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POST-TRANSLATIONAL INSERTION OF A SMALL TAIL-ANCHORED PROTEIN INTO THE MEMBRANE OF THE ENDOPLASMIC RETICULUM

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Finally, I would like to dedicate this work and efforts put into it to two beautiful angels that are always watching over me. I love you.

...volim vas...

Aleksandru i Maji

1. ABSTRACT

Proteins destined for membrane insertion can be targeted to and inserted into the endoplasmic reticulum (ER) either during synthesis (cotranslationally) or after their synthesis is completed (post-translationally). While the cotranslational pathway is well characterized, much less is known about a post-translational pathway for targeting and insertion of membrane proteins.

In this study post-translational targeting and insertion of the small, tail-anchored ER membrane protein RAMP4op was analyzed. RAMP4op has been chosen as a model because of its small overall length, carboxy terminal location of the transmembrane (TM) domain and the presence of the short cytoplasmic segment. This allows investigation to be focused on the significance of the TM domain in processes of ER targeting and insertion.

Membrane targeting and insertion of RAMP4op was analyzed in the rabbit reticulocytes lysate *in vitro* translation system supplemented with rough microsomal membranes (RM) post-translationally. Upon insertion into the membrane, RAMP4op becomes N-glycosylated. This allows clear discrimination between cytosolic and membrane inserted forms of the protein. In this assay system, RAMP4op can be efficiently targeted and inserted into RM using a post-translational pathway dependent on ATP hydrolysis.

In the absence of membranes and after release from the ribosome, RAMP4op was detected in a defined soluble cytosolic complex. Cytosolic RAMP4op could be maintained in an insertionally competent state for at least one hour. Chemical crosslinking was used to search for and analyze potential interacting partners of RAMP4op. In the absence of membranes, a single cytosolic, non-ribosomal protein of 40 kDa (p40) was discovered in the proximity of RAMP4op. The interaction with p40 is established via the TM domain of RAMP4op. This interaction is hydrophobic in nature since cross-linking between RAMP4op and p40 is abolished in the presence of a non-ionic detergent. In the presence of RM RAMP4op could not be cross-linked to p40. Cross-linking between these two proteins was re-established upon removal of membranes. This suggests that the interaction between RAMP4op and p40 is part of the pathway for post-translational targeting of RAMP4op.

Treatment of RM with trypsin resulted in significantly reduced efficiency of RAMP4op insertion. This shows that ER membrane proteins are required for the efficient post-translational insertion of RAMP4op. Treatment of RM with trypsin in lower concentrations, sufficient to inactivate SRP receptor, had no effect on the efficiency of RAMP4op post-translational insertion. Therefore, functional SRP receptor is not required for the ER insertion of RAMP4op.

In context of these findings, cytosolic factors that are possible candidates for p40 or may be involved in an ATP hydrolysis-dependent step during RAMP4op post-translational targeting/insertion are discussed.

ZUSAMMENFASSUNG

Integrale Membranproteine können entweder gleichzeitig mit ihrer Synthese, d. h. cotranslational, oder nach Abschluss ihre Synthese, d. h. posttranslational, in die Membran des endoplasmatischen Retikulums (ER) inseriert werden. Während der cotranslationale Insertionsprozess vergleichsweise gut charakterisiert wurde, ist nur wenig über die Vorgänge bei der posttranslationalen Membraninsertion bekannt.

In dieser Arbeit wurde die posttranslationale Membraninsertion des kleinen ER-Proteins RAMP4op untersucht, welches über seinen Carboxyterminus in die Membran inseriert wird. Dieses so genannte „tail-anchored“ RAMP4op wurde als Modellsubstrat ausgewählt, da es eine relativ kurze Aminosäuresequenz besitzt und außer seiner carboxyterminalen Transmembrandomäne (TMD) nur ein kleines cytoplasmatisches Segment besitzt. Diese Besonderheiten erlaubten die Fokussierung auf die Funktion der TMD bei der ER-Membraninsertion.

Die Lokalisierung und Insertion von RAMP4op in die Membran wurde in einem in vitro Retikulozyten-Lysat Translationssystem untersucht, dem posttranslational ribosomenbesetzte mikrosomale Membranen (RM) zugesetzt werden konnten. Die Membraninsertion von RAMP4op führt zur N-Glykosylierung des Proteins, die eine Unterscheidung von cytosolischen und membraninserierten Formen erlaubt. In dem verwendeten System kann RAMP4op effizient auf einem ATP-abhängigen, posttranslationalen Weg in die RM inserieren.

In der Abwesenheit von Membranen und nach der Freisetzung vom Ribosom konnte RAMP4op in einem löslichen, cytosolischen Komplex identifiziert werden. Dieses cytosolische RAMP4op befand sich in einem stabilen, insertionskompetenten Zustand. Mittels chemischer Quervernetzung wurde nach möglichen Interaktionspartnern von RAMP4op gesucht. In der Abwesenheit von Membranen konnte ein cytosolisches, nicht-ribosomales Protein mit einem Molekulargewicht von 40 kDa (p40) als Interaktionspartner identifiziert werden. Die Wechselwirkung von p40 mit RAMP4op wird über seine TMD vermittelt. Es handelt sich um eine hydrophobe Interaktion, da sie durch Zugabe von nicht-ionischen Detergenzien unterbunden werden konnte. In der Anwesenheit von RM erfolgte keine Quervernetzung von RAMP4op und p40; wurden jedoch die Membranen anschließend wieder entfernt, so konnte erneut eine Interaktion beobachtet werden. Diese Ergebnisse deuten darauf hin, dass eine Interaktion von RAMP4op und p40 Bestandteil des posttranslationalen Insertionswegs für RAMP4op ist.

