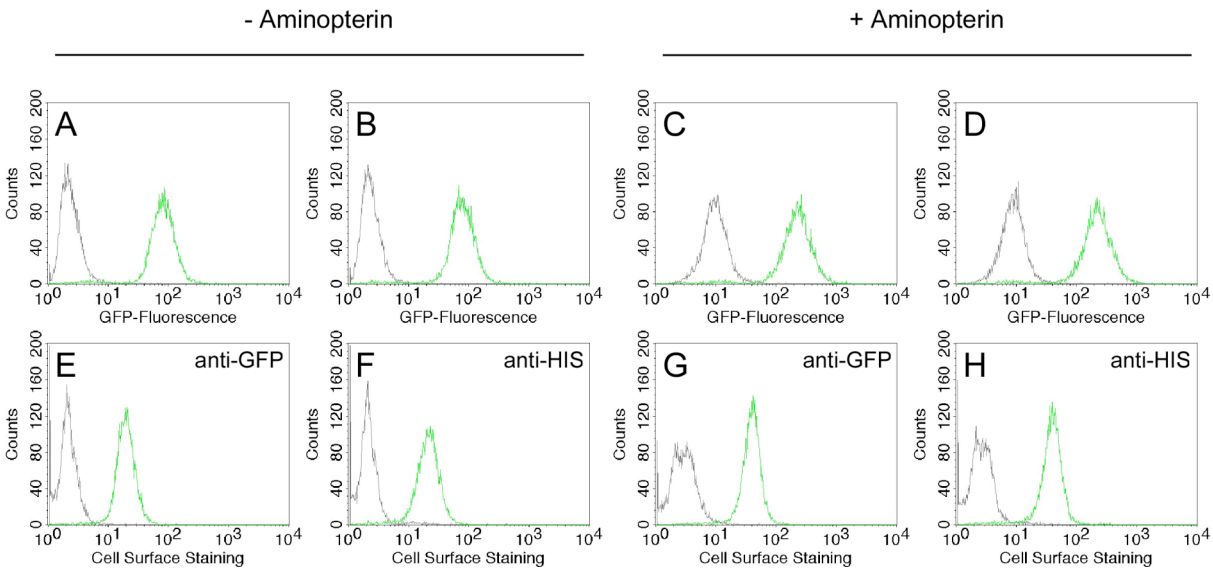

Fig. 3.12 Analysis of FGF2-GFP-DHFR export in the absence or presence of aminopterin employing *FACS*.

Non-induced (grey curves) and doxicycline-induced (green curves) FGF2-GFP-DHFR cells were grown in the absence (panels A, B, E and F) or presence of 50 mM aminopterin (panels C, D, G and H). Following detachment cells were processed for *FACS* analysis using either affinity-purified anti-GFP antibodies (panels A, C, E and G) or monoclonal anti-His_6-tag antibodies (panels B, D, F and H). Total GFP fluorescence (panels A to D) as well as cell surface staining (panels E to H) were measured simultaneously using a FACSCalibur flow cytometer (Becton Dickinson).

Grey curves represent cells grown in the absence of doxicycline and green curves cells grown in the presence of doxicycline. Regarding the GFP fluorescence a clear shift is visible (Fig. 3.12, panels A, B, C and D). Addition of aminopterin causes an increase in protein expression even in not induced cells compared to non-aminopterin conditions (Fig. 3.12, grey curves panels A and B compared to panels C and D).

The export analysis of the reporter construct reveals that in the absence of aminopterin the protein is detectable on the cell surface using externally added anti-GFP or anti- His_6-tag antibodies (Fig. 3.12, panels E and F, green curves). Upon addition of aminopterin the protein remains detectable on the cell surface in a comparable extent to non-aminopterin conditions employing both antibodies (Fig. 3.12, panels G and H, green curves). Therefore, being consistent with the data obtained by confocal microscopy, secretion of the reporter construct is not affected by aminopterin (see section 3.1.9).

Because of the elevated expression levels in the presence of aminopterin, the amount of doxicycline added to the cells to induce protein expression was titrated to

adjust the expression level to that of cells grown in the absence of aminopterin (analysis of the GFP fluorescence by *FACS*, data not shown). After adjusting the protein expression in the presence of aminopterin, the *FACS* analysis was repeated three times. The obtained values for cell surface staining were averaged and signals in the absence of aminopterin were set to 100% to compare export levels in the absence or presence of aminopterin.

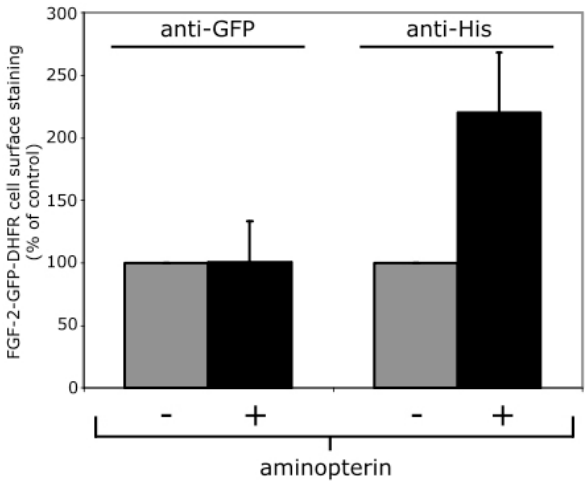

Fig. 3.13 Statistical analysis of FGF2-GFP-DHFR export in the absence or presence of aminopterin-based on expression corrected cell surface staining.

Since aminopterin causes a significant increase of FGF2-GFP-DHFR expression, the amount of doxicycline added to aminopterin treated cells was titrated until an expression level was reached similar to cells grown in the absence of aminopterin. Under these conditions, FGF2-GFP-DHFR export was determined based on cell surface staining. Cell surface staining measured in the absence of aminopterin was set to 100% and compared to cell surface staining in the presence of aminopterin (n=3).

When using anti-GFP antibodies the signal in the presence of aminopterin is not elevated or reduced demonstrating that FGF2-GFP-DHFR export is not influenced by aminopterin (Fig. 3.13, anti-GFP). Results obtained with the monoclonal anti-His_6-tag antibody show an about two-fold higher signal in cell surface staining in the presence of aminopterin (Fig. 3.13, anti-His_6). This might be due to an increased accessibility of the C-terminal His_6-tag fused to the DHFR domain when aminopterin binds and alters the conformation of the DHFR domain.

Taken together the analyses by confocal microscopy and the data obtained by flow cytometry revealed that the addition of aminopterin does not influence the export of the reporter constructs. FGF2-GFP-DHFR is efficiently exported in the presence of aminopterin.

3.1.11 Limitations of the DHFR Approach

The purpose of the DHFR system was to analyze if export of FGF2 requires protein unfolding. Fusion of FGF2 to a DHFR domain results in a reporter molecule, which can not be unfolded in the presence of aminopterin, a substrate analogon of DHFR that binds tightly to the active center.

The export of FGF2-GFP-DHFR was not inhibited in the presence of aminopterin. When analyzing the fusion construct by employing anti-GFP and anti-His_6-tag antibodies it could furthermore be shown that the reporter molecule is exported completely as analyzed by both flow cytometry and confocal microscopy (see sections 3.1.9 and 3.1.10). In contrast, the analysis of the control construct consisting of a mitochondrial targeting sequence (MTS) fused to DHFR revealed that the import into the mitochondrial matrix is blocked upon addition of aminopterin as revealed by both confocal microscopy and a biochemical analysis based on MTS processing (see sections 3.1.6 and 3.1.9). The mitochondrial import process consists of two membrane translocations steps and is dependent on the unfolding of the cargo proteins. By proving that the applied DHFR system is functional in the mitochondrial import translocation process, the results obtained for the FGF2 fusion construct show that FGF2 can be exported in a folded conformation since its export is not blocked by the addition of the drug. But it has to be taken into consideration that there are certain limitations applying to this system.

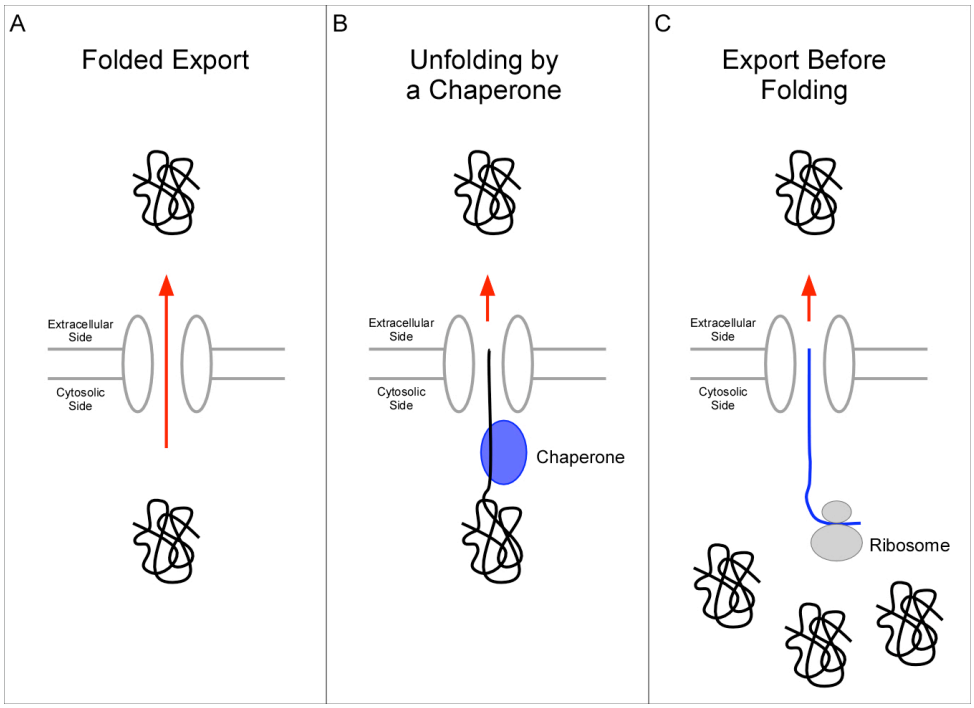

Fig. 3.14 Schematic overview: Limitations of the DHFR system.

The first and expected case would be that the fusion construct is exported in a fully folded state with aminopterin bound to the DHFR domain (Fig. 3.14 A). However, a second possibility could be that specific cytosolic chaperones function in the unconventional export pathway of FGF2 capable of unfolding the reporter molecule even in the presence of aminopterin. Unfolded material would be exported (Fig. 3.14 B). A third possibility could be that reporter molecules get exported before folding occurs. In this scenario only a fraction of total protein is exported consisting of nascent polypeptide chains, which directly function as substrates for the export machinery in a co-translational manner. These proteins are not folded when they are exported, so that aminopterin could not bind to the active since the DHFR domain does not reach its functional conformation and the active center of the enzyme is not formed. Proteins in the cytosol being folded on the other hand would not leave the cell. In that case unfolded material would be exported in the presence of aminopterin (Fig. 3.14 C).

Thus, to rule out these possibilities show that only folded molecules are exported a new approach is necessary. An experimental approach to overcome the limitations of the DHFR system is a *molecular piggyback export analysis system*, which is presented in the next part of the study.

3.2 Molecular Piggyback Export Analysis System

The rationale of the piggyback export analysis system is to investigate whether two proteins are exported attached to each other. Moreover, it addresses the question if a protein has to be folded correctly and maintain its conformation to be exported by the non-classical export pathway.

One protein is a cargo molecule destined for export, the other an interaction partner that is not exported on its own. The two molecules associate prior to export by non-covalent interactions. In case the conformation of the two molecules is maintained during membrane translocation they can be exported as a complex. Based on this rationale, the strategy monitors the folding state during membrane translocation by the fact that protein-protein interactions are lost upon unfolding or any other conformational change. The system is termed *piggyback export analysis system* since a non-exported protein is co-translocated 'on the back' of an exported one as observed in nuclear or peroxisomal import (Weil et al., 1999; Wu et al., 2000). Moreover, it shows parallels to bacterial protein export mediated by the twin-arginine (Tat) transporter. It was observed that this translocation system mediates export of oligomeric complexes, even if they contain additional co-factors (DeLisa et al., 2003).

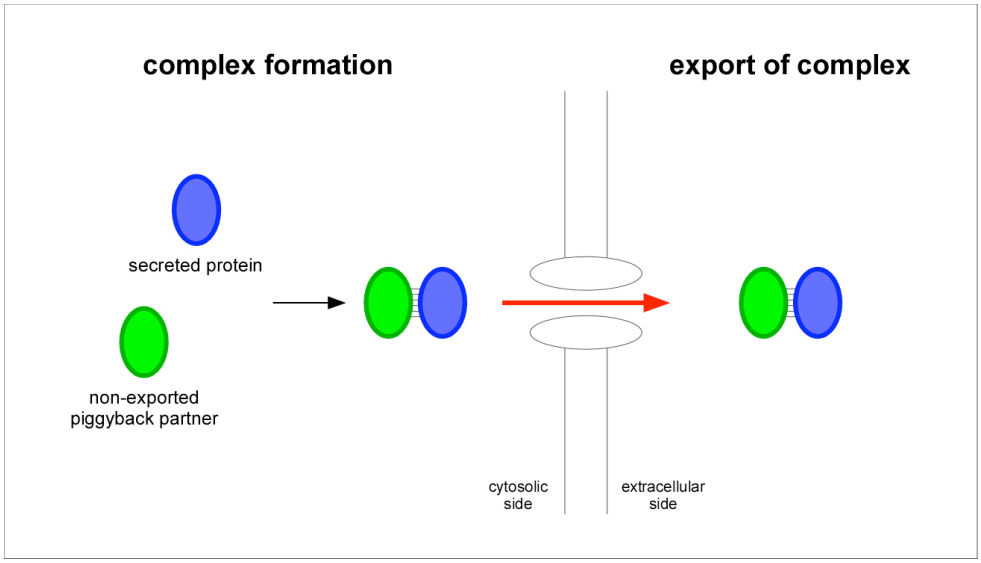

Fig. 3.15 Schematic overview: Piggyback export system (simplified).

The system overcomes the limitations of the DHFR approach since correct folding is a prerequisite and must be maintained during the whole export process in order to

transport the non-exported interaction partner out of the cell. To apply this system to the unconventional secretion of FGF2 one of two interacting protein domains, which are not exported on their own were either fused to FGF2 or to GFP. When both reporter molecules are expressed in the same cell it is possible to analyze if export of the complex formed by the interacting domains occurs.

3.2.1 Generation of Piggyback Reporter Cell Lines

To generate cell lines, which express the reporter proteins cDNA constructs, molecular cloning techniques were employed (see Material & Methods, section 2.2.15). To express both reporter proteins in one cell a bicistronic expression vector, termed pRTi, was used. The pRTi vector is based on the pRevTRE2 viral transduction vector (Clontech) which was modified in a way that an internal ribosome entry site (IRES) is inserted into the multiple cloning site. The MCS itself was extended with more restriction sites to be more flexible in the use of restriction enzymes. These modifications resulted in a vector with two multiple cloning site separated by the IRES element. After integration into the genome of target cells transcription leads to a bicistronic mRNA from which two proteins are translated. One derived from the first MCS upstream, and the other from the second MCS downstream of the IRES element. Following generation of the reporter constructs, $CHO_{MCAT\text{-}TAM2}$ cells were transduced using viral particles which carry the respective cDNAs (Engling et al., 2002) similar to the generation of cell lines used in the DHFR approach (see section 3.1.1, Fig. 3.2 and 3.3 as well as text and Material & Methods, sections 2.3.4 and 2.5.3). The sorting step to obtain clonal cell lines was performed using a FACSAria system (Becton Dickinson).

The interacting proteins used to establish the piggyback export analysis system are the F_c domain of the mouse immunoglobulin 2α (IgG2α) and the IgG binding domains of Protein A from *staphylococcus aureus*. They form a stable, non-covalently linked complex and are used in standard research techniques and purification procedures. A well known example is the twin affinity purification (TAP) method which is used to purifiy proteins present at low cellular level under native conditions which, in turn, allows purification of associated proteins or oligomeric complexes. It consists of fusion of the TAP-tag to the target protein and the introduction of the construct into

cells for expression. The TAP-tag consists of the IgG binding domains of Protein A and the calmodulin binding peptide (CBP). These two tags are fused in tandem separated by a TEV (tobacco virus etch) protease cleavage site (Carrington and Dougherty, 1988). The fusion protein and associated components are recovered from cell lysates by affinity purification using an IgG-matrix. After washing, TEV protease is added to release the bound material and removed in a final purification step (Puig et al., 2001; Rigaut et al., 1999). The IgG2α domain and Protein A were used to generate the following piggyback interaction constructs.

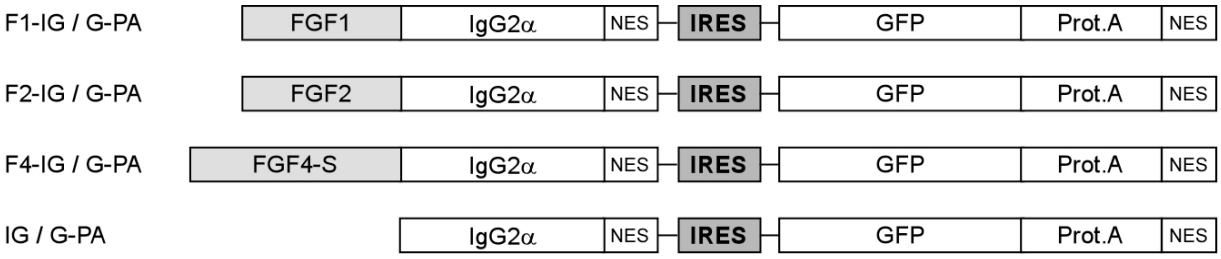

Fig. 3.16 Schematic overview of piggyback cDNA constructs.