Die Trypsinbehandlung von RM reduzierte die Membraninsertion von RAMP4op signifikant. Proteine der ER-Membran sind also für eine effiziente posttranslationale Insertion erforderlich. Niedrige Trypsinkonzentrationen, die für die Inaktivierung des SRP-Rezeptors hinreichend sind, besitzen jedoch keinen Effekt auf die posttranslationale Insertion. Ein funktioneller SRP-Rezeptor ist also für die ER-Insertion von RAMP4op nicht erforderlich.

In diesem Zusammenhang werden cytosolische Faktoren diskutiert, die als mögliche Kandidaten für p40 in Frage kommen oder die an einem ATP-abhängigen Schritt bei der posttranslationalen Insertion von RAMP4op beteiligt sind.

2. INTRODUCTION

2.1. Structure and topology of membrane proteins

Biological membranes consist of a continuous double layer of amphipathic lipid molecules with which membrane proteins are associated in different ways. Structural organization and functional properties of different cellular membranes are determined by their proteo-lipid composition.

Membrane proteins can be classified in two main categories on the basis of the nature of the lipid-protein interaction. *Integral membrane proteins* span the lipid bilayer and are therefore sometimes referred to as "transmembrane proteins". They can be solubilized by detergents. *Peripheral membrane proteins* are associated with membranes through an interaction with integral membrane proteins or via covalently attached lipid chain. As they do not span the membrane, detergent solubilization is not required for their release. Instead, peripherally associated proteins can be extracted using buffers with high or low pH, different ionic strength or by enzymatically breaking the bond between a protein and a membrane-embedded lipid anchor.

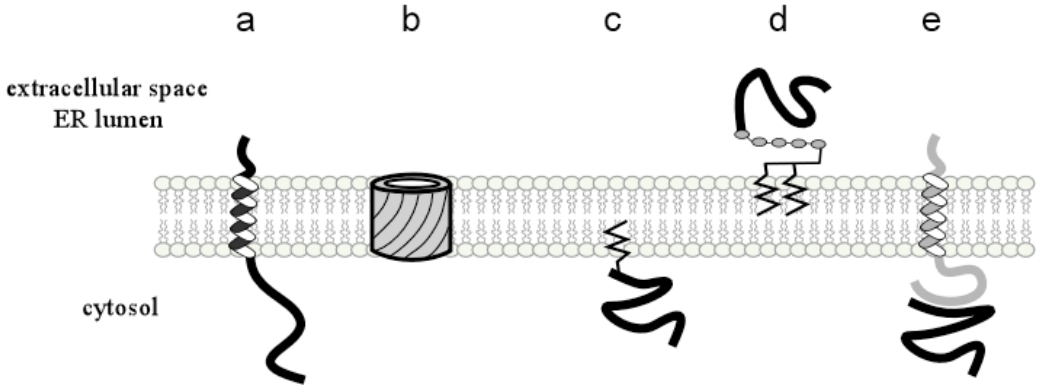

Fig. 1: Types of membrane protein associations with a lipid bilayer; a-integral, alpha helical transmembrane protein; b-integral transmembrane protein with the beta-barrel structure; c-peripheral membrane protein with a lipid-modified terminus; d-GPI-anchored protein; e-peripheral protein associated with a membrane indirectly via interaction with an integral membrane protein.

2.1.1. Integral membrane proteins

Proteins that are spanning a lipid bilayer and are inserted into a membrane have their water-soluble parts exposed to hydrophilic environments on both sides of a membrane. One or more polypeptide chain segments built from apolar residues are embedded within the hydrophobic core of a lipid bilayer. A membrane-spanning domain of integral membrane proteins most often consists of one or more α helices, but can also be organized from multiple β strands (Fig. 1a and 1b).

In the α helical conformation, hydrogen bonding between neighboring peptide bonds is maximized. This structure allows polar carbonyl and imino groups, involved in formation of the peptide backbone, to be shielded from the apolar lipid environment within a membrane. Hydrophobic side chains of these amino acids protrude outward from the helix and can form van der Waals interactions with fatty acyl chains in a lipid bilayer. The length of a membrane-embedded α helix is between 15 to 25 residues.

In a *β barrel* structure, a cylindrical transmembrane domain is formed from multiple antiparallel β sheets that have their polar side chains projected towards the hydrophilic interior of a barrel. Hydrophobic side chains are oriented toward the lipid environment. These structural elements are often found in outer membrane proteins of bacteria, mitochondria and chloroplasts.

2.1.1.1. Single-spanning integral membrane proteins

Proteins that are spanning a lipid bilayer with only one transmembrane domain are called single-spanning membrane proteins. Depending on orientation in the membrane these proteins are classified in two groups. **Type I** membrane proteins have their amino terminus in an extracytosolic compartment (lumen of the ER, bacterial periplasm) (Fig. 2a). Carboxy terminus of type I membrane proteins is located in the cytosol. **Type II** membrane proteins have the opposite orientation with amino terminus inside the cytosol and carboxy terminus in the extracytosolic compartment (Fig. 2b).

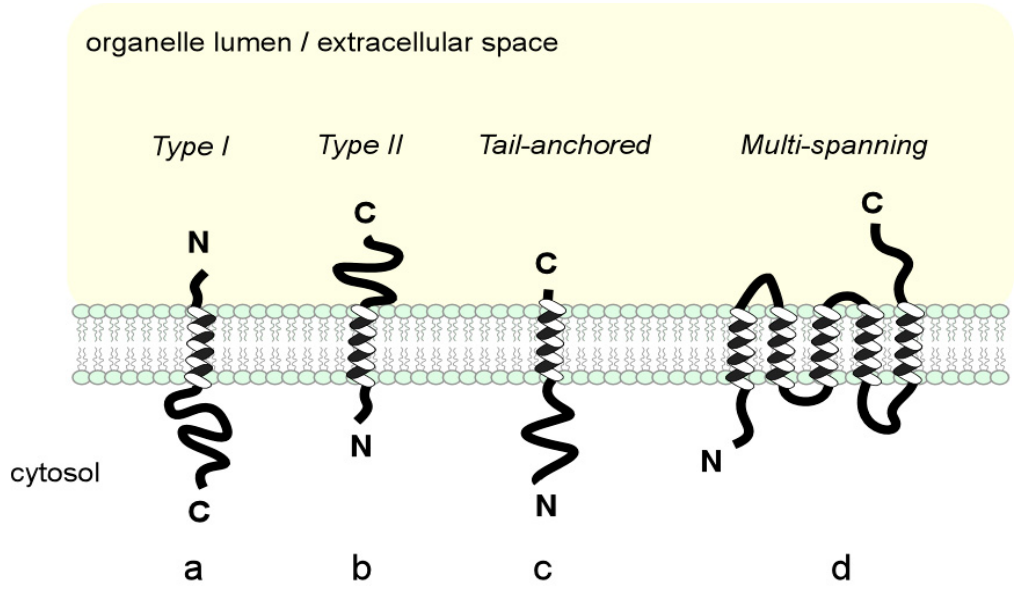

Fig. 2: Types of integral membrane proteins (MP); (a) Type I MP have their amino terminus in an extracytosolic compartment/lumen of the ER; (b) Type II MP have their carboxy terminus in an extracytosolic compartment/lumen of the ER; (c) transmembrane domain of tail-anchored proteins is located at the extreme carboxy terminus; (d) multi-spanning integral MP contains multiple hydrophobic segments that act as transmembrane anchors.

Proteins belonging to both of these groups can have their transmembrane domain located in different parts of a molecule. In extreme cases, the bulk of a polypeptide chain can be on one or the other side of a membrane. Members of the **tail-anchored** protein family, for example, have α-helical transmembrane domain located within carboxy terminal 30-40 residues. As these proteins are oriented in type II topology, only a very small number of carboxy terminal amino acids reside in the extracytosolic compartment (Fig. 2c).

2.1.1.2. Multi-spanning integral membrane proteins

Proteins that have more then one transmembrane domain and are spanning the membrane multiple times are called multi-spanning membrane proteins (Fig. 2d). Their termini can be located either in cytosol or in extracytosolic compartment. Hydrophilic domains of a protein form loops of different sizes. Hydrophobic α-helices of multispanning membrane proteins are often tightly bundled to compact structures from which lipids are excluded. These *intramolecular* helix-helix contacts are mainly based on hydrophobic interactions, as well as on interhelical hydrogen bonding (150). There are few examples where two helices are connected by a pair of charged residues (64, 117). *Intermolecular* contacts established between transmembrane domains of different multi-spanning membrane proteins form the basis for the formation of defined oligomeric complexes.

2.1.2. Peripheral membrane proteins

Majority of these proteins establish associations with membranes via an interaction with integral membrane proteins (Fig.1e). In addition, certain cytosolic proteins become attached to the cytosolic face of a membrane by fatty acyl group (myristate or palmitate) attached to the amino terminal glycine residue (Fig. 1c). Anchoring to the membrane can also be achieved by unsaturated fatty acyl group (farnesyl or geranylgeranyl) attached to a cysteine residue at or near the carboxy terminus.

Specific amino acid motifs found in some cytosolic proteins can mediate reversible association of these proteins with membranes. More common of these *lipid binding domains* are pleckstrin homology domain (PH domain - binds phosphorylated phosphatidylinositols), C2 domain (binds acidic phospholipids) and the ankyrin-repeat domain (binds phosphatidylserine).

Third type of membrane associations is found in certain proteins attached to the extracellular side of the plasma membrane. These, so-called *GPI anchored* proteins (Fig. 1d) have the glycolipid containing phosphoetanolamine and phosphatidylinositol (PI) attached to their carboxy terminus (89). Membrane association is established via PI fatty acid chains which are inserted into the lipid bilayer.

2.2. Biosynthesis of membrane proteins

Pathways of membrane protein biogenesis in organisms ranging from bacteria to higher eukaryotes share a common outline. Translation of all membrane proteins is initiated on cytosolic ribosomes. Targeting to the correct destination membrane within a cell can occur during ongoing synthesis (cotranslationally) or after protein has been released from the ribosome (post-translationally).