FGF1 = *fibroblast growth factor* 1, FGF2 = *fibroblast growth factor* 2, FGF4-S = *fibroblast growth factor* 4 containing a classical signal sequence, IgG2α = F_c domain of the mouse immunoglobulin 2α, GFP = *green fluorescent protein*, Prot.A = *Protein A* from staphylococcus aureus, NES = nuclear export signal derived from subunit 9 of the F_0 ATPase from neurospora crassa, IRES = internal ribosome entry site, orientation = left to right corresponds to 5' to 3'

The first reporter construct, termed F1-IG / G-PA, consists of FGF1 fused to the IgG2α domain with a C-terminal nuclear export signal (NES). FGF1 is an unconventionally secreted protein that belongs to the family of heparin binding fibroblast growth factors. The calculated molecular mass for this construct is 46 kDa. The second fusion protein, termed F2-IG / G-PA, is comprised of FGF2 fused to the IgG2α domain with the NES sequence at the C-terminus. It is also a member of the heparin binding growth factor family and closely related to FGF2. FGF2 is the main focus of the current study investigating its folding state during unconventional secretion. The calculated molecular mass is 46 kDa. The next construct, termed F4-IG / G-PA, consists of FGF4 with a classical signal sequence (FGF4-S) fused to GFP C-terminally extended with the NES sequence. FGF4 also belongs to the FGF protein family but is secreted via the classical secretory pathway mediated by the ER and the Golgi system. It was employed in the piggyback export analysis approach to function as a control since no co-export of the piggyback interaction partner is expected. FGF4-S is inserted co-translationally into the ER and transported to the

cell exterior by membrane-bound vesicles passing the Golgi apparatus. The non-secreted interaction partner GFP-Protein A is synthesized on free ribosomes in the cytosol so that the two molecules are intracellularly separated and can not associate to form the complex. The calculated molecular mass for FGF4-S-IgG2α-NES is 51 kDa. The fourth reporter construct is termed IG / G-PA and was designed without a FGF moiety to function as an additional control which should not be secreted since no targeting motif for the classical secretory pathway or an unconventionally secreted protein moiety is present. Unlike the FGF4-S-containing construct the complex of the two interacting molecules can form since both proteins are synthesized in the cytosol and none is sequestered into a membrane-bound organelle. The calculated molecular mass is 29 kDa.

The non-exported interaction partner used to generate the different reporter constructs consists of GFP fused to Protein A C-terminally extended by a NES sequence. It was cloned into the MCS downstream of the IRES element which results in expression of a discrete protein from the bicistronic mRNA. It has a calculated molecular mass of 44 kDa. GFP was used to generate the non-exported interaction partner because it can easily be monitored due to its fluorescence activity. Moreover, it can be detected by affinity-purified polyclonal anti-GFP antibodies as described by engling et al. (Engling et al., 2002) which are available in our laboratory (see section 3.1.1.1 and Material & Methods section 2.1.9). The reason for the addition of the NES (nuclear export signal) sequence (Fukuda et al., 1996) to all reporter molecules is to minimize the nuclear localization observed in the analysis of the DHFR fusion constructs. The addition of the NES sequence results in an increase of the pool of cytosolic reporter molecules being available for export and complex formation.

As a standard control a cell line which expresses FGF2-GFP (Engling et al., 2002) in a doxicycline-dependent manner was used. The reporter construct was also extended with a C-terminal NES sequence to reduce nuclear localization. The corresponding cell line was generated by modifying the construct in the vector pRevTRE2 by adding the C-terminal NES using molecular cloning techniques. Following viral transduction of $CHO_{MCAT\text{-}TAM2}$ cells (Engling et al., 2002) *FACS*-based sorting was performed to obtain single clones as described in section 3.1.1 (see also Material & Methods, sections 2.3.4 and 2.5.3). The generated clonal cell line was

termed CHO FGF2-GFP-NES and the reporter protein has a calculated molecular mass of 46 kDa.

3.2.2 Biochemical Analysis of Doxicycline Dependent Protein Expression

To test for doxicycline-dependent protein expression cells being transduced with the reporter constructs were grown in the absence or presence of 1 µg/ml doxicycline for 18 h. Cell lysates prepared in SDS sample buffer were analyzed by SDS-PAGE and Western blot. Fusion constructs containing the IgG2α domain were detected using Alexa 680-coupled goat anti-mouse IgG antibodies normally used as secondary antibodies. To detect the piggyback interaction partner containing the GFP moiety affinity-purified anti-GFP antibodies in combination with secondary Alexa 680-coupled antibodies were used. Alexa 680-labelled proteins were visualized using an *Odyssey infrared imaging system*.

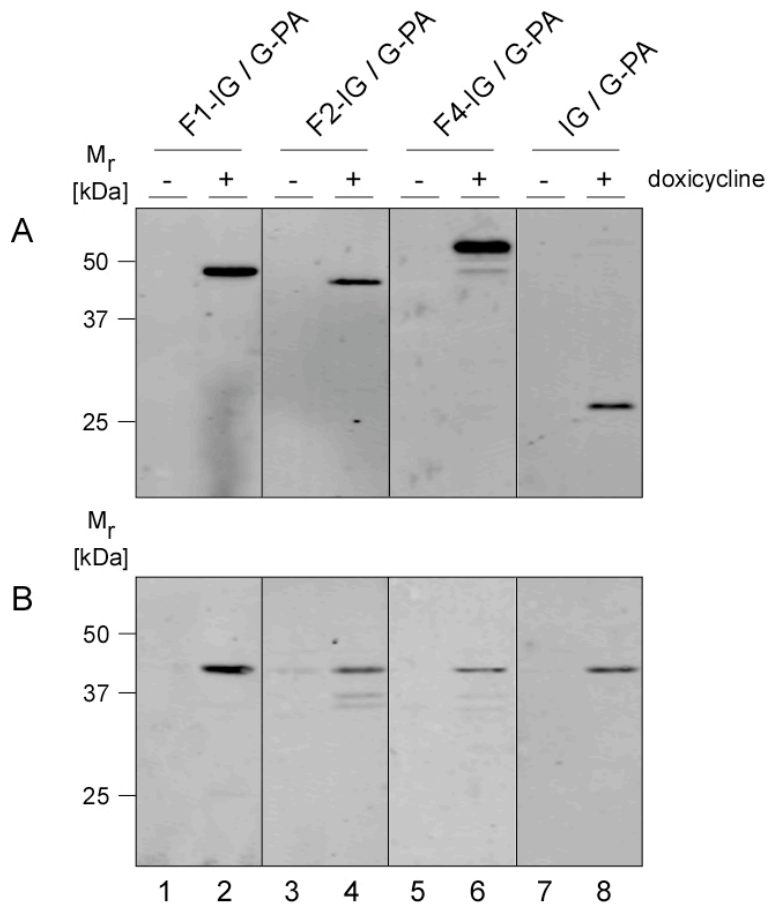

Fig. 3.17 Western Blot analysis of doxicycline-dependent protein expression.

Reporter cell lines were grown in the absence or presence of 1 µg/ml doxicycline for 18h. Cell lysates were separated on 13% SDS gels followed by Western blot transfer. Detection was performed applying either anti-mouse IgG antibodies (panel A) or affinity-purified anti-GFP antibodies (panel B). For the analysis of each cell line material derived from 10,000 cells was loaded.

The analysis revealed that the expression of the two discrete reporter proteins from one mRNA is functional. Detection of reporter proteins employing anti-mouse IgG antibodies showed doxicycline-dependent protein expression of the IgG2α-containing reporter molecules (Fig 3.17, panel A, lanes 2, 4, 6 and 8). Additionally, if the samples are analyzed using anti-GFP antibodies doxicycline-dependent protein expression of GFP-containing reporter proteins is observed (Fig 3.17, panel B, lanes 2, 4, 6 and 8). All GFP-containing fusion proteins migrate corresponding to their calculated molecular mass of 44 kDa as compared to the protein standard (Fig. 3.17, panel B, lanes 2, 4, 6 and 8) although being expressed in the different piggyback cell lines. Some samples contain post lysis degradation products visible as faint bands migrating faster than the reporter construct.

The IgG2α-containing fusion proteins also migrate corresponding to their calculated molecular masses as compared to the protein standard. The only exception is the

FGF1 construct, which was expected to migrate alike the FGF2 fusion protein because the polypeptide chains are of the same length (Fig. 3.17, panel A, lanes 2 and 4). The observation made might be due to the different amino acid composition of the two molecules, which can result in a different migration behaviour in the electric field during SDS-PAGE. The analysis of the FGF4-containing fusion constructs revealed additional bands migrating faster than the one with the highest intensity (Fig. 3.17, panel A, lane 6). These might correspond to differently glycosylated forms of the molecule which result from ER- and Golgi-associated posttranslational modifications providing first evidence that the FGF4 construct indeed enters the classical secretory pathway.

3.2.3 Analysis of Doxicycline Dependent Protein Expression Based on Flow Cytometry

Cells grown in the absence or presence of 1 µg/ml doxicycline were prepared for *FACS* analysis using the plate labelling technique (see Materials & Methods, section 2.5.2). To detect the IgG2α fusion proteins on the cell surface, cells were labelled using anti-mouse IgG allophycocyanin-coupled antibodies. Dead cells were stained using propidium iodide to exclude them from the subsequent data evaluation. Samples were analyzed in a FACSCalibur flow cytometer (Becton Dickinson).

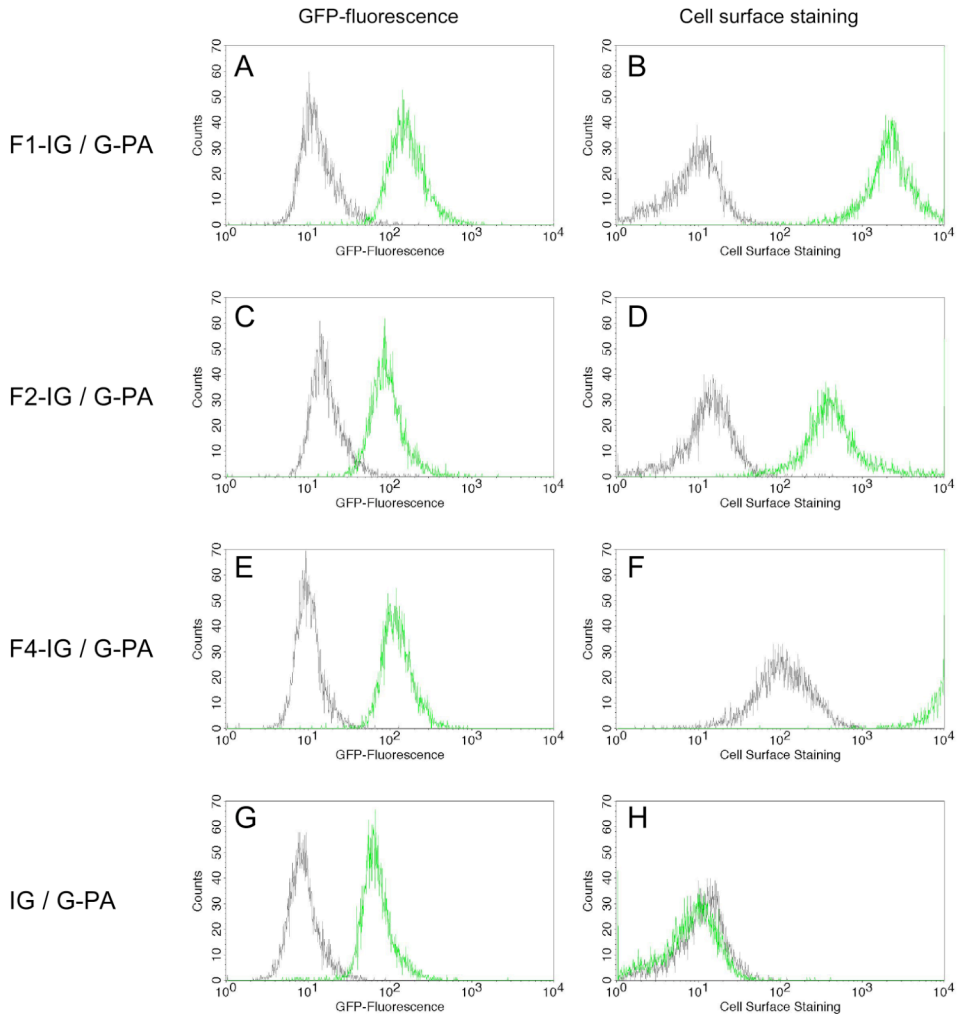

Fig. 3.18 Quantitative analysis of doxicycline-dependent protein expression and secretion based on *FACS*.

F1-IG / G-PA cells (A, B), F2-IG / G-PA cells (C, D), F4-IG / G-PA cells (E, F) and IG / G-PA (G, H) were grown in the absence (grey curves) and presence (green curves) of 1 µg/ml doxicycline for 18h. Cells were processed employing plate labelling using anti-mouse IgG allophycocyanin-coupled antibodies (B, D, F and H). Living cells were analyzed for GFP fluorescence and cell surface staining simultaneously in a FACSCalibur flow cytometer (Becton Dickinson).

All cell lines show doxicycline-dependent protein expression detectable by an increase of the GFP fluorescence upon addition of doxicycline. Grey curves correspond to cells grown in the absence of doxicycline, green curves to cells grown in the presence of doxicycline (Fig. 3.18, panels, A, C, E and G)

The analysis of cell surface staining revealed that FGF1, FGF2 and FGF4 fusion constructs are exported. There is a significant increase in the allophycocyanin-derived fluorescence detectable when comparing grey to green curves. This demonstrates that protein export occurs since extracellular, cell surface bound material is detectable (Fig 3.18, panels B, D, and F). As expected the cell line

expressing the IG / G-PA fusion proteins exhibits no cell surface staining since the reporter protein IgG2α-NES is not exported due to the absence of a FGF moiety (Fig. 3.18, panel H).
The F4-IG / G-PA construct shows an elevated export efficiency compared to the FGF1- and FGF2-containing reporter construct. Moreover, the cell surface staining signal is already increased compared to the other cell lines in the absence of doxicycline. The reason for this increase under doxicycline-free conditions might be that the cells express the reporter construct on a very low basic level even without being induced. Since the export via the ER/Golgi pathway occurs efficiently a part of this basic level population might already be exported. Upon addition of doxicycline the obtained signal shifts to the upper limit of the *FACS* detection range so that the green curve starts at the end of the x-axis and is out of range and thus can not be depicted.

The analysis of the generated cell lines employing Western blot and flow cytometry revealed that the reporter constructs are expressed in a doxicycline-dependent manner. Furthermore, both piggyback interaction proteins are expressed simultaneously from one mRNA in all cell lines. A *FACS*-based analysis revealed that all FGF-containing reporter constructs are exported and localize to the cell surface.

3.2.4 Rationale of the Piggyback System

The basis for the piggyback export system is the formation of a complex consisting of the exported fusion protein FGF2-IgG2α and the interaction partner GFP-Protein A. The complex is formed based on the correct three-dimensional structure of the reporter molecules which leads to a non-covalent interaction of the IgG2α domain with the IgG binding domains of Protein A. This association has to occur prior to export inside the cell.

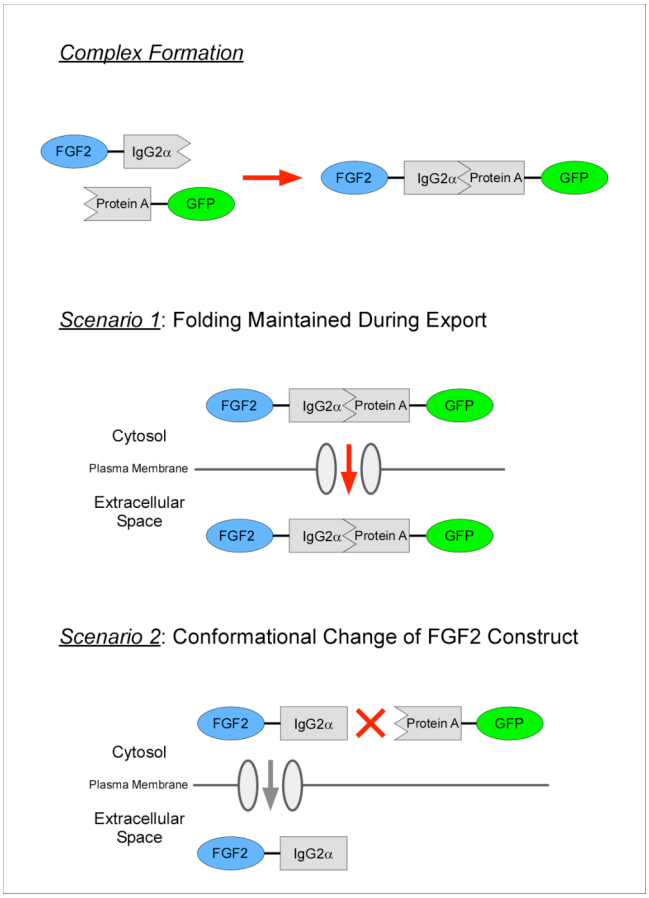

Fig. 3.19 Schematic overview: Rationale of the piggyback export system.

FGF2 = *fibroblast growth factor* 2, IgG2α = F_c domain of the mouse immunoglobulin 2α, GFP = *green fluorescent protein*, Protein A = *Protein A* from staphylococcus aureus.

When the reporter molecules associate the unidentified targeting motif of FGF2 directs the complex to the unconventional export machinery which mediates membrane translocation. In scenario 1 export occurs without conformational changes of the reporter molecules. The interaction between IgG2α and Protein A is not disrupted and GFP-Protein A is detectable outside of the cell (Fig. 3.19, Scenario 1). As depicted in scenario 2 the conformation of the FGF2-containing fusion protein is changed. The complex is disrupted and reporter construct FGF2-IgG2α is exported alone. GFP-Protein A can not be detected in th extrcellular space.

To be able to analyze the co-export of the interacting molecules the formation of the complex inside the cell has to be verified. If the two reporter proteins do not interact prior to export no clear conclusions can be drawn regarding the folding state of FGF2 during membrane translocation. To analyze complex formation two independent experimental strategies were employed. First, a co-immunoprecipitation analysis was

performed and in second approach the reporter proteins were subjected to gel filtration employing FPLC analysis techniques.

3.2.5 Co-Immunoprecipitation Analysis of Piggyback Complex Formation

In a co-immunoprecipitation experiment one component of the piggyback complex is selectively bound by specific antibodies immobilized on sepharose-beads whereas the second component is not recognized and can not bind to the beads on its own. This assay analyzes complex formation because only if an interaction between the two complex building molecules takes place both are co-precipitated by this approach. Cell lysates of the piggyback cell lines grown in the presence of 1 µg/ml doxicycline for 18 h were prepared. A detergent-free buffer and mechanical lysis employing sonication and sample clearance by centrifugation were used in order not to disrupt the complexes. The samples were incubated with Protein A coupled to sepharose beads to which the IgG2α reporter molecules can bind. To precipitate F1- and F2-IG / G-PA from samples additionally anti-FGF2 antibodies immobilized on sepharose beads were employed. These antibodies recognize both, FGF2 and FGF1, with high specificity (see Materials & Methods, sections 2.1.9, 2.4.10 and 2.4.11).

After extensive washing steps the bound material was eluted from the beads using SDS sample buffer and analyzed by SDS-PAGE and Western blot. IgG2α-containing reporter constructs were detected using anti-mouse IgG Alexa 680-coupled antibodies which were visualized using the *Odyssey infrared imaging system*. The detection of GFP-containing constructs was performed employing primary anti-GFP and secondary RG16-HRP-coupled antibodies, which only detect native antibody molecules. The use of the RG16 secondary antibodies excludes the detection of antibodies derived from immunoprecipitation procedure so that only precipitated reporter proteins are visualized on the Western blot using the chemiluminescence-based ECL system and x-ray film detection. Additionally, input samples consisting of cell lysates not incubated with beads were analyzed in order to probe for expression of the interacting reporter molecules and to have an intrinsic marker for each reporter construct to compare precipitated material.

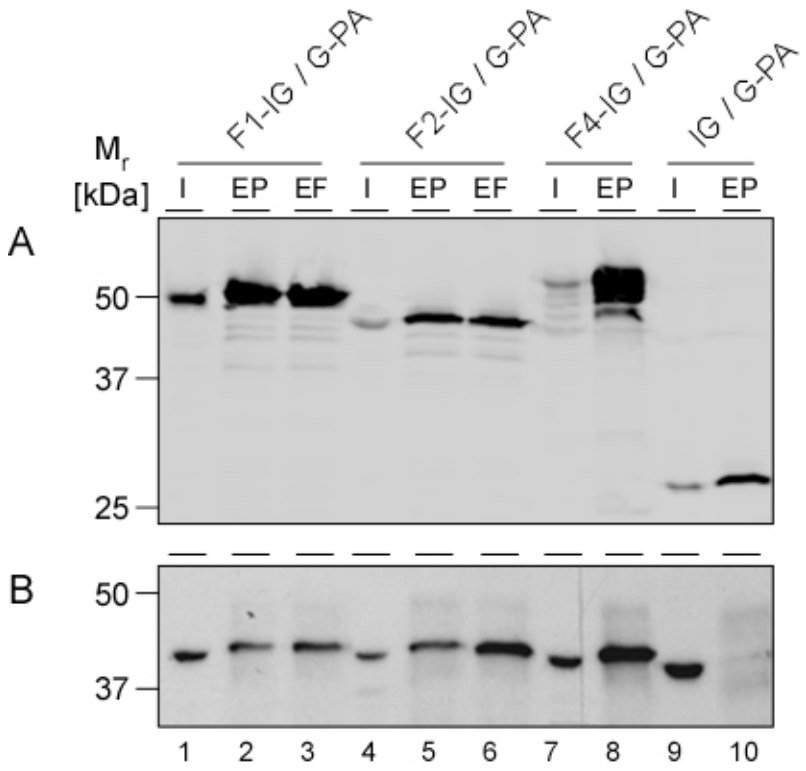

Fig. 3.20 Co-immunoprecipitation analysis of piggyback interactions.

Reporter cell lines were grown in the presence of 1 µg/ml doxicycline for 18 h. Cell free supernatants were prepared employing detergent-free buffer combined with mechanical lysis and cleared by centrifugation. Samples were incubated with Protein A-sepharose-beads or in the case of F1- and F2-IG / G-PA with Protein A-sepharose beads decorated with affinity-purified anti-FGF2 antibodies for 3 h at 4°C. Bound material was eluted using SDS sample buffer and analyzed by SDS-PAGE (13% gels) and Western blotting. Reporter molecules were detected applying anti-mouse IgG HRP-coupled antibodies (panel A) or primary affinity-purified anti-GFP and secondary RG16-HRP-coupled antibodies (panel B). Input samples, not incubated with beads, were loaded additionally. I = input samples (1% of total protein), EP = eluates from Protein A beads (10% of total protein), EF = eluates from sepharose beads decorated with anti-FGF2 antibodies immobilized (10% of total protein).

The analysis employing anti-mouse IgG antibodies revealed that all piggyback constructs containing the IgG2α domain bind to Protein A-sepharose beads (Fig. 3.20, panel A, lanes 2, 5, 8 and 10) or to beads decorated with anti-FGF2 antibodies in the case of F1- and F2-IG / G-PA (Fig. 3.20, panel A, lanes 3 and 6) when compared to the untreated input samples (Fig. 3.20, panel A, lanes 1, 4, 7 and 9). This demonstrates that it is possible to specifically precipitate the IgG2α-containing reporter molecules.

For the construct F1-IG / G-PA it could be shown that the interacting reporter constructs form a complex because the interaction partner GFP-Protein A-NES can be co-precipitated using Protein A-sepharose beads or sepharose beads decorated with anti-FGF2 antibodies (Fig. 3.20, panels A and B, lanes 2 and 3).

The analysis of the F2-IG / G-PA reporter construct also confirms an interaction between the two molecules demonstrated by co-precipitation of the GFP-containing reporter protein using Protein A-sepharose beads or beads decorated with anti-FGF2 antibodies (Fig. 3.20, panels A and B, lanes 5 and 6).
The formation of the complex consisting of FGF4-S-IgG2α and GFP-Protein A could also be verified since the GFP-containing piggyback interaction protein is co-precipitated using protein A-sepharose beads (Fig. 3.20, panels A and B, lane 8).
When analyzing the IG / G-PA reporter construct, binding of the IgG2α domain to the Protein A-sepharose beads was detectable (Fig. 3.20, panel A, lane 10). However, co-precipitation of GFP-Protein A-NES could not be observed. Thus, it is not possible to analyze the formation of the complex IgG2α and GFP-Protein A in this experiment. On the other hand this demonstrates specificity of the employed method with regard to the results obtained for the other reporter constructs. The detected co-precipitation must be due to complex formation and can not be an experimental artefact or the result of unspecific binding.

3.2.6 Probing Complex Formation Employing Gel Filtration

In order to analyze complex formation using an independent method, cell lysates of the reporter cell lines were subjected to gel filtration using a size exclusion column and an analytical fast performance liquid chromatography (FPLC) system (SMART, Amersham Biosciences, see Material & Methods, section 2.4.11).
The cell lines expressing the piggyback interaction reporter proteins and, as a control, cells expressing FGF2-GFP-NES were grown in the presence of 1 µg/ml doxicycline for 18h. Cell free supernatants were prepared avoiding the use of detergent to keep the proteins under native conditions. A mechanical lysis procedure was employed consisting of a sonication step using a low pulse-intensity and freeze-thaw-cycles (see Material & Methods, section 2.4.2). 50 µl of each protein sample were separated according to the molecular mass of the proteins by the use of superdex 200 beads in a Tricorn 5/150 column (Amersham Pharmacia, length: 150 mm, diameter: 5 mm, bed volume: 3 ml). Superdex 200 beads retain proteins from 10 to 600 kDa on the column.

To allocate proteins of different molecular masses to respective fractions a premixed marker protein solution (Gel Filtration Chromatography Standard, Bio-Rad) was subjected to separation by gel filtration using the freshly prepared column.

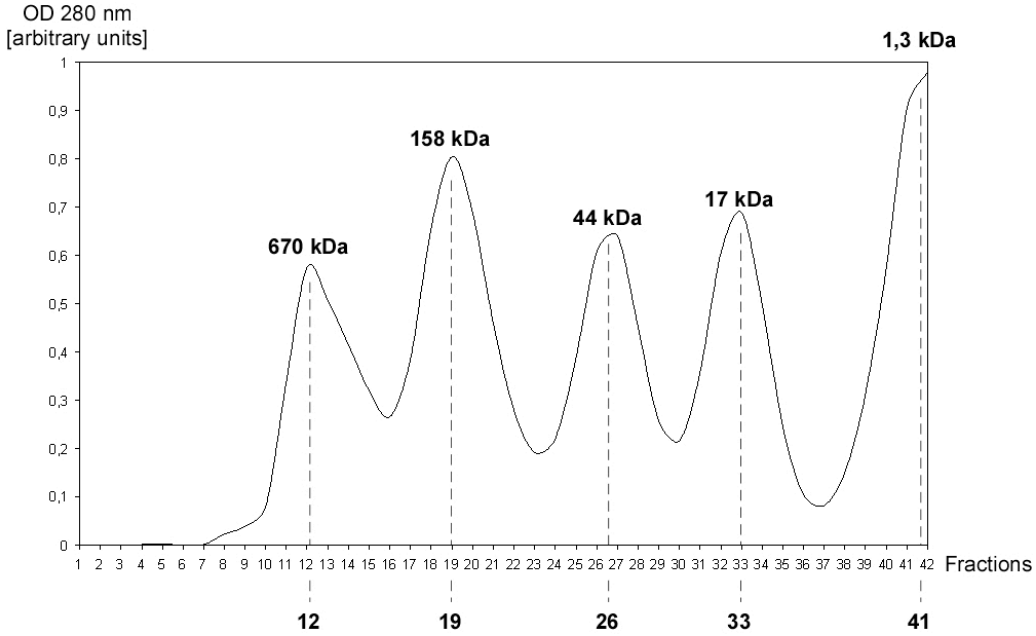

Fig. 3.21 Elution profile of marker proteins separated on a Superdex 200 column.

10 µl of a premixed marker proteins solution with defined sizes (Gel Filtration Chromatography Standard, BioRad) were fractionated by gel filtration in an analytical FPLC system (SMART, Amersham Pharmacia). Superdex 200 beads were used as matrix material to separate proteins by molecular mass in a range of 10 to 600 kDa. A column of 150 mm length and a diameter of 5 mm with a bed volume of 3 ml (Tricorn 5/150, Amersham Pharmacia) was employed. The absorbance at 280 nm is plotted against the eluted fractions.

The separation of the marker protein mixture showed that the proteins are retained differently on the gel filtration column according to their molecular masses and therefore elute in different fractions:

Protein	Size [kDa]	Fraction
Thyroglobulin	670	12
IgG	158	19
Ovalbumin	44	26
Myoglobin	17	33
Cyanocobalamin	1,3	41

Fig. 3.22 Table: Molecular masses of marker proteins (Gel Filtration Chromatography Standard, BioRad) and retention by Superdex 200 beads (Amersham Pharmacia) using a Tricorn 5/150 column (Amersham Pharmacia) in an FPLC system.

The obtained data were used as a standard in order to estimate molecular masses of proteins of the unknown samples when these elute from the column in their respective fractions.

The correlation of protein size and retention behaviour depending on the molecular mass (Fig.3.22) is shown graphically in the following diagram displayed as an exponential function corresponding to the curve fitting equation: $y = 8482{,}2e^{-0{,}2041x}$.

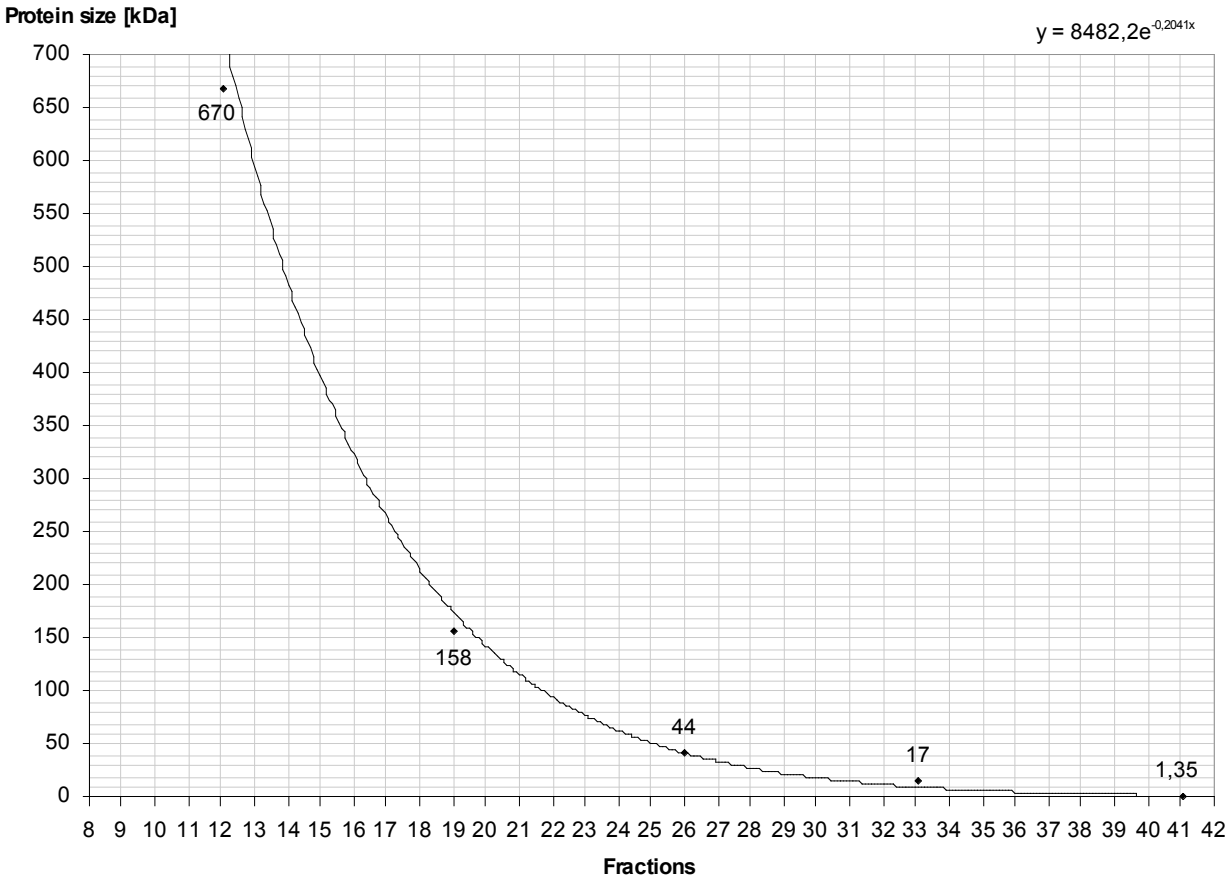

Fig. 3.23 Diagram: Relation of molecular masses of marker proteins to retention behaviour employing Superdex 200 beads.

Molecular masses of marker proteins in correlation to retention on a Superdex 200 matrix and elution in respective fractions is displayed as an exponential function (curve fitting equation: $y = 8482{,}2e^{-0{,}2041x}$). The calculation is based on the data obtained from the analysis of a marker protein mixture (Gel Filtration Chromatography Standard, BioRad) as described.

After determination of the standard 50 µl of the different cell free supernatants were run separately over the column and 42 fractions of each run were collected. Every second fraction starting from fraction 10 through fraction 28 was analyzed employing SDS-PAGE and Western blot analysis. To detect the IgG2α-containing reporter proteins anti-mouse IgG antibodies coupled to the fluorophore Alexa 680 were used. The GFP fusion constructs were detected by the use of primary anti-GFP and Alexa

680-coupled secondary antibodies. Visualization was performed using an *Odyssey infrared imaging system*.