Delivery to the correct location in cell depends on a targeting signal present within a membrane protein itself (123). Most often a targeting signal is represented by hydrophobic sequence of amino acids that can be part of a transmembrane region. Choice of the pathway used for targeting of membrane proteins depends on physico-chemical properties of a signal (length, hydrophobicity/amphipathicity, location of a signal within a polypeptide chain). In some proteins, targeting signals are cleavable and can be proteolytically removed upon successful delivery to the destination membrane.

Delivery of newly synthesized membrane proteins to correct locations is of special importance for eukaryotic cells which have a complex system of membrane-enveloped organelles containing different sets of proteins. Targeting information contained within a signal sequence is decoded by cytosolic factors that transiently associate and escort membrane proteins to correct destinations. During cotranslational targeting, these cytosolic factors associate with a protein substrate to be delivered while it is still being synthesized on cytosolic ribosomes. Post-translationally targeted proteins associate with cytosolic components after termination of translation. Besides having a role in delivery of a substrate to the specific location, members of cytosolic targeting complexes can be involved in maintenance of an insertionally competent folding state of delivered cargo. This is particularly important for post-translationally targeted membrane proteins that contain one or more hydrophobic domains prone to aggregation in the hydrophilic environment of the cytoplasm. In order to be protected from misfolding and aggregation, these proteins may associate with members of different families of molecular chaperones or chaperone-like factors (151).

Cytosolic targeting factors, associated with proteins to be delivered, are recognized and bound by membrane associated receptor complexes located at a correct destination site within a cell. In order to be inserted into a target membrane, delivered proteins are transferred from a receptor complex to a proteinaceous channel that is embedded in the lipid bilayer.

Translocation of a polypeptide chain through the channel and across a membrane is a process that requires energy (123). It is provided by the action of molecular machines that can either “push” the translocating protein from one side, or “pull” it from the other side of the membrane. The actual insertion into the lipid bilayer is a consequence of lateral polypeptide movement within the plane of a

membrane. This enables the hydrophobic transmembrane domain to move out of the channel and associate with surrounding lipids.

Folding of membrane proteins into the final conformational state can occur during the translocation phase or after insertion into the bilayer. This process can include oligomerization of proteins that are part of membrane complexes. Improperly folded or damaged membrane proteins are destined for degradation or are collected as a cellular debris in form of aggregates (21).

2.2.1. Topological determinants for insertion of proteins into the bacterial or ER membranes

The topology of membrane proteins with α-helical transmembrane segments is established during the membrane insertion phase. Single-spanning membrane proteins can be arranged in either type I (N-terminus faces the lumenal/exocytosolic side) or type II orientation (N-terminus faces the cytosol). Due to the presence of multiple transmembrane domains, multi-spanning membrane proteins can adopt a number of different topological states. In this case multiple transmembrane domains separate hydrophilic parts of a protein that can form loops of different sizes located on either side of a membrane.

In addition to their function in targeting, signals contained within the amino acid sequence of a membrane protein have an important role in protein topogenesis (76, 136). Three types of signals can influence the membrane protein topology.

Cleavable signal sequences are thought to insert in a loop like fashion, with the amino terminus facing the cytosol. Only in this topology carboxy terminal cleavage site of a signal sequence becomes exposed to signal peptidase located on the lumenal/exocytosolic face of a membrane. A membrane protein that is targeted to the ER by cleavable signal sequence becomes anchored to the lipid bilayer by hydrophobic *stop-transfer* sequence. This topogenic element, which induces arrest of further translocation, acts as the transmembrane domain upon insertion into the lipid bilayer. Signal sequences that cannot be cleaved, *signal-anchor sequences*, as their name implies serve a dual purpose. In the cytosol they act as membrane targeting signals. In the ER membrane, the hydrophobic domain of a signal-anchor sequence inserts into the lipid bilayer becoming a transmembrane anchor.

Membrane orientation of signal-anchor sequences depends on multiple factors. Experiments conducted by different groups (118, 122, 134) have pointed out the role of the hydrophobic segment length in determination of the topology. Based on these studies it appears that longer apolar segments facilitate translocation of the amino terminus (type I orientation). Shortening of the TM domain has the opposite effect: more efficient translocation of the carboxy terminal part of a transmembrane protein. Another factor is the hydrophobicity of the apolar core of a signal. More hydrophobic sequences have higher tendency for translocation of an amino terminus into the ER lumen (32).

One of the membrane insertion determinants very often used for topology prediction is the distribution of charged residues around the hydrophobic core of signal sequences and transmembrane domains. So-called "positive-inside rule" was first established after statistical analysis of bacterial proteins where positive residues were found to be about three times more abundant in cytoplasmic parts of transmembrane proteins (139). This has generally been attributed to the transmembrane potential (negative in the cytoplasm relative to the bacterial periplasm) and to the fact that negatively charged lipids are more abundant in the cytoplasmic bilayer leaflet (137). For ER membrane proteins, the net difference in charge between polypeptide segments flanking the hydrophobic core may influence orientation of a signal sequence (50). A flanking segment with greater positive charge is generally cytoplasmic.

It was recently reported that specific charged residues in lumenal and cytoplasmic loops of Sec61α translocon component contribute to orienting signal sequences according to the positive-inside rule in the ER (43). The authors of this study proposed that the more positively charged part of a topogenic signal becomes cytoplasmically oriented because of its interaction with negatively charged residues in the cytoplasmic loop of Sec61α. Introduction of point mutations that caused charge inversions in critical Sec61α amino acids (R67, R74 and E382) weakened the positive-inside charge rule and have led to increase in the amount of the protein inserted with inverted topology.