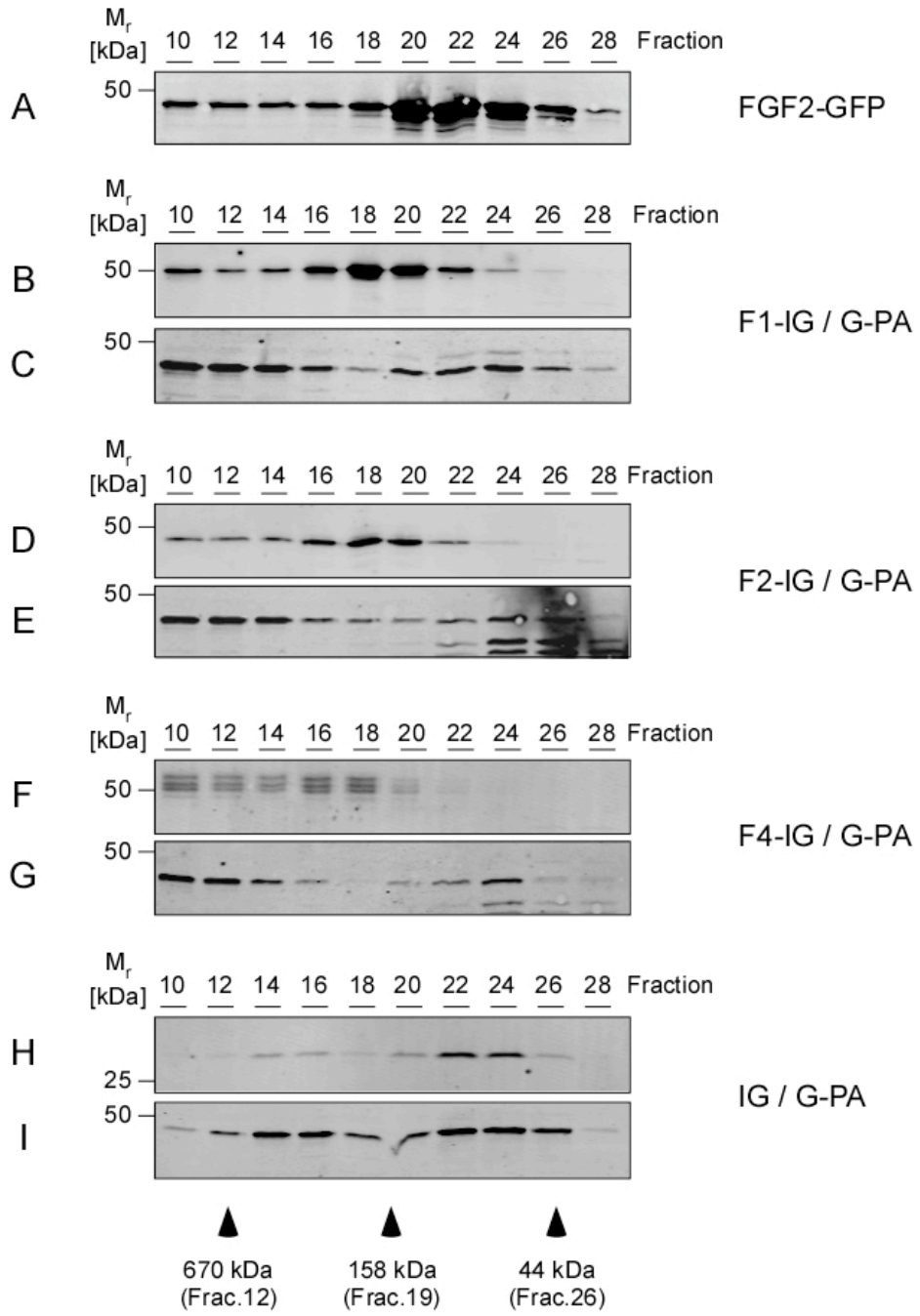

Fig. 3.24 Biochemical analysis of cell lysates fractionated by gel filtration.

Cell free supernatants of induced reporter cell lines were prepared using a detergent-free buffer, sonication and clearance by centrifugation (100,000 g, 45 min, 4°C). 50 µl of the samples were fractionated according to molecular mass using superdex 200 beads by FPLC (SMART system, Amersham). Every second fraction from 10 to 28 was analyzed on 13% SDS gels and Western blot. Per fraction 15% of eluted material was loaded. Detection of the reporter molecules was performed applying anti-mouse IgG Alexa 680-coupled antibodies (panels B, D, F, H) or affinity-purified anti-GFP and secondary Alexa 680-coupled antibodies (panels A, C, E, G, I). Visualization was performed using an *Odyssey infrared imaging system*.

To be able to interpret the data obtained from the gel filtration analysis it is important to known what are the calculated molecular masses of the reporter proteins and the putative complexes. If the reporter proteins associate into complexes they are retained on the column according to the molecular mass of the complex. Therefore, they elute in fractions which correspond to the molecular mass of the complex not in fractions which correspond to the molecular mass of the monomeric reporter protein. The putative complexes could be on the one hand heterodimers consisting of the IgG2α-containing reporters and the interaction partner GFP-Protein A-NES or on the other hand heterotetramers consisting of two heterodimers since the GFP moiety has the tendency to dimerize what would result in a tetramer formation. FGF2-GFP is expected to be either monomeric (46 kDa) or to form homodimers (92 kDa). The possible combinations and calculated molecular masses for the piggyback pairs are summarized in the following table.

Sizes [kDa]	monomer	heterodimer	heterotetramer
FGF1-IgG2α-NES	46	90	180
FGF2-IgG2α-NES	46	90	180
FGF4-S-IgG2α-NES	51	95	190
IgG2α-NES	29	73	146
GFP-Protein A-NES	44	-	-

Fig. 3.25 Table: Calculated molecular masses of reporter molecules and putative complexes.

Based on the amino acid sequence the molecular masses of the reporter molecules were calculated (Editseq software, DNA Star). Molecular masses of the putative heterodimers were calculated by addition of the molecular mass of an IgG2α-containing reporter molecule and GFP-Protein A-NES. Molecular masses of putative heterotetramers were determined by multiplying the values of the heterodimers by a factor of two.

The fractionation according to molecular mass of the construct FGF2-GFP, when detected with anti-GFP antibodies, showed the highest amount of protein in fraction 22. There is a clear elution-peak detectable compared to the other fractions (Fig. 3.24, panel A). This corresponds to a molecular mass of 80 to 90 kDa when compared to the retention behaviour of the marker proteins suggesting that most of the protein is found in a homodimeric state with a calculated molecular mass of 92 kDa.

When analyzing the fractions obtained from the sample F1-IG / G-PA employing anti-mouse IgG antibodies most of the protein elutes in fraction 18 (Fig. 3.24, panel B). This demonstrates a shift of the elution-peak compared to FGF2-GFP from fraction

22 to fraction 18 implying an increase in molecular mass. Fraction 18 corresponds to a molecular mass of 180 to 200 kDa which would match the calculated mass of a heterotetramer complex of 180 kDa. Also F2-IG / G-PA and F4-S-IG / G-PA exhibit an elution peak in fraction 18 when analyzed by anti-mouse IgG antibodies (Fig. 3.24, panels D and F). This shows that most of the proteins also seem to form heterotetramers because these have a calculated molecular mass of 180 or 190 kDa what would fit to the observed retention behaviour on the column. For the cell line F4-IG / G-PA the appearance of the elution peak in this mass range is an unexpected result. The construct FGF4-S-IgG2α-NES is expected to be co-translationally inserted into the ER due to the presence of a classical hydrophobic signal sequence. The interaction partner GFP-Protein A-NES is translated on free ribosomes in the cytosol and a complex is not expected to form because the molecules are spatially separated. However, a shift of the elution peak, which corresponds to the molecular mass of the complex, is observed and can only be explained by post lysis complex formation. If upon cell lysis the ER/Golgi system and the plasma membrane are disrupted the two proteins can associate since they are no longer sequestered in membrane-bound compartments. The construct IgG2α-NES without FGF moiety exhibits the elution-peak in fraction 23 corresponding to a molecular mass of 60 to 80 kDa (Fig. 3.24, panel H). This result implies that IgG2α-NES forms a heterodimer with the piggyback interaction partner which is expected to have a molecular mass of 73 kDa.

The analysis of the piggyback interaction partner GFP-Protein A-NES using anti-GFP antibodies showed an unexpected result. A large amount of protein is detectable in high molecular weight fractions. Especially F1-, F2- and F4-S-IG / G-PA show intense signals in fractions 10 and 12 which corresponds to a size of 670 kDa or even more (Fig. 3.24, panels C, E and G). This might be due to the formation of high molecular weight aggregates as a result of the lysis procedure that are not retained on the column. They are found in the exclusion volume, which is defined by the range of molecular masses that are retained. Proteins larger than 600 kDa and smaller than 10 kDa are not retained on the Superdex 200 matrix and pass through the column without associating to the beads. Therefore, they elute in the very first fractions. High amounts of protein are also found in the low molecular weight fractions 24 and 26 corresponding to a size of 60 to 40 kDa (Fig. 3.24, panels C, E and G). These fractions represent GFP-containing proteins not attached to other molecules but

rather being monomeric. Another quite unexpected finding is that the elution peaks for the IgG2α-containing reporter constructs and GFP-Protein A-NES are not present in the same fractions. This would have been expected if they were attached to each other in complexes. It might be possible that only a small portion of the overall GFP reporter molecules is actually present in the piggyback complexes and that the rest are free monomers or form high molecular weight aggregates as detected by the Western blot analysis visualized using GFP antibodies.

The analysis of the complex formation by a co-immunoprecipitation method and gel filtration shows that the piggyback complexes are formed to a significant extent. It was possible to co-precipitate GFP-Protein A-NES with Protein A-sepharose beads or with sepharose beads decorated with anti-FGF2 antibodies. The gel filtration analysis shows a shift of the elution peak to higher molecular weight fractions compared to FGF2-GFP-NES representing an increase in molecular mass as a result of complex formation. The elution peaks of the IgG2α-containing reporter constructs match with the expected molecular masses of the putative complexes demonstrating a stable interaction between the piggyback reporter proteins.

3.2.7 Export of Reporter Molecules as Analyzed by Flow Cytometry

Cells were cultivated in the absence or presence of 1 µg/ml doxicycline for 18 h and prepared for *FACS* analysis using the plate labelling technique (see Materials & Methods, section 2.5.2). Doublets of each sample were processed in order to simultaneously analyse the export of the IgG2α-containing reporter constructs and a possible co-export of the piggyback interaction partner GFP-Protein A-NES. The detection of the IgG2α-containing reporter constructs was performed using anti-mouse IgG allophycocyanin-coupled antibodies. The interaction partner GFP-Protein A-NES was detected employing primary anti-GFP antibodies and secondary allophycocyanin-coupled antibodies. Dead cells were stained using propidium iodide and thus could be excluded from the following evaluation. Immuno-labelled cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

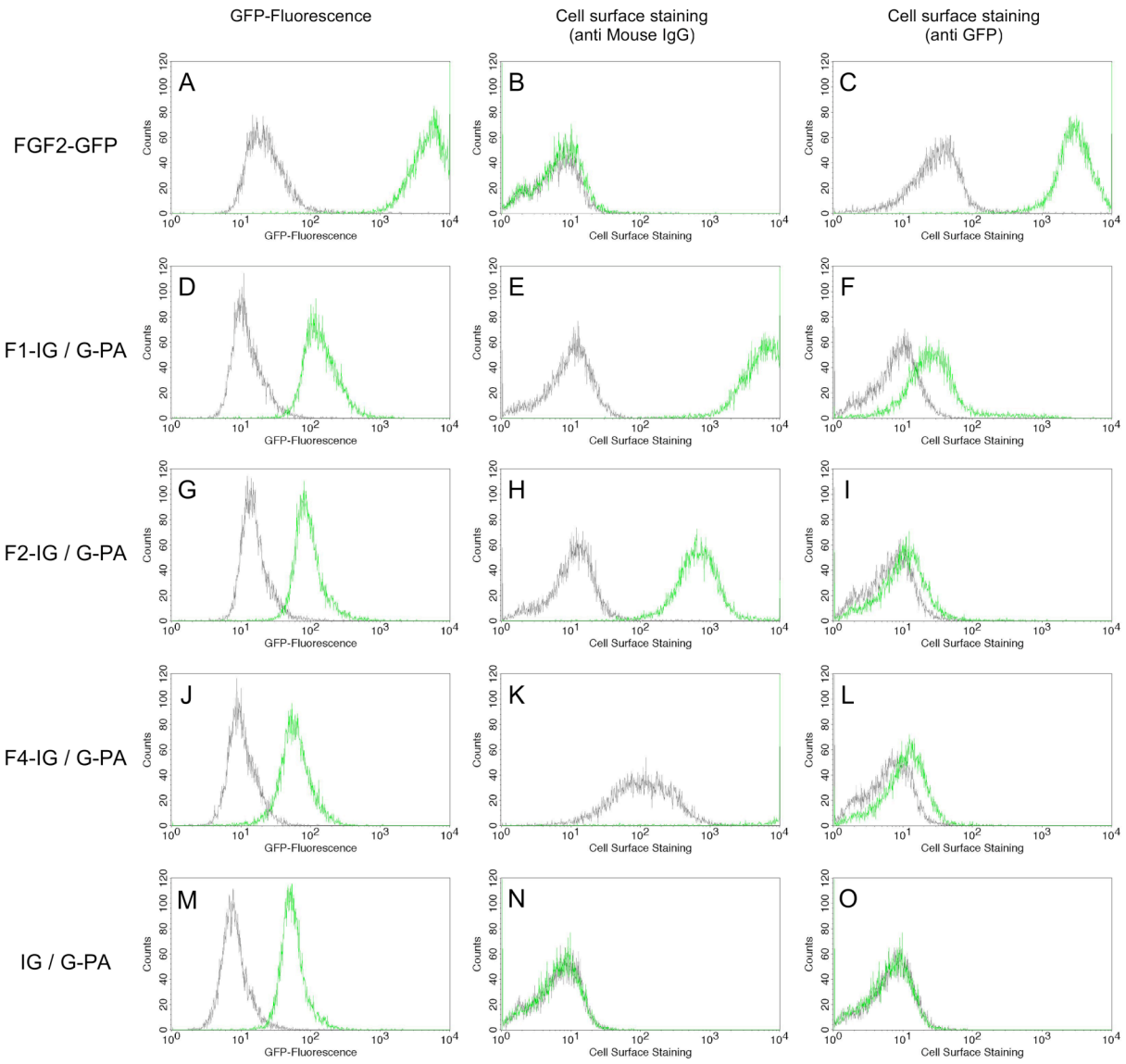

Fig. 3.26 Export of piggyback complexes analyzed by Flow Cytometry.

Cells grown in the absence (grey curves) and presence (green curves) of 1 µg/ml doxicycline for 18 h were prepared for *FACS* analysis applying plate labelling technique. Exported reporter molecules on the cell surface were detected by anti-mouse IgG allophycocyanin-coupled antibodies or primary anti-GFP and secondary allophycocyanin-coupled antibodies. Samples were analyzed in a FACSCalibur flow cytometer (Becton Dickinson).

All cell lines express their respective reporter constructs in the presence of doxicycline as shown by GFP fluorescence. Grey curves represent non-induced cells whereas green curves represent cells grown in the presence of doxicycline (Fig. 3.26, panel A, D, G, J and M).

Probing for cell surface staining using anti-mouse IgG antibodies showed no significant signal for the control cell line FGF2-GFP since no IgG2α domain is present in the reporter construct (Fig. 3.26, panel B, grey versus green curve). The cell lines F1-, F2- and F4-IG / G-PA instead show significant cell surface staining

signals in the presence of doxicycline (Fig. 3.26, panel E, H and K, green curves,) demonstrating an efficient export of their respective IgG2α-containing reporter constructs. The cell line F4-IG / G-PA shows export with a higher efficiency compared to F1- and F2-IG / G-PA as observed previously (see section 3.2.3, Fig. 3.18). The obtained values are at the upper limit of the *FACS* detection range higher than 10,000 arbitrary fluorescence units. The green curve is therefore not visible in the diagram as it begins at the end of the x-axis. The cell line IG / G-PA shows no cell surface staining signal when employing anti-mouse IgG antibodies. This findings suggest that the construct IgG2α-NES is not exported as expected because it does not contain a protein moiety with a targeting signal (Fig. 3.26, panel N).

When employing anti-GFP antibodies the FGF2-GFP control cell line shows a significant cell surface staining signal representing efficient export of the reporter construct (Fig. 3.26, panel C). F1-IG / G-PA exhibits a low cell surface staining signal of the piggyback reporter GFP-Protein A compared to FGF2-GFP although a shift of the peak in the presence of doxicycline is detectable (Fig. 3.26, panel F). Here one has to take into consideration that the piggyback reporter is expressed at a much lower level in comparison to FGF2-GFP-NES. This is a known phenomenon of the pRTi vector, since proteins translated from the MCS upstream of the IRES element are expressed with reduced efficiency compared to proteins being expressed from the MCS downstream of the IRES element for an unknown reason. The difference is also visible in the diagrams representing the GFP fluorescence. When comparing signals of the FGF2-GFP cell line (Fig. 3.26, panel A, green curve) to any of the piggyback cell lines (Fig. 3.26, panels D, G, J or M, green curves) one can see an enormous difference in fluorescence intensity of nearly two decades. This shows that the overall protein amount of GFP-containing reporter molecules is much lower. Therefore, it is problematic to directly compare the obtained cell surface staining signals, however those of the piggyback interaction partners are probably significant with regard to the low expression level. The analysis of the cell line F2-IG / G-PA shows nearly the same result. The observed signal using the anti-GFP antibody is even lower compared to F1-IG / G-PA (Fig. 3.26, panel I). It is slightly above background fluorescence in the absence of doxicycline but displays a shift of the green curve. Also the F4-IG / G-PA cell line shows a low signal of exported GFP-containing interaction partner comparable to F2-IG / G-PA (Fig. 3.26, panel L). This is an unexpected finding because FGF4-S-IgG2α-NES is exported via the classical

secretory pathway. The complex should not be formed because the IgG2α-containing reporter molecule is cotranslationally inserted into the ER and the GFP-containing interaction partner is translated in the cytosol. The control cell line IG / G-PA shows no cell surface staining at all employing anti-GFP antibodies. The green and grey curve match for induced and not induced cells and no difference can be observed (Fig. 3.26, panel O). This finding demonstrates specificity of the assay. The signals obtained when analyzing the other cell lines must be a result of co-exported material on the cell surface.

To statistically analyze export of the different reporter molecules the *FACS* experiment was repeated three times. The obtained values for cell surface staining analyzed with either anti-mouse IgG or anti-GFP antibody were averaged. The background fluorescence of untransfected $CHO_{MCAT\text{-}TAM2}$ cells was subtracted from all values to obtain data representing the fluorescence derived from the expression of the reporter constructs. In the case of negative values after subtraction of the background fluorescence the values were set to zero. This shows that absolutely no export occurred.

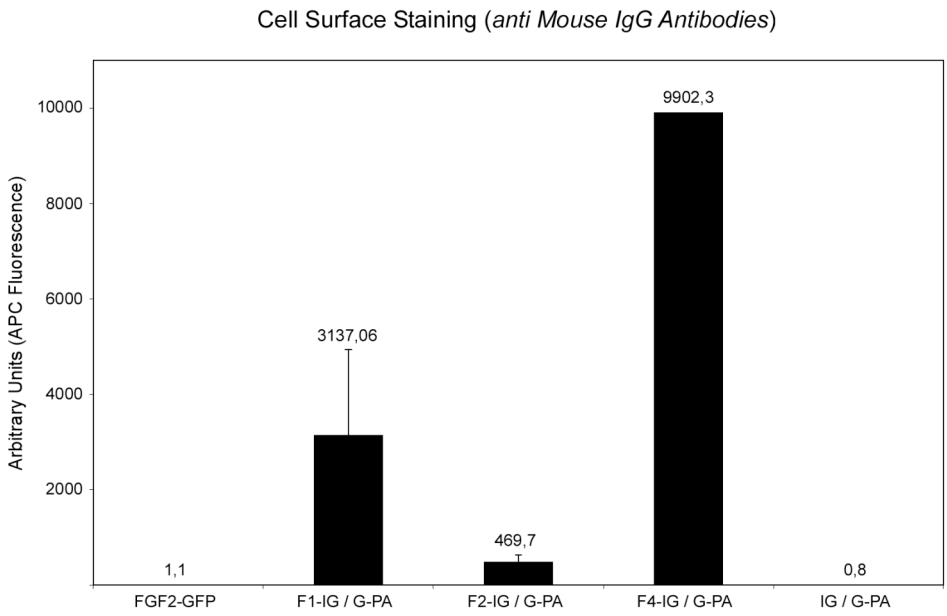

Fig. 3.27 Statistical analysis of secreted IgG2α-containing reporter molecules.

The *FACS* analysis was performed three times. Export of the reporter molecules was determined by measuring cell surface staining using anti-mouse IgG allophycocyanin-coupled antibodies. The background fluorescence of untransfected $CHO_{MCAT\text{-}TAM2}$ was subtracted and the values were averaged. Negative values after subtraction were set to zero. Standard deviation was calculated and is displayed as error bars (n=3).

The diagram in Fig. 3.27 displays cell surface staining of the different reporter cell lines detected with the anti-mouse IgG antibody. By the use of this antibody the secretion of the actively exported piggyback partner is measured.

FGF2-GFP cells exhibit no fluorescence since the fusion protein does not contain the mouse IgG2α domain. This also demonstrates specificity of the antibody used since no background signal is observed and functions as a negative control. F1-IG / G-PA cells show a significant cell surface staining signal of about 3100 arbitrary fluorescence units demonstrating efficient export of the FGF1 construct. F2-IG / G-PA cells also show a significant surface staining signal of about 470 arbitrary fluorescence units. This construct is also actively exported by the cells. The cell line F4-IG / G-PA exhibits the highest cell surface staining signal of the IgG2α-containing reporter constructs. The obtained values of all three experiments are at the limit of the *FACS* detection range of about 10,000 arbitrary fluorescence units. Since the FACS does not display signals higher than 10,000 arbitrary fluorescence units the values obtained in the three independent experiments were displayed as 9910,46 arbitrary fluorescence units. The FACS system calculates the median values for each sample which take into account the signal of each cell measured and the number of cells showing the signal. Therefore, the signal is not shown as the maximum of 10,000 arbitrary fluorescence units. After background subtraction the values are exactly 9902,3 arbitrary fluorescence units and, therefore, calculation of the standard deviation is not possible. The construct consisting only of the IgG domain (IG / G-PA) is not detectable on the cell surface resembling the fact that it is not secreted since it lacks a FGF fusion domain to mediate export or binding.

The analysis employing anti-mouse IgG antibodies demonstrates that all constructs consisting of a growth factor moiety fused to the IgG2α domain are actively exported by the cells and can be detected on the cell surface.

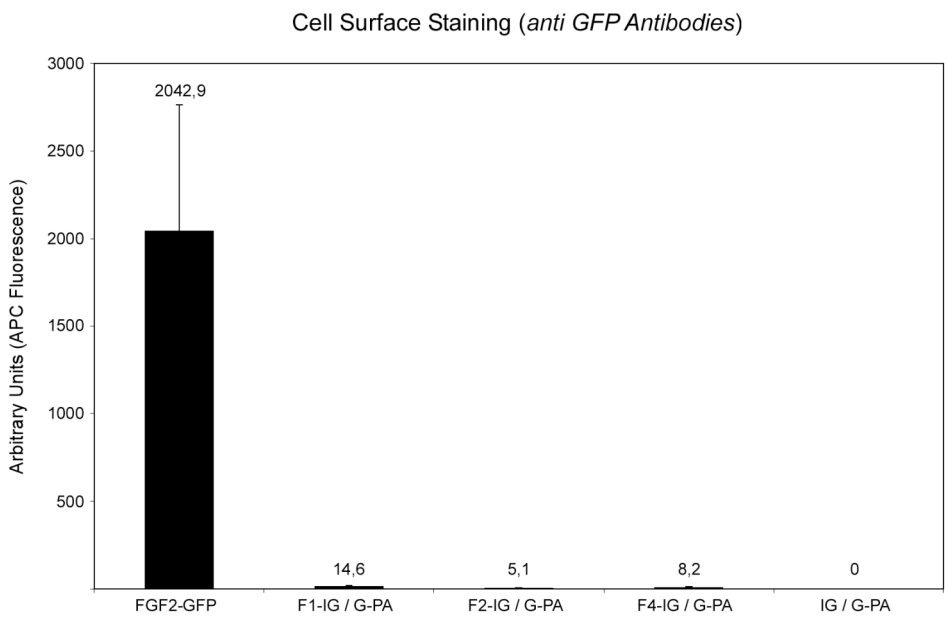

Fig. 3.28 Statistical analysis of secreted GFP-containing reporter molecules.

The *FACS* analysis was performed three times. Export of the reporter molecules was determined by measuring cell surface staining using anti-GFP antibodies and allophycocyanin-coupled secondary antibodies. The background fluorescence of untransfected $CHO_{MCAT\text{-}TAM2}$ cells was subtracted and the values were averaged. Negative values after subtraction were set to zero. Standard deviation was calculated and is displayed as error bars (n=3).

The analysis of the cell lines employing anti-GFP antibodies revealed that the reporter construct FGF2-GFP is exported efficiently and can be detected on the cell surface (Fig. 3.28).

Analysis of the piggyback cell lines shows that only a small portion of the GFP-containing piggyback interaction partner is present on the cell surface. The cell lines which are expected to export the complex, F1-IG and F2-IG / G-PA, and the cell line F4-IG / G-PA which should not show co-export display low signals of cell surface bound GFP-Protein A-NES. The control cell line IG / G-PA shows no export of the GFP-containing interaction partner (Fig. 3.28). Against the background of the strongly different expression levels discussed before this findings suggest that the piggyback interaction partner GFP-Prot A-NES is co-exported to a certain extent.

3.2.8 Biochemical Analysis of Piggyback Export

To further probe for cell surface localization of the reporter molecules employing a biochemical method, cells expressing the various reporters were analyzed in a biotinylation assay. For this purpose cells expressing the reporter proteins were

incubated with a membrane impermeable biotinylation reagent (EZ-link Sulfo-NHS-SS-biotin, Pierce) after washing and removal of the growth medium. The biotinylation reagent binds covalently to all surface proteins via the ε-amino group of all accessible lysine residues. After preparing cell lysates using a detergent-containing buffer, biotinylated proteins can be purified applying streptavidin beads. It is then possible to compare the biotinylated and non-biotinylated fraction of the protein employing SDS-PAGE and Western blot analysis. Biotinylated proteins, which can be recovered from streptavidin beads, represent exported material which was associated with the cell surface. The non-biotinylated proteins separated from the biotin-reagent inside the cell correspond to non-secreted material. For this purpose samples taken before (input) and after streptavidin purification (eluate) were analyzed. To detect the reporter constructs either anti-mouse IgG Alexa 680-coupled antibodies or primary anti-GFP and secondary Alexa 680-coupled antibodies were used. All Western blots were analyzed in an *Odyssey infrared imaging system*.

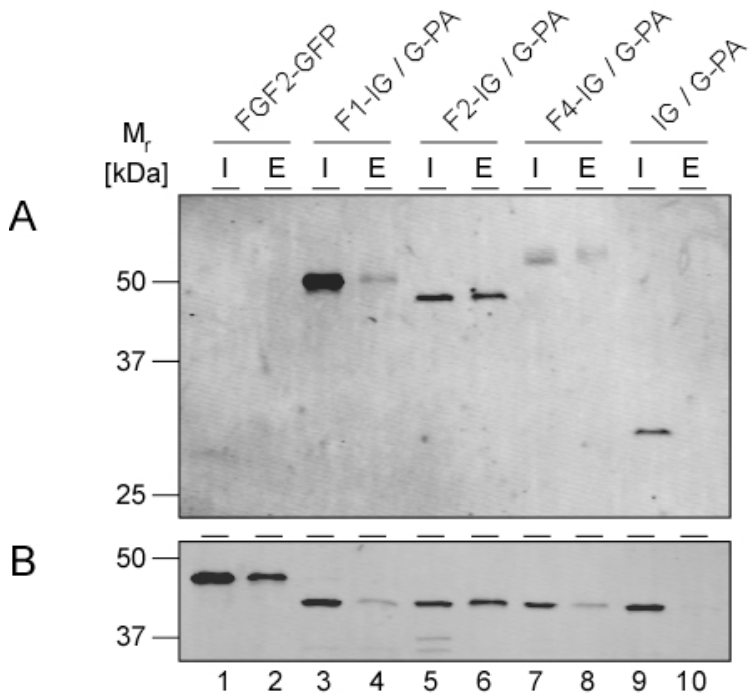

Fig. 3.29 Biochemical analysis of piggyback export applying biotinylation.

Cells expressing the reporter constructs were incubated with a membrane impermeable biotinylation reagent (30 min, 4°C). Lysates were prepared using a detergent-containing buffer, sonication and cleared by centrifugation. An input sample was saved and the total protein concentration was determined. Samples were incubated with streptavidin beads (1 hour, room temperature, constant shaking), followed by extensive washing steps. Bound material was eluted using SDS sample buffer. Input (1% of total protein) and eluate (10% of total protein) were analyzed on 13% SDS gels followed by Western blot transfer. Detection was performed using anti-mouse IgG Alexa 680-coupled antibodies (panel A) or primary anti-GFP and secondary Alexa 680-coupled antibodies (panel B). Visualization was performed in an *Odyssey infrared imaging system*.

The control cell line FGF2-GFP exhibits export of the reporter construct shown by a significant signal of biotinylated protein in the eluate fraction when using anti-GFP antibodies (Fig. 3.29, panel B, lane 2). The band of biotinylated protein displays half the intensity compared to the band of the non-biotinylated fraction observed when analyzing the input sample. This corresponds to an amount of 0.5% of total cellular protein because when analyzing the input sample 1% of total cellular protein was loaded as quantified by protein concentration determination (Fig. 3.29, panel B, lane 1). The analysis employing anti-mouse IgG antibodies displays no signal since no IgG domain is present in the reporter construct (Fig. 3.29, panel A, lanes 1 and 2). This shows a high specificity of the anti-mouse IgG antibody since no cross-reactivity can be observed.

When analyzing the F1-IG / G-PA reporter construct one can see a low signal of the reporter protein on the cell surface when using anti-mouse IgG antibodies compared to the overall protein in the input sample (Fig. 3.29, panel A, lane 3 and 4). A small population of the reporter construct seems to be exported compared to the input sample. The analysis using anti-GFP antibodies revealed that probably also a small fraction of the piggyback reporter GFP-Protein A-NES is co-exported visible as a faint band in the eluate fraction (Fig. 3.29, panel B, lane 4).

The reporter construct F2-IG / G-PA shows signals of the same intensity in the input and eluate fraction detectable by anti-mouse IgG antibodies (Fig. 3.29, panel A, lanes 5 and 6). This demonstrates that a high amount of the reporter construct is exported. The same observation is made when analyzing the piggyback interaction partner (Fig. 3.29, panel B, lanes 5 and 6). The interaction partner seems to be co-exported to a high extent.

The reporter molecule FGF4-IgG2α-NES shows signals of low intensity in the input and eluate fractions. The bands are not distinct and represent differently glycosylated forms of the molecule (Fig. 3.29, panel A, lanes 7 and 8) as observed previously (see section 3.2.2, Fig. 3.17). The amount of expressed protein seems to be reduced when compared to the other reporter molecules. Also the GFP interaction partner is partially exported visible as a faint band (Fig. 3.29, panel B, lane 8).

The construct IG / G-PA displays no export when comparing the input and eluate fraction. Neither the IgG2α construct nor the GFP construct is detectable on the cell surface (Fig. 3.29, gels anti-mouse IgG and anti-GFP, lanes 9 and 10). This is an expected results since no FGF moiety is present.

Compared on a qualitative basis the biotinylation assay and the *FACS* analysis show co-export of the GFP-containing piggyback interaction partner to a certain extent (see section 3.2.7). However, when compared on a quantitative basis the data obtained in the two experimental approaches differ. In the biotinylation analysis the FGF2-containing construct shows the highest signal of exported, cell surface attached material (Fig. 3.29, panel A, lane 6) which is not observed in *FACS* analysis. When using flow cytometry the cell line F4-IG / G-PA exhibits the highest cell surface staining signal (see section 3.2.7, Fig 3.26, panel K). Also the analysis of the co-export of the GFP-containing interaction partner displays differences. F2-IG / G-PA cells seem to co-export a high amount of the piggyback interaction partner in comparison to the other cell lines as analyzed by biotinylation. The *FACS* analysis revealed only a minor portion of GFP-Protein A-NES being co-exported for all cell lines which express FGF-containing reporter constructs (see section 3.2.7, Fig. 3.28). To be sure that the results of the biotinylation are representative and to rule out experimental artifacts the analysis was repeated three times. Within the independent experiments the obtained results were not consistent concerning the amounts of exported IgG2α constructs and potentially co-exported GFP-Protein A-NES (data not shown). This might be due to the long and complicated experimental procedure consisting of biotinylation, cell lysate preparation, binding to streptavidin beads, elution, SDS-PAGE analysis and numerous washing steps in between. Taken together the *FACS* analysis is the more accurate, precise and stable analysis method and should be preferred when investigating export processes and piggyback effects.

3.2.9 Analysis of GFP-Protein A-NES Release into Culture Medium

In order to analyze whether GFP-Protein A-NES is released from the cells into the culture medium and could thus falsify the results obtained when analyzing export of the piggyback complexes an immunoprecipitation assay was performed. Culture medium from cells expressing the reporter molecules was incubated with Protein A-sepharose beads decorated with anti-GFP antibodies. As a control for the immunoprecipitation procedure recombinant GFP was titrated into medium taken from $CHO_{MCAT\text{-}TAM2}$, which were not transfected. Bound material was analyzed by

SDS-PAGE and Western blot applying primary anti-GFP antibodies and secondary RG16 HRP-coupled antibodies as described in 3.2.5.

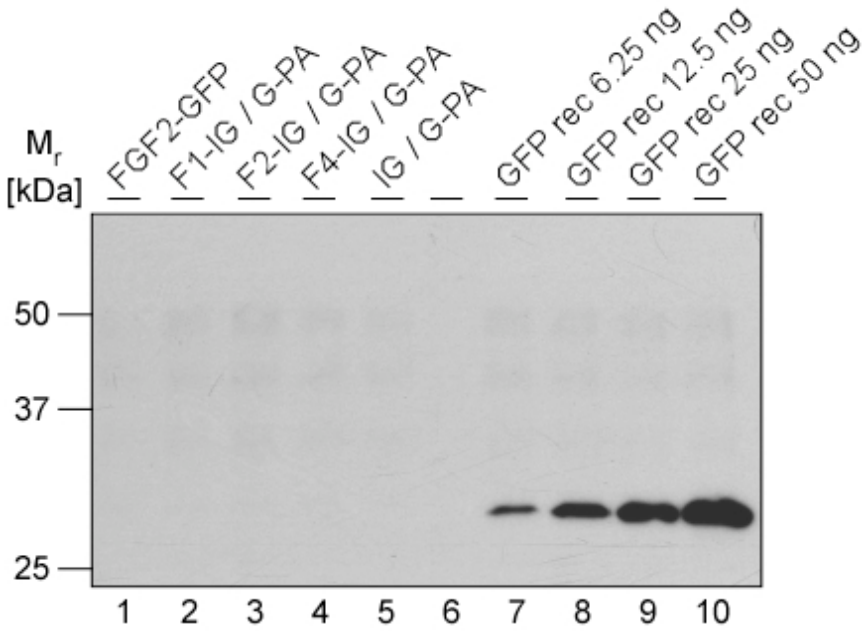

Fig. 3.30 Immunoprecipitation of GFP-containing reporter molecules unspecifically released from the reporter cells.

Medium from induced reporter cells was incubated with Protein A-sepharose beads decorated with anti-GFP antibodies. As a control recombinant GFP was titrated into medium obtained from untransfected $CHO_{MCAT-TAM2}$ cells and incubated with anti-GFP antibodies coupled to Protein A-sepharose beads. After washing bound material was eluted in SDS sample buffer. Samples were analyzed by SDS-PAGE (13% gels) and Western blot transfer. Precipitated material was detected using anti-GFP antibodies and RG16 HRP-coupled secondary antibodies. 50% of eluted material was loaded in each lane. Visualization was performed using the ECL system and x-ray film detection.