Although several studies have shown that mutations of charged residues flanking a hydrophobic region affect orientation of a protein, an asymmetric distribution of charges is often not sufficient to inverse topology of a protein (5, 11). It is thus clear that additional factors contribute to topogenesis of membrane proteins. One such factor is the folding of the part of a polypeptide located amino terminally from a targeting signal. Such a folding event may sterically prevent translocation of an amino terminus irrespectively of charge distribution.

2.2.2. Protein targeting and insertion into the bacterial plasma membrane

Targeting to the membrane of bacterial secretory and integral membrane proteins is accomplished using different pathways. Both classes of proteins contain signal sequences that are recognized by cytosolic factors that mediate membrane delivery. Subtle differences in the hydrophobicity of a signal sequence determine the pathway that will be followed during the targeting.

Protein insertion into/translocation across the membrane of bacteria depends on number of membrane-associated protein factors, usually organized in multimeric complexes. Different substrates require different sets of these factors for the efficient insertion. Membrane translocation in bacteria also requires presence of proton motive force (PMF).

2.2.2.1. Cytosolic factors that mediate targeting to the bacterial membrane

Targeting of bacterial membrane proteins is dependent on *signal recognition particle* (SRP) which consists of the 48 kDa GTPase called Ffh and the 4.5S RNA molecule (69, 95). Carboxy terminal M-domain of Ffh, together with the specific region of the 4.5S RNA (domain IV), forms the surface to which a signal sequence is attached by the combination of hydrophobic and electrostatic interactions. SRP binds to a nascent chain during protein elongation, after about 70 amino acids have been synthesized. The ribosome-nascent chain complex is targeted by SRP to the membrane-bound GTPase called FtsY which functions as SRP receptor (37, 93). In *E. coli*, FtsY is distributed between the cytoplasm and the membrane (84). It is thought that membrane association of FtsY is maintained through its amino terminal 200 amino acids comprising A domain (108, 149). Computer analysis of the FtsY homologues in Gram-positive bacteria from the order Actinomycetales shows presence of a putative transmembrane domain at the amino terminus of the protein (13).

In vitro studies have shown that SRP and FtsY stably interact with each other when both proteins are in the GTP-bound form. Interaction between the ribosome-nascent chain-SRP complex and FtsY serves the purpose of localizing the targeted protein in vicinity of the membrane embedded system that conducts insertion into the lipid bilayer. Interaction between SRP and FtsY also induces reciprocal activation of GTPase domains of both proteins (109). GTP hydrolysis leads to dissociation of SRP from FtsY which allows recycling of SRP to the cytosol where it can engage in another round of targeting.

Recent *in vivo* studies conducted by Bibi and co-workers show that in addition to suggested SRP-dependent pathway for targeting of membrane proteins in *E. coli*, an alternative pathway may exist in which FtsY plays a central role (55, 56). The postulated mechanism of this pathway assumes that translating ribosomes are targeted to the bacterial membrane in the complex with FtsY. According to this model, SRP has no role in targeting itself, but is required at later stages for transfer of a nascent chain to the membrane-embedded translocation apparatus and/or release of the ribosome/FtsY from the membrane.

Bacterial proteins destined for secretion are preferentially delivered to the membrane after being released from the ribosome (25, 76, 115). These proteins contain amino terminal signal sequences which are significantly less hydrophobic than the signal sequences of bacterial inner membrane proteins. Due to this decreased hydrophobicity SRP cannot bind to such signal sequences (80). Ribosome-bound nascent chains that escape in this way SRP recognition become associated with the cytosolic chaperone SecB after about 150-200 residues have been synthesized (111). SecB is a protein of 17 kDa that functions as a homotetramer, organized as dimer of dimers (29). It is believed that signal sequence does not contribute directly to the interaction of preproteins with SecB. Instead, a signal sequence could indirectly affect SecB binding by retarding the folding of the mature domain of

a preprotein (83, 112). *In vivo*, SecB shows binding selectivity towards proteins that are rich in beta-sheet structures and are prone to aggregation. A typical SecB binding motif is about nine amino acids long and enriched in aromatic and basic residues, while acidic residues are strongly disfavored ((70) and references therein).

After termination of translation, a complex between newly synthesized protein and SecB is delivered to the bacterial membrane, where it engages in translocation.

2.2.2.2. Components of the bacterial membrane required for protein insertion

The majority of proteins targeted to the bacterial membrane via SRP- and SecB-dependent pathways utilize membrane-embedded, heterooligomeric complex called "translocon" for both insertion into and translocation across the lipid bilayer. Bacterial translocon consists of a protein conducting channel formed by set of transmembrane proteins. SecY and SecE form the core of this channel (70). These two subunits are essential for the translocation. *In vivo* SecYE associate with SecG to form SecYEG translocon complex. SecG, although not essential, enhances the efficiency of protein translocation, in particular at lower temperatures and when the proton motive force is low or absent (47, 101). The motor of the translocation in bacteria is represented by the ATPase SecA ((70) and references therein). This protein can cycle between the cytosol and the membrane where it associates with the SecYEG complex. SecYEG - bound SecA can drive nascent chain translocation at the expense of ATP hydrolysis. It functions as a homodimer that can accept nascent chains brought to the membrane by the SRP-ribosome complex or proteins to be secreted delivered in the complex with SecB. In the absence of bound substrate, SecA is in the ADP-bound form. Upon binding of a protein to be translocated, SecA exchanges ADP for ATP. In its ATP-bound state, SecA converts to a more extended conformation and becomes partially inserted into the translocon channel. During this conformational change, a stretch of the polypeptide of about 2-2.5 kDa in mass becomes inserted into the translocon. Upon hydrolysis of ATP, SecA reverses the conformational change, releasing the translocated part of a protein to SecYEG. SecA can then rebind to the partially translocated substrate and the whole cycle starts from the beginning. Recently, another model was proposed that is not based on cycles of SecA membrane insertion (127). According to this model, ATP binding would result in movement of only a small SecA region that binds to a nascent chain. This movement would be directed towards the SecYEG channel, allowing the polypeptide translocation to occur. Upon the ATP hydrolysis this SecA domain would be returned to its original position and the cycle would repeat.

Recently, another component of the bacterial membrane was discovered that is involved in the assembly of membrane proteins. YidC is a 60 kDa protein with six transmembrane domains. It was initially identified as the sole factor that, in the presence of the proton motive force, mediates membrane insertion of two small bacteriophage proteins previously thought to insert spontaneously

(26, 120, 121). The coat protein of the filamentous phage Pf3 has 44 amino acids and the single transmembrane domain. The coat protein of M13 phage is 73 amino acids long and contains cleavable signal sequence and two TM domains (74). In the absence of YidC, these two proteins associate hydrophobically with the membrane in a manner that allows partial partitioning of a polypeptide into the lipid bilayer, without the translocation of a hydrophilic segment across the membrane. YidC supports the translocation event and promotes folding of a hydrophobic segment into a transmembrane conformation (126). Therefore, YidC is proposed to function as a membrane chaperone that can support folding reactions within the lipid environment, as well as to mediate Sec-independent membrane protein insertion.

A portion of YidC molecules in the bacterial membrane can also be found in association with the SecYEG translocon (125). Certain inner membrane proteins that require Sec translocon for the insertion (single-spanning FtsQ, multispanning leader peptidase - Lep and manitol permease - MtlA) can be cross-linked to YidC in later stages of translocation, after the nascent chain has already been inserted into the translocon ((27, 28, 75) and references therein). However, depletion of YidC has a relatively mild effect on the membrane insertion of these proteins. It has been proposed that SecYEG-associated YidC is involved in catalyzing insertion into the lipid bilayer after the transmembrane regions have interacted with the SecYEG complex. YidC could also have a role in packing and assembly of TM domains before their coordinated release into the lipid bilayer.

Homologues of YidC exist in mitochondria (Oxa1) and chloroplasts (Alb3) (75). Oxa1 appears to be a part of the oligomeric complex involved in the insertion of mitochondrial inner membrane proteins from the matrix of the organelle in a process that requires presence of the transmembrane potential (53, 62, 96). Alb3 is involved in the insertion of proteins into the membranes of the chloroplast thylakoid system (63, 94).

Another Sec-independent pathway which is used for translocation of fully folded proteins, often with bound cofactors, is mediated by membrane proteins TatA, TatB and TatC. These proteins are proposed to form a different type of a protein conducting channel in the membrane (12). Signal sequences directing proteins into this pathway resemble typical bacterial signal sequence, but include the conserved double arginine motif and appear to have less hydrophobic H-region. A source of energy required for the Tat-mediated translocation is not known, but homologous components mediate the ΔpH dependent import of folded proteins in the thylakoid system of the chloroplast.

2.2.2.3. Complexity of membrane protein biogenesis in bacteria

A thorough analysis of targeting and insertion of membrane proteins characterized by different topologies and presence/absence of large periplasmic/cytosolic domains has been conducted by Fröderberg and coworkers (38).

In these experiments, SRP and YidC were shown to be involved in the assembly of all analyzed membrane proteins, although significant differences were detected in YidC dependence. Two of analyzed proteins (one with two TM segments and the large cytosolic domain and the other with the single TM segment and the large periplasmic domain) were inserted independently of SecYE. SecA was involved in translocation of large periplasmic domains of analyzed membrane proteins. The requirement for SecA was linked to the requirement for SecY and SecE.

Taken together it can be concluded that, although certain trends are present, targeting and assembly of membrane proteins in bacteria occur using multiple mechanisms and overlapping sets of factors.

2.2.3. Protein targeting and insertion into the endoplasmic reticulum

Eukaryotic proteins destined for secretion or membrane incorporation are first inserted into the endoplasmic reticulum. Cells posess multiple pathways for the delivery of proteins to the ER (66).

Proteins that are targeted to the ER during translation and in a complex with the synthesizing ribosome are defined as *cotranslationally* targeted proteins. Pathway that mediates cotranslational targeting is well characterized and appears to be unique for all eukaryotes (115, 130).