The analysis revealed that no GFP fusion protein can be found in the medium of the cell lines expressing the various reporter constructs (Fig. 3.30, lanes 1-5). The control were recombinant GFP was added to the medium of untransfected cells shows that GFP with a minimal concentration of 6,25 ng/ml medium is detectable by immunoprecipitation (Fig. 3.30, lane 7). Neither the control cell line FGF2-GFP nor the four piggyback cell lines show release of GFP-containing reporter constructs into the medium. This result implies that any signal of GFP-containing fusion proteins obtained in previous experiments which analyze cell surface staining must be due to an actively occurring export process.

3.2.10 Summary of the Piggyback Export Approach

The goal of the piggyback export analysis system was to monitor the folding state of FGF2 during unconventional secretion. The reporter system is based on a non-covalent interaction between the F_c domain of the mouse IgG2α and Protein A. Actively exported FGF2 was fused to the IgG2α domain and GFP which can not leave the cell on its own to Protein A. The two reporter constructs are expressed from one mRNA in cells stably transfected with the bicistronic expression vector pRTi. Additionally, three other cell lines were successfully generated in which FGF2 was substituted by FGF1, FGF4 with a classical signal sequence (FGF4-S) or no FGF moiety. It could be shown that all cell lines express both reporter molecules simultaneously in a doxicycline-dependent manner.

Upon expression of the reporter constructs a complex between the IgG2α domain fused to the respective FGF moiety and Protein A fused to GFP is expected to form. Complex formation was analyzed by co-immunoprecipitation and gel filtration in which proteins are retained on a column according to their molecular mass in a FPLC system. The analyses revealed that the piggyback complexes are formed in all reporter cell lines to a significant extent.

The analysis of the co-export of the reporter protein GFP-Protein A-NES proved to be problematic. A *FACS*-based analysis revealed that the FGF-containing reporter constructs are exported but only a small amount of the GFP-containing interaction partner seems to be co-exported. By contrast, the biochemical biotinylation assay showed a different result compared on a quantitative basis. However, when compared qualitatively both analysis techniques revealed that co-export occurs to a certain extent. A possible explanation might be that it can not be excluded that the export machinery mediating non-classical protein export is not capable of translocating the piggyback partners associated in a complex but can only export the FGF fusion constructs alone.

Taken together the obtained data do not allow a clear interpretation. Because of the inconsistency of the independent experimental systems it is not possible to draw an unambiguous conclusion. The system needs to be further optimized in order to finally answer the question if FGF2 is exported in a folded conformation.

4 Discussion

More than 15 years ago the phenomenon of unconventional protein secretion was discovered and an alternative protein export pathway that mediates secretion by an ER/Golgi-independent mechanisms was proposed (Muesch et al., 1990; Rubartelli et al., 1990). Although it was initially supposed that release occurs unspecifically in association with cell damage (McNeil et al., 1989) more and more evidence was provided that non-classical export is a controlled release mechanism actively regulated by the cell (Cleves, 1997; Engling et al., 2002; Florkiewicz et al., 1995; Hughes, 1999; Nickel, 2005; Rubartelli et al., 1990).

The classical secretory pathway is the main export route of secretory proteins and characterized in great detail at the molecular level. Nascent secretory proteins are directed to the translocon by their N-terminal, hydrophobic signal sequence and are co-translationally inserted into the ER (Walter et al., 1984). They are modified and incorporated into membrane-bound vesicles which mediate transport to the Golgi apparatus where the protein maturation continues (Halban and Irminger, 1994; Rothman and Wieland, 1996). Additionally, the classical secretory pathway provides a high level of quality control to guarantee the production and release of functional proteins (Arvan et al., 2002; Hammond and Helenius, 1995). Transport to the plasma membrane is mediated by membrane-bound vesicles which travel along the cytoskeleton (Goodson et al., 1997). Upon fusion with the plasma membrane they release their content to the extracellular space (Pelham, 1996).

By contrast, unconventional protein secretion starts with the synthesis of proteins on free ribosomes in the cytosol. All known examples are soluble protein, which do not contain a hydrophobic signal sequence and are, therefore, not directed to the ER/Golgi pathway (Muesch et al., 1990). Nevertheless, they are found in the extracellular space but plasma membrane translocation is not dependent on ER/Golgi transport since it is not affected by inhibitors of the classical secretory pathway (Rubartelli et al., 1990; Sato et al., 1993). Moreover, non-classically secreted proteins are not modified by, e.g. ER/Golgi-dependent glycosylation, despite bearing corresponding consensus sites (Hughes, 1999). The group of unconventionally secreted proteins is heterogeneous in terms of function, however, it is comprised of proteins which are in part structurally related. Among these are

growth factors like the proangiogenic mediator FGF2, lectins of the ECM such as galectin-1, inflammatory cytokines like IL-1β and MIF, viral proteins such as HIV-tat and stage-regulated parasitic proteins like HASPB of *Leishmania*. They do not share one unconventional secretion pathway but their release occurs by mechanistically distinct, vesicular and non-vesicular processes independent of the ER/Golgi-system (Nickel, 2005). Unconventionally secreted proteins are of high biomedical relevance and elucidating the molecular basis of their release mechanism is of great interest with regard to drug development and new therapeutical methods (Nickel, 2003; Nickel, 2005).
In particular, FGF2 which acts as a direct stimulator of angiogenesis is of great interest since it is involved in tumor-associated neovascularization which contributes to elevated supply with nutrients and to spreading of metastases throughout the body (Nugent and Iozzo, 2000; Smith et al., 2001). The soluble, cytosolic 18 kDa isoform of FGF2 is released by non-classical means (Florkiewicz et al., 1995; Nickel, 2003) but the involved machinery remains elusive at the molecular level. Recent findings point to a direct translocation across the plasma membrane (Nickel, 2005; Schäfer et al., 2004). FGF2 contains spatially separated binding sites for association with high affinity tyrosine kinase receptors (FGFRs) and heparan sulfate side chains of HSPGs (Raman et al., 2003; Schlessinger et al., 2000) which, following secretion, allow binding of FGF2 to the cell surface (Engling et al., 2002).

To further elucidate the mechanism of unconventional secretion of FGF2 two experimental *in vivo* systems were established using CHO cells. These cells are particularly suitable for the analysis of FGF2 export since they do not express FGF receptors so that FGF2 induced signalling and induction of differentiation processes does not occur. However, the cells possess HSPGs on their surface which allow binding of secreted FGF2 and, thus, direct analysis of exported material. By using $CHO_{MCAT\text{-}TAM2}$ (Engling et al., 2002) proteins of interest can be introduced into the genome by retroviral transduction due to the constitutive expression of the murine cationic amino acid transporter MCAT-1 (Albritton et al., 1989; Davey et al., 1997) on the cell surface. Additionally, expression of the reporter constructs can be controlled by the addition of doxicycline because the doxicycline-sensitive transactivator rtTA2-M2 (Urlinger et al., 2000) is also constitutively expressed in this cell line. The different

model cell lines expressing the reporter constructs were analyzed for non-classical export using independent read-out systems.

1) *Confocal microscopy.* To qualitatively analyze export of reporter molecules non-permeabilized cells were processed employing externally added antibodies and analyzed by confocal microscopy. This method allows the analysis of the subcellular localization of GFP-containing reporter proteins due to fluorescence. Simultaneously, export of FGF-containing reporter proteins can be analyzed by labelling of surface bound material using appropriate antibodies.

2) *Flow cytometry.* To quantitatively analyse export of the various reporter proteins employing *FACS* exported material bound to the cell surface was labelled using antibodies directed against different epitopes of the various reporter constructs. Additionally, expression levels can be measured simultaneously by fluorescence of the GFP moiety present in various reporter constructs. This can be used to normalize the overall expression of reporter constructs with regard to different experimental conditions.

3) *Cell surface biotinylation.* To analyze export of reporter proteins by an independent biochemical method a membrane-impermeable biotin reagent was used to label cell surface exposed proteins. Following detergent-based lysis the biotinylated population was recovered employing immobilized streptavidin. Biotinylated proteins corresponding to exported material and non-biotinylated proteins which represent the intracellular population were compared using Western blot and, thus, export of reporter molecules was analyzed.

Additionally, experimental analyses were performed which did not directly analyze export processes but which were important to establish the systems, like protease protection assays, gel filtration using FPLC and co-immunoprecipitation. They are discussed in more detail in the respective sections.

As already mentioned, two independent analysis systems were established during this study to analyze the folding state of FGF2 during membrane translocation. In the first approach FGF2 export was reconstituted by expression of a fusion construct,

which is comprised of FGF2, GFP and a C-terminal DHFR domain (Backhaus et al., 2004). The DHFR domain allows aminopterin-dependent stabilization of the molecule to analyze a potential need for unfolding (Eilers and Schatz, 1986). The second experimental approach is based on the association of an FGF2 fusion construct with an interaction partner dependent on the three-dimensional structure of both molecules. It monitors co-export which can only occur if the conformation of both proteins is maintained during membrane translocation to answer the question whether FGF2 is exported in a folded state. Both systems were exploited using the described analytical techniques in various modifications.

4.1 Analysis of the Need for Unfolding Employing a DHFR Fusion Protein System

To establish the DHFR fusion protein system, two cDNA constructs, FGF2-GFP-DHFR and MTS-GFP-DHFR respectively, were generated by molecular cloning and verified by sequencing (see sections 3.1.1 and 2.2.14). The reporter construct MTS-GFP-DHFR contains an N-terminal mitochondrial targeting sequence and serves as a positive control for the experimental system. Mitochondrial import is known to be dependent on protein unfolding and import of DHFR fusion proteins can be blocked by addition of aminopterin (Eilers and Schatz, 1986). Additionally, the DHFR domain contains a C-terminal His_6-tag which facilitates antibody-specific detection of both reporter proteins. To generate model cell lines virally transduced cells were subjected to a *FACS*-based sorting procedure. Three rounds of sorting were performed in the presence, absence and presence of doxicycline to obtain cells which express the reporter constructs in a doxicycline-dependent manner (see section 3.1.1, Fig. 3.3 as well as text). After generation of the model cell lines they were characterized by Western blot analysis, confocal microscopy and flow cytometry to verify doxicycline-dependent protein expression and export of the FGF2-containing reporter constructs. Additionally, a cell line expressing the fusion protein FGF2-GFP without the DHFR domain was analyzed as a control.

4.1.1 Characterization of Model Cell Lines Expressing DHFR Fusion Proteins

To characterize generated model cell lines biochemically, a Western blot analysis was employed using affinity-purified anti-GFP antibodies. It revealed that all cell lines express the reporter constructs in a doxicycline-dependent manner. Moreover, the reporter molecules migrate according to their calculated molecular masses in the SDS-PAGE when compared to a protein standard (see section 3.1.2, Fig. 3.4). An interesting observation was made when analyzing the reporter construct MTS-GFP-DHFR which was designed to contain a mitochondrial targeting sequence. Three forms of the protein were detectable. The slow migrating precursor form, the intermediate form and the fully processed form which correspond to the mitochondrial membrane translocation steps upon transport to the mitochondrial matrix during which the MTS is cleaved twice (Cavadini et al., 2002; Daum et al., 1982; Eilers et al., 1988; Schmidt et al., 1984) (see section 3.1.2, Fig. 3.4, lane 6). The detection of the three different forms of the fusion protein provided first evidence that the MTS-containing construct is imported into mitochondria because this specific cleavage of the MTS only occurs during mitochondrial import (see section 3.1.2, Fig. 3.4, lane 6).

To verify the results obtained in the Western blot analysis, the cell lines were analyzed by confocal microscopy. It was confirmed that protein expression is induced upon addition of doxicycline as analyzed by total GFP fluorescence (see section 3.1.3, Fig. 3.5, panels A, B, D, E, G and H). Regarding the subcellular localization of the FGF2-containing reporter constructs cytosolic and nuclear staining distribution was observed. The latter may results from the presence of the GFP moiety which is known to equilibrate between the nucleus and the cytoplasm upon expression in CHO cells. When expressed as a fusion protein with FGF2 the two proteins might also form a bipartite nuclear localization signal (NLS). Analysis of corneal endothelial cells revealed that the fusion protein FGF2-GFP is targeted to the nucleus in this cell line (Choi et al., 2000). Moreover, FGF2 is known to translocate to the nucleus upon receptor binding followed by internalization, however, this is rather unlikely in CHO cells since they do not possess FGFRs. Recent findings point to a role of HSPG binding to nuclear localization of externalized FGF2 (Hsia et al., 2003). These studies also made use of CHO cells so that the observations are directly comparable to the

in vivo system used in this experimental approach. The reporter protein MTS-GFP-DHFR is excluded from the nucleus and localizes to dot-like structures throughout the cytoplasm which corresponds to a mitochondrial localization pattern (see section 3.1.3, Fig. 3.5, panel H). Furthermore, it is not found in association with the plasma membrane.
When analyzing exported material by staining with externally added anti-GFP antibodies only the FGF2-containing reporter proteins are detectable on the cell surface. This observation shows that FGF2-GFP and FGF2-GFP-DHFR are translocated across the plasma membrane and can rebind to the cell surface since non-permeabilized cells were used for the analysis so that only exported material is detected. The MTS-containing reporter is not detectable on the cell surface showing that it is not exported.

To complete characterization of the model cell lines, a *FACS* analysis was performed employing affinity-purified anti-GFP and anti-His_6-tag antibodies. By the use of these antibodies the GFP moiety in the central region of the reporter constructs or in case of the DHFR-containing fusion proteins the extreme C-termini can be detected which is of great importance for later localization studies. The analysis by flow cytometry verified the doxicycline-dependent protein expression since cells grown in the presence of doxicycline show a shift of GFP-derived fluorescence upon addition of doxicycline (see section 3.1.4, Fig. 3.6). The analysis of the cell surface staining by externally added antibodies revealed that FGF2-GFP and FGF2-GFP-DHFR are exported and rebind to the cell surface (see section 3.1.4, Fig. 3.6, panels C, G and H). The use of the anti-His_6-tag antibodies demonstrates specificity of the observed signal when comparing cells expressing FGF2-GFP and cells expressing FGF2-GFP-DHFR since detection of the reporter protein is only possible when the DHFR domain with the C-terminal His_6-tag is present (see section 3.1.4, Fig. 3.6 panels D and H). Moreover, the DHFR domain does not influence export efficiency as both FGF2-containing reporter construct show similar cell surface staining signals when using anti-GFP antibodies (see section 3.1.4, Fig. 3.6 panels C and G). The construct MTS-GFP-DHFR does not exhibit cell surface staining neither employing anti-GFP nor anti-His_6-tag antibodies (see section 3.1.4, Fig. 3.6 panels K and L). This shows that it is not exported and again demonstrates specificity of the analysis method when compared to the other constructs. Additionally, one can conclude that export is

directly related to the FGF2 moiety since only FGF2-containing reporter proteins are exported.

Taken together, the observations made when analyzing the model cell lines by biochemical methods, confocal microscopy and *FACS* showed that protein expression is dependent on the addition of doxicycline. FGF2-GFP and FGF2-GFP-DHFR are translocated across the plasma membrane and bind to the cell surface. Exported material is specifically detectable employing anti-GFP or anti-His_6-tag antibodies. The reporter construct MTS-GFP-DHFR is not exported from CHO cells but mitochondrial import could be demonstrated by Western blot analysis and confocal microscopy.

4.1.2 Analysis of Aminopterin Binding to DHFR Fusion Constructs

In order to apply the DHFR system to mitochondrial import and to the unconventional secretion of FGF2, it was of great importance to show that aminopterin actually binds to the DHFR domain in the reporter constructs. Therefore, a protease protection assay was performed and analyzed employing monoclonal DHFR antibodies. Cell lysates obtained from model cell lines expressing FGF2-GFP-DHFR or MTS-GFP-DHFR were incubated with trypsin in the absence or presence of aminopterin (see section 3.1.7, Fig. 3.9). In the absence of trypsin and aminopterin the reporter constructs are detectable as full length proteins migrating corresponding to their calculated molecular masses as compared to a protein standard. Additionally, the DHFR moiety is detectable as a fragment migrating at 20 kDa which results from post lysis degradation (see section 3.1.7, Fig. 3.9, lanes 1 and 4). In the presence of trypsin, but in the absence of aminopterin, all reporter molecules are completely degraded (see section 3.1.7, Fig. 3.9, lanes 2 and 5). In the presence of trypsin and aminopterin the full length reporter molecules and the DHFR fragment are detectable to a significant extent. This shows that aminopterin binds to the DHFR moiety and thereby mediates protection against proteolysis. Even the full length reporter proteins MTS-GFP-DHFR and FGF2-GFP-DHFR are protected as a result of aminopterin-induced stabilization (see section 3.1.7, Fig. 3.9, lanes 4 and 6). These observations

are consistent with other reports showing that ligand binding to DHFR stabilizes proteins and protects them against proteolysis (Salvador et al., 2000).

In summary, it was shown by protease protection analysis that aminopterin actually binds to the DHFR domain of both fusion proteins and mediates stabilization against proteolysis.

4.1.3 Influence of Aminopterin on the Mitochondrial Import of MTS-GFP-DHFR

To show that protein translocation across a membrane can be blocked in living cells under conditions preventing protein unfolding (Eilers and Schatz, 1986; Rassow et al., 1989), cells expressing the construct MTS-GFP-DHFR were grown in the absence or presence of aminopterin and analyzed by confocal microscopy. In the absence of aminopterin the reporter protein localizes to mitochondria shown by the mitochondrial staining pattern observed previously (see section 3.1.5, Fig. 3.7). Upon addition of aminopterin the subcellular localization changed drastically and cytoplasmic and nuclear staining was observed. The mitochondrial localization pattern is not detectable anymore. This demonstrates that aminopterin blocks import of the reporter construct into mitochondria so that the molecule is distributed throughout the cytoplasm and additionally localizes to the nucleus due to the GFP moiety as discussed above.

To analyze the influence of aminopterin on mitochondrial membrane translocation using an independent method, a biochemical analysis of cell lysates was performed following incubation of cells in the absence or presence of aminopterin. The previously observed forms of the reporter proteins corresponding to the mitochondrial import steps are detectable in the absence of aminopterin on an SDS gel as the slow migrating precursor form, the intermediate form resulting from the first membrane translocation into the intermembrane space of the mitochondria and the prevailing, fully processed form. The predominance of the latter demonstrates efficient import into the mitochondrial matrix (see section 3.1.6, Fig. 3.8, lane 2). Upon addition of aminopterin the amount of the fully processed from and the precursor form is largely

reduced demonstrating inhibition of mitochondrial import. The intermediate form remains unchanged (see section 3.1.6, Fig. 3.9, lane 3). These observations suggest degradation of the membrane-arrested precursor form in the presence of aminopterin to keep the mitochondrial import complexes in a functional state. However, a small portion of the reporter protein seems to pass to the intermembrane space even in the presence of aminopterin and is rapidly translocated to the mitochondrial matrix. These observations are consistent with earlier reports showing that mitochondrial import is dependent on unfolding prior to membrane translocation (Eilers and Schatz, 1986; Wienhues et al., 1991).

In summary, the analysis of the influence of aminopterin on mitochondrial import as a control for the DHFR system *in vitro* demonstrates that import of the fusion construct MTS-GFP-DHFR into mitochondria can be blocked efficiently. Thus, the system is functional and can be used in the background of the FGF2 reporter construct to analyze the need of unfolding for FGF2 plasma membrane translocation.

4.1.4 Analysis of FGF2-GFP-DHFR Membrane Translocation in the Presence of Aminopterin

To analyze the effect of aminopterin on FGF2-GFP-DHFR export, it was necessary to monitor whether plasma membrane translocation of the reporter protein occurs completely. Therefore, it was important to detect various domains of the reporter molecule independent of the GFP moiety located in the N-terminal region like, e.g., the extreme C-terminus using anti-His_6-tag antibodies. Since the FGF2 moiety which mediates non-classical export is located at the N-terminus of the reporter construct potential detection of the extreme C-terminus in the extracellular space would confirm complete translocation of the molecule. This is because in case the reporter protein would be stuck in a putative plasma membrane-resident transporter detection of the N-terminal segment only could result in misinterpretation as it does not clearly demonstrate complete translocation of the reporter molecule.

To qualitatively analyze the influence of aminopterin on the export of FGF2-GFP-DHFR, reporter protein-expressing cells grown in the absence or presence of

aminopterin were analyzed by confocal microscopy. Cells were processed without permeabilization employing anti-GFP and anti-His_6-tag antibodies for the reason given above. Detection employing both antibodies showed that export of the reporter protein is not affected by aminopterin as both the GFP moiety and the extreme C-terminus were detectable on the cell surface. This shows that FGF2-GFP-DHFR is exported in the presence of aminopterin and suggests that unfolding is not required for FGF2 membrane translocation.

To verify the results obtained using a quantitative system, a *FACS* assay was performed under the same experimental conditions. Again, both antibodies were used to analyze complete membrane translocation of the reporter molecule. When analyzing expression levels a threefold increase in the presence of aminopterin was detectable (see section 3.1.10, Fig. 3.12, green curves in panels A, B compared to C, D). This effect might be due to aminopterin-dependent stabilization of the reporter molecule which might lead to a prolonged half-life of the protein so that the pool of molecules available for export increases. This, in turn, leads to elevated cell surface staining levels since more protein is present in the same time period compared to non-aminopterin conditions. Remarkably, the cell surface staining signal is only increased about twofold suggesting a saturation of the export machinery with substrate (see section 3.1.10, Fig. 3.12, green curves in panels E, F compared to G, H). Nevertheless, export of the reporter protein is not inhibited in the presence of aminopterin. To directly compare cell surface staining signals derived from exported material the amount of doxicycline used to induce protein expression in the presence of aminopterin was titrated down to establish conditions where expression levels are comparable. The experiment was repeated three times with comparable expression levels in the absence and presence of aminopterin and the data were analyzed statistically. The analysis revealed that FGF2-GFP-DHFR export is not affected by aminopterin as analyzed by anti-GFP antibodies. When employing anti His_6-tag antibodies the cell surface staining signal was found to be increased by a factor of two (see section 3.1.10, Fig. 3.13). This effect is probably the result of an enhanced accessibility of the C-terminal His_6-tag due to the stabilization of the DHFR domain in the presence of aminopterin (Backhaus et al., 2004).

The results obtained from the analysis of the DHFR fusion protein system show that membrane translocation of FGF2 is likely to occur in a folded state. The export of the reporter construct is not blocked in the presence of aminopterin and secretion occurs as efficiently as under non-aminopterin conditions or as observed for the construct FGF2-GFP. Importantly, by employing antibodies directed against the N-terminal part of the fusion protein (anti-GFP) and against the extreme C-terminus (anti-His_6-tag) complete translocation of the reporter molecule could be verified. The analysis of the control construct MTS-GFP-DHFR in the same cellular background and expression system showed that import into mitochondria is almost completely inhibited in the presence of the drug. The precursor form and, therefore, all subsequent isoforms are largely reduced in the presence of aminopterin due to degradation of the membrane-arrested precursor to keep the mitochondrial import complexes in a functional state. By the protease protection assay it was shown that aminopterin stabilizes the DHFR moiety of the reporter constructs demonstrating that it not only binds to DHFR but also prevents unfolding. Taken together, these observations show that the DHFR system is functional and unfolding can be prevented *in vivo*. Therefore, it can be concluded that FGF2 export does not require protein unfolding (Backhaus et al., 2004).

However, there are certain limitations of the system that have to be taken into consideration. The system does not rule out that specific chaperones potentially involved in unconventional secretion are able to unfold the reporter molecule even in the presence of aminopterin. Another possibility would be that secreted reporter proteins are directly translocated as nascent polypeptide chains comparable to cotranslational insertion into the ER (Pfeffer and Rothman, 1987; Rapoport, 1992b; Rothman and Orci, 1992). In this case, aminopterin could not bind to this population of reporter molecules since the DHFR domain is not folded when export occurs. In both scenarios FGF2-GFP-DHFR would be exported in an unfolded state even though aminopterin does not block membrane translocation. To overcome these limitations a new experimental system was established which directly monitors the folding state of FGF2 during non-classical membrane translocation.

4.2 Piggyback Export Analysis System

To further analyse the folding state of FGF2 during export, a piggyback export analysis system (see section 3.2 introducing text and 3.2.4) was established which monitors whether the conformation of FGF2 is maintained during membrane translocation. It is based on the rationale that FGF2 fused to the mouse IgG2α F_c domain can form a non-covalent complex with GFP fused to Protein A inside cells. Complex formation is mediated by the interacting domains IgG2α and Protein A and depends on the correct three-dimensional structure of both fusion proteins. If GFP-Protein A would be detectable extracellularly, export of the complex would have been demonstrated. This, in turn, would establish that FGF2 is exported in a folded state because otherwise any protein-protein interactions would be lost. The system also addresses the question whether quality control means can apply during unconventional secretion. If correct folding is a prerequisite for non-classical export, release of non-functional proteins would be prevented. This would suggest that the cell is able to monitor correct folding of unconventional export cargo by an unknown mechanism and can reject proteins which do not fulfill quality control requirements.

To generate model cell lines, the respective reporter constructs were cloned and verified by sequencing (see sections 2.2.15 and 3.2.1). The reporter constructs are organized in two ORFs separated by an IRES-element in the bicistronic expression vector pRTi. The transcription of the mRNA leads to translation of two distinct proteins, an IgG2α-containing FGF reporter molecule and GFP-Protein A. The IgG2α-containing reporter constructs were designed with FGF1, FGF2, FGF4-S (with a signal sequence) or no FGF moiety to analyze the folding state of FGF2 and FGF1 during export and to generate control constructs which are classically secreted (FGF4-S) or not exported (no FGF moiety). All fusion constructs contain a nuclear export signal (NES) at the C-terminus to reduce nuclear localization as observed during the analysis of the DHFR system and to increase, in turn, the pool of cytosolic reporter molecules available for export. The cDNA constructs were introduced into $CHO_{MCAT\text{-}TAM2}$ cells by retroviral transduction (Engling et al., 2002). As described when establishing the DHFR system, transduced cells were subjected to three rounds of FACS sorting to generate clonal cell lines expressing the reporter proteins in a doxicycline-dependent manner. The generated piggyback model cell lines were

characterized using Western blot analysis and flow cytometry. Additionally, a cell line expressing FGF2-GFP-NES was analyzed as a control.

4.2.1 Characterization of Piggyback Model Cell Lines

The Western blot analysis of cell lysates employing anti-mouse IgG and anti-GFP antibodies revealed that all reporter proteins are expressed in a doxicycline-dependent manner. Furthermore, the expression of two distinct proteins from one mRNA is functional in the model cell lines (see section 3.2.2, Fig. 3.17). The IgG2α-containing reporter proteins migrate in an SDS gel according to their calculated molecular masses as compared to a protein standard. An unexpected observation was that the FGF1- and FGF2-containing reporter proteins do not show the same migration behaviour although they are similar in size (see section 3.2.2, Fig. 3.17, panel A, lanes 2 and 4). This can be explained by the different amino acid compositions of the FGF moieties, which might influence the migration behaviour in the electric field during SDS-PAGE. When analyzing FGF4-S-IgG2α-NES additional bands migrating faster than the predominant one are detectable (see section 3.2.2, Fig. 3.17, panel A, lane 6). These bands are likely to represent glycosylated forms of the molecule. Since glycosylation is an ER- and Golgi-associated modification (Colley, 1997; Ernst and Prill, 2001; Helenius and Aebi, 2004; Rothman and Lodish, 1977) this observation confirms that FGF4-S-IgG2α-NES enters the classical secretory pathway as expected.

The analysis employing flow cytometry confirmed doxicycline-dependent expression of the various reporter proteins as detected by a strong increase of GFP-derived fluorescence upon addition of doxicycline (see section 3.2.3, Fig. 3.18, panels, A, C, E and G, grey versus green curves). To analyze export of the various FGF-containing reporter proteins, which is the basis for the subsequent analysis of co-export of the piggyback interaction partner GFP-Protein A, cells were processed employing externally added anti-mouse IgG antibodies. The analysis revealed that the FGF-containing reporter constructs are exported to a significant extent and can be detected bound to the cell surface. The reporter construct IgG2α-NES which does not contain a FGF moiety is not detectable extracellularly. This demonstrates

specificity of the antibodies used because cell surface staining signals are directly dependent on an FGF moiety. As expected, FGF4-S-IgG2α-NES shows elevated cell surface staining levels as compared to the FGF1- and FGF2-containing reporter proteins since it is exported via the classical secretory pathway. When expression was induced the cell surface staining signal shifts to the upper limit of the detection range of the flow cytometer because the construct is exported in large amounts (see section 3.2.3, Fig. 3.18, panel F, green curve).

Taken together, the characterization employing Western blot and *FACS* analysis showed that both interaction partners are expressed from a bicistronic mRNA as two distinct proteins in a doxicycline-dependent manner. Furthermore, constructs containing an FGF moiety are exported in the *in vivo* system and can be detected on the cell surface.

4.2.2 Verification of Complex Formation Employing Biochemical Methods

The association of FGF-containing reporter proteins and GFP-Protein A in non-covalent complexes is a prerequisite for the piggyback export system to be functional. Therefore, experimental evidence had to be obtained to verify that the molecules do form cytoplasmic complexes. To analyze complex formation two independent biochemical methods were applied.

For the analysis employing co-immunoprecipitation, cell free supernatants which were prepared without detergent to keep proteins under native conditions were incubated with Protein A-sepharose beads to allow binding of the reporter constructs mediated by the IgG2α domain. Additionally, beads decorated with anti-FGF2 antibodies were employed. The analysis revealed that reporter proteins containing the IgG2α domain can be sedimented bound to Protein A-sepharose beads and that FGF1- and FGF2-IgG2α-NES can be recovered using anti-FGF2 antibodies (see section 3.2.5, Fig. 3.20, panel A). When analyzing the samples employing anti-GFP antibodies it could be shown that GFP-Protein A is efficiently co-purified with FGF1-, FGF2 and FGF4-S-IgG2α-NES (see section 3.2.5, Fig. 3.20, panel B). These

findings demonstrate that the reporter proteins do form cytoplasmic complexes. In the case of the reporter molecule IgG2α-NES, binding to Protein A-sepharose beads was verified but co-purification could not be observed (see section 3.2.5, Fig. 3.20, panel A and B, lane 10). Thus, complex formation could not be analyzed employing this experimental approach. On the other hand, this observation demonstrates specificity of the method as compared to the other reporter molecules because co-purification must be due to complex formation and can not result from unspecific binding or be an experimental artefact.

To confirm complex formation using an independent method, an FPLC gel filtration analysis was performed. Cell free supernatants prepared without detergent were separated according to molecular mass employing a superdex 200 size exclusion column. Superdex 200 beads retain molecules from 10 to 600 kDa on the column to various extents and, therefore, allow fractionation dependent on molecular mass. The fractionation of a marker protein mixture containing proteins with defined molecular masses was used as a standard to correlate retention on the column to molecular mass based on the elution profile (see section 3.2.6, Fig. 3.21, 3.22 and 3.23). Following standardization of the column, cell free supernatants of the piggyback model cell lines and FGF2-GFP-NES-expressing cells were separated using the FPLC system and the obtained fractions were analyzed by Western blot employing anti-mouse IgG and anti-GFP antibodies. In order to interpret the read-out of the experimental system the molecular masses of the putative complexes which could result from the potential oligomerization combinations were calculated (see section 3.2.6, Fig 3.25). The Western blot analysis of FGF2-GFP-NES revealed that a peak of protein elution is detectable in fraction 22 which corresponds to a molecular mass of 90 kDa. This observation suggests that most reporter molecules are present in homodimers which have a calculated molecular mass of 92 kDa (see section 3.2.6, Fig. 3.24, panel A).

The analysis of cell free supernatants obtained from FGF1- and FGF2-IgG2α-NES-expressing cells employing anti-mouse IgG antibodies showed that a shift of the elution-peak to fraction 18 occurs (see section 3.2.6, Fig. 3.24, panels B and D). Elution in this fraction corresponds to a molecular mass of 180 to 200 kDa, which matches the molecular mass of a heterotetramer consisting of two FGF-containing reporter molecules and two GFP-Protein A molecules. The observed shift of the

elution profile confirms that the complex between FGF1- or FGF2-IgG2α-NES and Protein A-GFP is formed. The same observation was made when analyzing FGF4-S-IgG2α-NES-expressing cells (see section 3.2.6, Fig. 3.24, panel F). This is a rather unexpected finding since FGF4-S-IgG2α-NES is expected to enter the classical secretory pathway and should thereby be segregated from GFP-Protein A which does not contain a signal peptide. A possible explanation would be that complex formation occurs during the preparation of cell free supernatants. When the plasma membrane and the ER/Golgi system are disrupted the two interacting molecules are not segregated anymore and association might occur. Fractionation of supernatants of IgG2α-NES-expressing cells showed the highest amount of protein to elute in fraction 23 (see section 3.2.6, Fig. 3.24, panel H). This corresponds to a molecular mass of 60 to 80 kDa which is consistent with the formation of a heterodimeric complex expected to have a molecular mass of 73 kDa. This finding implies that the construct IgG2α-NES associates with GFP-Protein A as expected.

The analysis of the interaction partner GFP-Protein A employing anti-GFP antibodies revealed that a large amount of protein is found in weight fractions 10, 12 and 14 corresponding to high molecular complexes or aggregates (see section 3.2.6, Fig. 3.24, panels C, E, G and I). These correspond to molecular mass of 600 kDa or more and are likely to represent protein aggregates which are not retained by superdex 200 beads and elute in the exclusion volume. These high molecular weight aggregates might from during cell free supernatant preparation. Additionally, high amounts of protein were found in the low molecular weight fractions 24 and 26 (see section 3.2.6, Fig. 3.24, panels C, E, G and I). These fractions correspond to monomeric GFP-Protein A molecules with a molecular mass of 44 kDa not associated with any interaction partner. Most remarkably, the elution-peaks of the fusion proteins FGF1- FGF2-, FGF4-S-IgG2α-NES and IgG2α-NES and their respective interaction partner GFP-Protein A are not found to be similar. This is an unexpected finding since complex formation implies that both reporter molecules are present in the same fractions. The observation suggests that only a subpopulation of GFP-Protein A is involved in complex formation and that a large amount is present as free monomers or high molecular weight aggregates as observed.

In summary, the analysis employing co-purification and gel filtration revealed that the complexes consisting of IgG2α-containing reporter molecules and GFP-Protein A are formed to a significant extent. The interaction partner GFP-Protein A could be co-purified although it can not bind to beads on its own. Furthermore, the elution profiles obtained by the gel filtration analysis suggest that the IgG2α-containing reporter molecules are found in fractions which correspond to the calculated molecular masses of the proposed complexes. These findings demonstrate that a stable association of the piggyback reporter molecules results in the formation of defined complexes.