Proteins that are delivered to the ER after being synthesized in the cytosol and released from ribosomes are said to be *post-translationally* targeted (15, 66, 114, 147). The term "post-translational", however, is more technical in nature and refers to the fact that a protein can be targeted to the ER in a ribosome-independent way, after the termination of translation. Factors and conditions required for the post-translational targeting to the ER are less defined and not well characterized. Based on current knowledge, the term "post-translational targeting pathway" appears not to represent a single pathway, but instead encompass multiplicity of mechanisms used for post-translational delivery of different classes of proteins. Ribosome-independent post-translational targeting to the ER was mainly characterized in *Saccharomyces cerevisiae* and in *in vitro* translation experiments using mammalian cell extracts supplemented with ER membranes.

Sufficiently hydrophobic sequences in both cotranslationally and post-translationaly delivered ER proteins act as targeting signals (98, 105, 114, 123). During the cotranslational delivery, cytosolic components that mediate targeting and help in maintenance of the insertionally competent state interact with a nascent chain during elongation (69, 95, 130). These associations are preserved during the targeting of the ribosome-nascent chain complex and are important for the first steps of the insertion into the ER. Post-translationally targeted proteins are compeletely exposed to the cytosolic environment after termination of translation. In order to prevent misfolding or modifications, these proteins interact with different cytosolic factors (90, 105, 151). This is particulary important for the post-translationally delivered membrane proteins that contain one or more hydrophobic domains prone

to aggregation in the hydrophilic environment. Maintenance of the insertionally competent state of these proteins is acquired by their association with cytosolic chaperones or chaperone-like factors. These cytosolic targeting complexes can include proteins acting to decode a targeting signal, as well as additional factors required for the docking to the ER.

2.2.3.1. Cotranslational targeting to the ER

Proteins that are targeted cotranslationally to the endoplasmic reticulum (ER) (Fig. 3) contain an amino terminal signal sequence that has the same tripartite structure as a bacterial one (positively charged amino terminal region, 6-12 hydrophobic residues of the H-region and the slightly polar C-region) (85). In case of cotranslationally targeted membrane proteins which lack a cleavable signal sequence, the first transmembrane domain serves as a hydrophobic targeting signal.

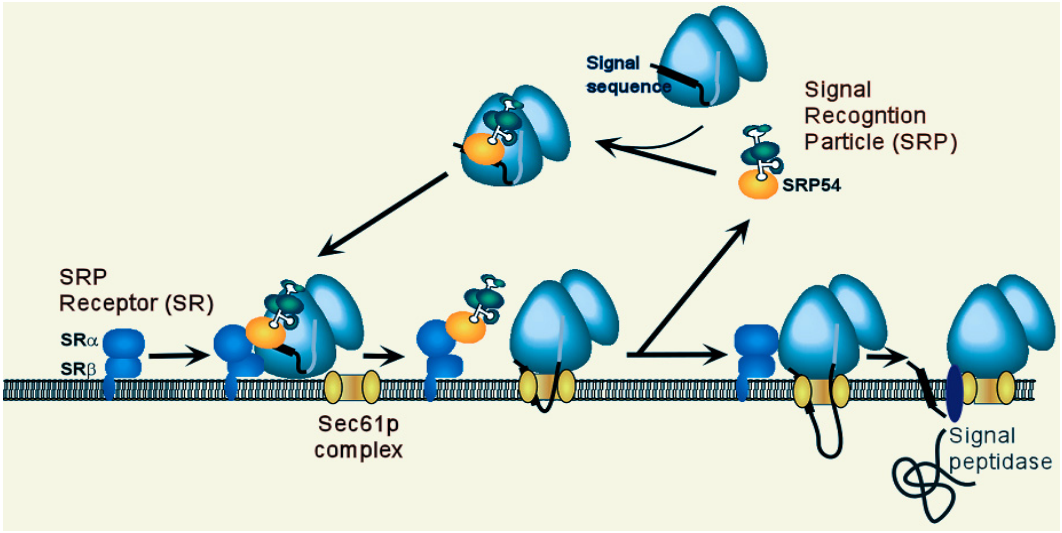

Fig. 3: Cotranslational targeting to the ER; The complex between the ribosome and a synthesizing nascent chain is recognized in the cytosol by SRP. SRP binds both to the hydrophobic part of a signal sequence and to the ribosome. SRP binding causes arrest in elongation. The ribosome-nascent chain-SRP complex is then delivered to the ER where SRP associates with SRP receptor. After transfer of a nascent chain onto the Sec61 translocon, SRP dissociates from its receptor and is recycled to the cytosol. Elongation of a nascent chain continues coupled with the simultaneous translocation across the ER membrane.

Eukaryotic signal recognition particle (SRP), which recognizes and binds to the hydrophobic core of a signal sequence, is a ribonucleoprotein complex assembled of six proteins and 7S RNA molecule (69, 95). Protein components of the SRP complex are named according to their apparent molecular mass (SRP9, SRP14, SRP19, SRP54, SRP68 and SRP72). 7S RNA molecule is a scaffold onto which protein components are assembled. SRP binds to the signal sequence and the ribosome, causing stalling of further translation. This elongational arrest is mediated by *Alu domain* of SRP, comprised of SRP9 and SRP14 subunits. Alu domain binds near A-site of the translating ribosome. This binding in turn prevents access of aminoacylated-tRNAs to the ribosome (46). Signal sequence is

bound to both SRP54 (the homologue of bacterial Ffh) and to the specific region of 7S RNA. Hydrophobic core of a signal sequence interacts with the methionine-lined pocket of SRP54 M-domain, while polar amino or carboxy terminal regions of a signal sequence make contacts with the RNA (9).