4.2.3 Analysis of the Folding State of FGF2 Employing Piggyback Export Analysis

To analyze whether co-export of GFP-Protein A associated with FGF-containing reporter proteins occurs, the piggyback model cell lines were analyzed by flow cytometry and cell surface biotinylation. Additionally an immunoprecipitation analysis of the cell culture medium was employed to exclude unspecific release of the reporter molecules.

The analysis by flow cytometry was performed using anti-mouse IgG and anti-GFP antibodies. The experiment was repeated three times to statistically evaluate the data obtained. When employing anti-mouse IgG antibodies no signal was observed for FGF2-GFP-NES-expressing cells since no IgG2α domain is present in this reporter construct. The analysis of FGF1-, FGF2 and FGF4-S-IgG2α-NES-expressing cells using the same antibodies showed that the reporter molecules are significantly exported (see section 3.2.7, Fig. 3.26, panels B, E, H, K and Fig. 3.27). As discussed in section 4.2.1, the FGF4-S-containing reporter construct is exported to a higher extent due to secretion via the classical secretory pathway as shown by the elevated cell surface signals. The reporter construct IgG2α-NES did not exhibit any cell surface staining demonstrating that it is not exported since it lacks an export-mediating FGF domain. This finding together with the analysis of FGF2-GFP-NES export demonstrates specificity of the antibodies employed.

The analysis employing anti-GFP antibodies displayed a strong cell surface signal of the construct FGF2-GFP-NES showing efficient export. The analysis of the potential co-export of GFP-Protein A in association with FGF1-IgG2α-NES revealed a significant cell surface staining signal suggesting that export of the complex occurs (see section 3.2.7, Fig. 3.26, panel F and Fig. 3.28). The signal is not directly comparable to the cell surface signal of FGF2-GFP-NES. The expression levels as detected by GFP-fluorescence differ strongly when comparing the piggyback model cell lines and the cell line expressing FGF2-GFP-NES (see section 3.2.7, Fig. 3.26, GFP-fluorescence). For unknown reasons, proteins expressed upstream of the IRES element in the pRTi vector exhibit reduced expression levels as compared to proteins expressed of the downstream multiple cloning site. This contributes to a reduction of the amount of GFP-containing reporter proteins in the piggyback model cell lines. Nearly the same results were obtained when analyzing the reporter construct FGF2-IgG2α-NES. The observed cell surface signals are lower as compared to the FGF1-containing reporter construct but an increase of the signal in the presence of doxicycline is detectable (see section 3.2.7, Fig. 3.26, panel I and Fig.3.28). This shows that export of the complex FGF2-IgG2α-NES / GFP-Protein A occurs to a certain extent. An unexpected observation was made when analyzing FGF4-S-IgG2α-NES. The cell line displays co-export although FGF4-S-IgG2α-NES is externalized via the classical secretory pathway and, therefore, should be segregated from the piggyback partner GFP-Protein A. For an unknown reason assembly into the complex seems to occur. The analysis of the reporter construct IgG2α-NES revealed that no co-export occurs. Cell surface staining signals are similar in induced and non-induced cells. Again, this demonstrates specificity of the read-out method and suggests that cell surface staining signals detected using anti-GFP antibodies might result from export of the complexes formed by the interacting molecules. However, the observations made when analyzing co-export in the model cell line expressing FGF4-S-IgG2α-NES allow the assumption that these low range cell surface signals could also result from unspecifically released GFP-Protein A which binds to FGF-containing reporter molecules associated with the cell surface. Taken together, the data are not consistent and co-export could not be verified to a significant extent, although a signal which is dependent on extracellular GFP-Protein A was detectable.

In order to employ an independent method, potential co-export was analyzed using a cell surface biotinylation assay. To label cell surface bound reporter molecules, induced cells were incubated with a membrane-impermeable biotinylation reagent. Biotinylated proteins which correspond to exported material were recovered from cell lysates employing streptavidin beads and compared to non-biotinylated proteins which represent intracellular material using a Western blot analysis. To distinguish between IgG2α-containing reporter proteins and co-exported GFP-Protein A the analysis was performed employing anti-mouse IgG and anti-GFP antibodies. The analysis of the reporter protein FGF2-GFP-NES revealed that export occurs to a significant extent as analyzed by anti-GFP antibodies. In the eluate from the streptavidin beads the reporter protein was detectable in high amounts. This fraction represents exported and, therefore, cell surface exposed material, which is accessible to the biotin reagent (see section 3.2.8, Fig 3.29, panel B, lanes 1 and 2). No signals could be observed when employing anti-mouse IgG antibodies which again demonstrates antibody specificity. The analysis of FGF1-IgG2α-NES using anti-mouse IgG antibodies revealed that only a small fraction of the IgG2α-containing reporter molecules seems to be exported as compared to the signal of the input sample. The same observation was made when analyzing co-export of GFP-Protein A employing anti-GFP antibodies (see section 3.2.8, Fig 3.29, panel A and B, lanes 3 and 4). The analysis of the FGF2-containing reporter construct provided different evidence concerning the amounts of exported material. An intense signal of exported FGF2-IgG2α-NES and of co-exported GFP-Protein A could be observed (see section 3.2.8, Fig 3.29, panel A and B, lanes 5 and 6). These findings suggest that the export of both interaction partners associated in a complex occurs more efficient as compared to the FGF1-containing reporter construct. The data obtained by the analysis of the FGF4-S-containing reporter construct showed lower signals with regard to the overall protein amount. Nevertheless, export of the IgG2α-containing reporter molecule and the GFP-containing interaction partner was detectable (see section 3.2.8, Fig 3.29, panel A and B, lanes 7 and 8). These observations are not consistent with the data obtained by *FACS* analysis especially when regarding the export efficiency of the construct FGF4-S-IgG2α-NES. The reporter construct IgG2α-NES and the interaction partner GFP-Protein A showed no export in the biotinylation assay (see section 3.2.8, Fig 3.29, panel A and B, lanes 9 and 10). This again confirms the previously observed finding that export is dependent on an FGF moiety.

To exclude unspecific release of the reporter molecule GFP-Protein A or inability of the complexes to bind to the cell surface, an immunoprecipitation analysis of cell culture medium was performed. Medium obtained from induced model cell lines was incubated with anti-GFP antibodies immobilized on sepharose-beads. Bound material was eluted and analyzed by Western blot employing anti-GFP antibodies. As a control different amounts of recombinant GFP were added to conditioned medium of $CHO_{MCAT\text{-}TAM2}$ cells, which are not transfected with a reporter construct. The analysis revealed that no GFP-containing reporter molecules can be found in cell culture medium. Neither FGF2-GFP-NES nor the piggyback reporter construct expressing cell lines showed signals of GFP-containing reporter molecules whereas the control of recombinant GFP allowed detection of 6.25 ng of protein. These findings suggest that unspecific release does not seem to occur although it could be possible that a small population which is not detectable by this method is still released. Additionally, the assay does not analyze to which extent exported molecules which are not bound to the cell surface are prone to degradation. Therefore, unspecific release could occur to a certain extent but the reporter molecules are not detectable in the medium.

The analysis of the piggyback export system revealed that GFP-Protein A seemed to be co-exported and was detectable on the cell surface to a certain extent. Compared on a qualitative basis the *FACS*-based analysis and the biotinylation assay showed that co-export occurs. However, when compared on a quantitative basis the data obtained by the two independent analysis methods are not fully consistent. The analysis by biotinylation suggests that FGF2-IgG2α-NES is exported most efficiently and that co-export of GFP-Protein A also occurs to a high extent. The analysis by flow cytometry showed that FGF4-S-IgG2α-NES exhibits the highest cell surface signal and FGF1-IgG2α-NES shows the most efficient co-export of GFP-Protein A as compared to the other piggyback constructs. These findings are not consistent within the two experimental approaches. Therefore, the biotinylation analysis was repeated three times but the results showed a high variation with regard to the amounts of proteins detected maybe due to the complicated experimental procedure (data not shown). These findings suggest that flow cytometry is more adequate to analyze co-export of the piggyback analysis system. Nevertheless, the results obtained show that co-export of the piggyback interaction partner occurs although not to such an extent in order to make the experiments fully conclusive.

4.3 Concluding Remarks

The aim of the present study was to analyze the folding state of FGF2 during unconventional secretion. Therefore, two experimental systems, namely the DHFR fusion protein system to prevent unfolding during membrane translocation and the piggyback export analysis system to investigate whether FGF2 is exported in a folded conformation, were employed. The DHFR system revealed that under conditions where unfolding is prevented FGF2 export is not affected. This suggests that FGF2 export does not seem to require unfolding (Backhaus et al., 2004). To overcome the limitations of the DHFR system a piggyback export analysis system was employed. The purpose was to address the question whether export of folded FGF2 can be mediated by the unconventional export machinery and to monitor the folding state of FGF2 during the actual export process. However, the data obtained were not fully conclusive. It could be shown that the complexes between the interacting molecules form as expected but the analysis of co-export did not provide consistent results. It is likely that co-export occurs to a certain extent but only a small amount of the interaction partner seems to be co-exported. These findings allow the proposal that FGF2 export might occur in folded state, especially when taking the evidence provided by the DHFR fusion protein analysis into account (Backhaus et al., 2004).

Additionally, the observations made have an impact on quality control issues during unconventional secretion. Since the cell possesses elaborate quality control mechanisms for the classical secretory pathway like ER-resident chaperones, the calnexin/calreticulin system and unfolded protein response (Helenius et al., 1997; Lyman and Schekman, 1996; Sidrauski and Walter, 1997) it is rather unlikely that there are no such mechanisms associated with unconventional secretory processes. A general mechanism could be to restrict export to correctly folded proteins which would provide a high level of quality control. Another system could involve specific cytosolic chaperones which bind to incorrectly folded molecules to retain them inside the cell comparable to the calnexin/calreticulin system in the ER (Tatu and Helenius, 1997).

Another hypothesis that is based on the export of folded molecules is that cell surface counter receptors, e.g. HSPGs, are directly involved in FGF2 plasma membrane translocation. Since the heparin binding site of FGF2 is comprised of

spatially separated amino acid residues in different loop regions of the molecule (Raman et al., 2003) folding into the correct three-dimensional structure is obligatory to form a functional binding site. Another finding supporting this view is that only correctly folded FGF2 can bind to heparin *in vitro* (Raman et al., 2003; Seddon et al., 1991). It could be shown in our laboratory that a mutant CHO cell line deficient in HSPGs (Esko et al., 1985) does not export FGF2 (unpublished data of Christoph Zehe). Export could be rescued by co-cultivation with cells not deficient in HSPGs (unpublished data of Christoph Zehe) which demonstrates the involvement of HSPGs in FGF2 export and, in turn, requires correctly folded FGF2 molecules which are able to bind to heparan sulfates. Another finding concerning the heparin binding site and structural characteristics is that C-terminally truncated versions of FGF2 are impaired in both heparin binding and export (data of André Engling (PHD thesis) and Christoph Zehe). These findings are consistent with the hypothesis that HSPGs are involved in FGF2 export and that this process is restricted to correctly foldedcFGF2 molecules.

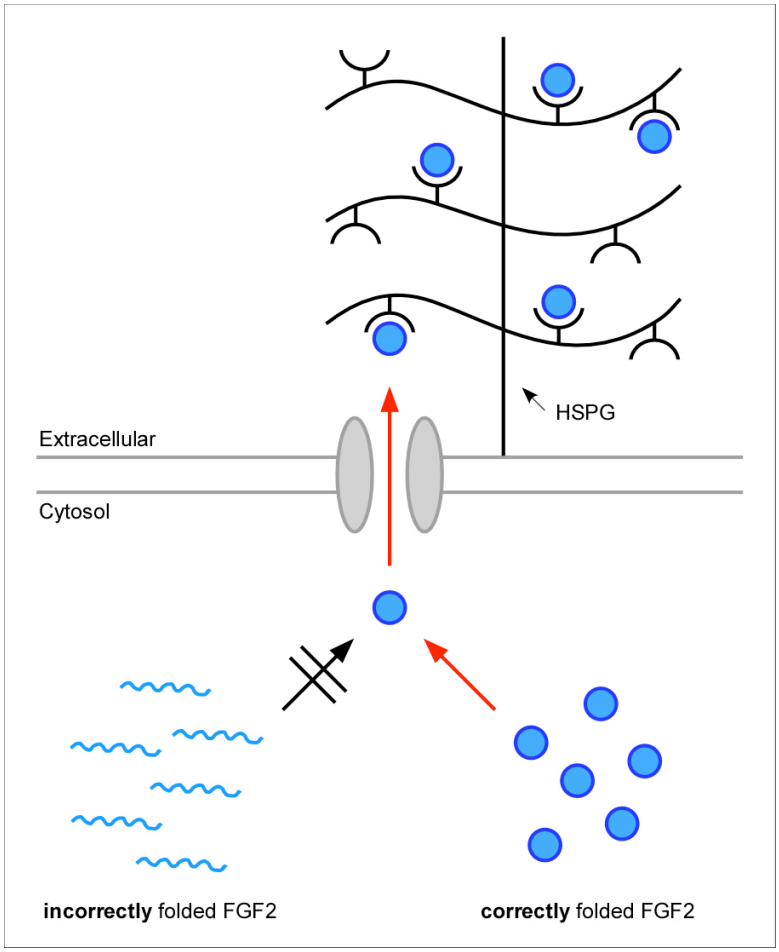

Fig. 4.1 Schematic overview: Model of FGF2 export dependent on correctly folded molecules involving HSPGs.
FGF2 = fibroblast growth factor 2, HSPG = heparan sulfate proteoglycan

As depicted in Fig. 4.1 potential quality control mechanisms in unconventional secretion of FGF2 could be rejection of improperly folded or aggregated molecules by the translocation machinery. If binding to HSPGs, which, in that case, function as counter receptors, is necessary, incorrectly folded molecules could not enter the non-classical export pathway.

As proposed for galectin-1, counter receptors could act as a molecular trap which removes secreted molecules from the equilibrium between the intra- and extracellular pool of FGF2 (Seelenmeyer et al., 2005). Another possibility could be that they might exert a pulling force at the extracellular side of a putative translocation pore. In that case, HSPGs would actively contribute to the export process which was also proposed for counter receptors of galectin-1 (Seelenmeyer et al., 2005). Consistent with the proposed models, Schäfer et al. provided evidence for a direct plasma membrane translocation of FGF2. It could be shown in an *in vitro* assay which

reconstitutes FGF2 secretion by employing plasma membrane-derived inside-out vesicles that FGF2 and galectin-1 are selectively translocated in a time- and temperature-dependent manner (Schäfer et al., 2004). These findings suggest the existence of a plasma membrane-resident transporter which directly mediates translocation being consistent with the proposed models.

Taken together, the data obtained by the two experimental systems presented in this study suggest that unconventional secretion of FGF2 does not require protein unfolding (Backhaus et al., 2004) and that non-classical export of FGF2 could depend on correctly folded cargo molecules to apply means of quality control to the unconventional secretory process.

5 Literature

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6 Abbreviations

αMEM	α-modification of Minimal Essential Medium
°C	degrees celsius
ABC	ATP binding cassette
APC	allophycocyanin
APS	ammonium peroxo disulphate
ATP	adenosin triphosphate
BFA	brefeldin A
bp	basepairs
CDB	cell dissociation buffer
cDNA	complementary DNA
CHO	chinese hamster ovary (cells)
CRD	carbohydrate recognition domain
C-terminal	carboxy terminal
ddH_2O	double destilled water
DHFR	dihydrofolate reductase
DMSO	dimethyl sulphoxide
DNA	desoxyribonucleic acid
E.coli	*escherichia coli*
e.g.	*exempli gratia*
ECL	enhanced chemoluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
En2	engrailed 2
ER	endoplasmatic reticulum
et al.	*et altera*
EtBr	ethidium bromide
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FGF1	fibroblast growth factor 1
FGF2	fibroblast growth factor 2

FGF4-S	fibroblast growth factor 4 with a classical signal sequence
FGFR	fibroblast growth factor receptor
FV	foamy virus
g	gravitation
Gal-1	galectin-1
GFP	green fluorescent protein
GTP	guanosine triphosphate
h	hour
HASPB	hydrophilic acylated surface protein B
HCl	hydrochlorid acid
HEK	human endothelial kidney (cells)
HIV	human immunodeficiency virus
HMGB	high mobility group protein
HRP	horse raddish peroxidase
HSPG	heparan sulfate proteoglycans
ICE	interleukin converting enzyme
IgG	immunoglobulin G
IgG2α	immunoglobulin 2 α
IL	interleukin
IRES	internal ribosome entry site
kDa	kilo Dalton
M	Molar
mA	milliampere
MAP	microtubule associated protein
MCAT	murine cationic amino acid transporter
MCS	multiple cloning site
mg	milligramme
MIF	migration inhibtory factor
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
MTS	mitochondrial targeting sequence
NaCl	natrium chloride

NES	nuclear export signal
ng	nanogramm
NLS	nuclear localization signal
nm	nanometer
NSF	N-ethyl-maleimid sensitive factor
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
pH	$-\log_{10} [H^+]$
PKC	proteinkinase C
PVDF	polyvinyliden fluoride
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SNAP	soluble NSF attachment protein
SNARE	soluble NSF attachment protein receptor
SRP	signal recognition particle
Taq	*thermus aquaticus*
Tat	HIV transactivator protein
TEMED	N,N;N´,N´-tetramethylethylenediamine
Tris	tris [hydroxymethyl] aminoethane
Tween 20	polyoxethylene sorbitane monolaureate
u	units (enzyme activity)
uPA	urokinase-type plasminogen activator
V	volt
v/v	volume per volume ratio
VEGF	vascular endothelian growth factor
w/v	weight per volume ratio

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Something unknown is doing we don't know what.

Sir Arthur Eddington (1882 - 1944)

The important thing is not to stop questioning.

Albert Einstein (1879 - 1955)