The ribosome-nascent chain-SRP (RNC-SRP) complex is targeted to the cytosolic side of the ER membrane via specific interaction between SRP and SRP receptor (SR). SR is a heterodimeric protein complex found exclusively in the rough ER membrane (37, 93). The larger SRα subunit (which is a homologue of bacterial FtsY) is peripherally associated with the ER via SRβ subunit, an integral single-spanning membrane protein. The transmembrane domain of the β subunit can be deleted without the loss of function, suggesting that SRP receptor has to be only transiently associated with the ER membrane in order to carry out its function.

Cycles of GTP binding and hydrolysis regulate the cotranslational targeting to the ER. Three GTPases involved in the process are SRP54, SRα and SRβ. GTP-binding site of SRP54 is empty when it binds to signal sequence. Affinity of SRP54 for GTP increases upon contacting the ribosome (7). The GTP-bound form of SRα contributes to the stability of the SRP-SR complex (113). GTP-bound SRβ is presumably required for the coordination with the presence of the translocon (40). Signal sequence is transferred from SRP54 to the translocon only when all three GTPases are in the GTP-bound form. After that, reciprocal stimulation of GTPase activities of SRP54 and SRα causes dissociation of SRP from its receptor (8). SRP is recycled to the cytosol and used for another round of targeting.

The major constituent of the ER membrane translocon is the Sec61 protein complex (44, 88). It is composed of three subunits: Sec61α, Sec61β and Sec61γ. Sec61α (the homologue of bacterial SecY) is 476 amino acids in length and spans the membrane 10 times. Sec61β and Sec61γ (the homologue of bacterial SecE) are of 12 kDa and 10 kDa, respectively, and have one membrane-spanning segment located at their carboxy termini.

Sec61α subunit is the core component of the channel itself and is indispensable for the process of cotranslational translocation. Experiments conducted in yeast (31) have shown that Sec61γ (Sss1p in yeast) is an essential gene. The yeast homologue of Sec61β (Sbh1p) is not required for viability (35). Mammalian Sec61β subunit has been shown to facilitate cotranslational translocation at the ER membrane and may also recruit signal peptidase complex into the transient association with the translocon (65). A reporter protein that contained the cytosolic domain of Sec61β was shown to be able to bind ribosomes in a salt-dependent manner (82). Another report proposes that Sec61β might function as a guanine nucleotide exchange factor (GEF) for the SRP receptor β subunit (54).

Three dimensional reconstruction of the ribosome-Sec61 complex in the absence of a signal sequence shows perfect alignment between the central pore of Sec61α and the exit tunnel of the

ribosome (10). Therefore a nascent chain probably moves directly from the aqueous channel within the ribosome to the aqueous pore within the membrane. A recent report describing the structure of the archeal homologue of the Sec61 complex shows presence of the funnel-like cavity on the cytoplasmic side of the complex (135). A small helix originating from the transmembrane domain 2 of Sec61α acts as a mobile plug blocking access into the translocon channel. The proposed model suggests that the channel opens for translocation by displacement of this helical plug. At the narrowest point, channel is lined with hydrophobic residues („pore ring“) that can form a gasket-like seal around a translocating polypeptide, maintaining in this way the permeability barrier. This would then explain why a tight sealing between the ribosome and the translocon is not necessary for maintenance of the permeability barrier.

The Sec61 translocon is also the site of membrane protein insertion into the lipid bilayer. Transmembrane helices integrate into the membrane by partitioning between the proteinaceous translocon and lipid surrounding (57). According to this model, sufficiently hydrophobic helices would prefer the lipid bilayer, whereas more polar helices would favor the translocon and the aqueous phase (135).

A number of other proteins transiently associate with the Sec61 translocon (6, 115). TRAM is a multispanning ER membrane protein found in proximity of the transmembrane segments of nascent polypeptides. Its exact function is unknown, but it is believed to be important for translocation of a specific subset of membrane proteins (52). The tetrameric translocon-associated protein complex (TRAP) is found in proximity of nascent chains and membrane-bound ribosomes (36, 51). The signal peptidase complex consists of 5 subunits and cleaves off a signal peptide of most secretory and membrane proteins as soon as the cleavage site becomes exposed on the lumenal side of the ER. The oligosacharyl transferase complex is responsible for transfer of oligosaccharyl moieties to asparagine residues located within N-glycosylation signals of translocating polypeptides. BiP is another protein found to be transiently associated with the translocon. It is a lumenal ER protein that belongs to the Hsp70 chaperone family and is primarily involved in protein folding and assembly.

2.2.3.2. Post-translational Sec-dependent targeting to the yeast ER

Most of the studies on eukaryotic Sec61-dependent post-translational targeting to the ER has been accomplished in yeast *Saccharomyces cerevisiae* using as the model prepro-alpha factor (ppαF), a secretory protein of 165 amino acids with the cleavable signal sequence (114).

During synthesis *in vitro* in RRL, ppαF can be cross-linked to SRP and both NAC (nascent chain-associated complex) subunits (105, 116). After release of the synthesized polypeptide from the ribosome, only cross-links to Hsp70 and TCP1 (a member of the eukaryotic chaperonin TRIC/CCT complex) could be identified.

Hsp70 molecular chaperones and their co-chaperones work together to guide folding of proteins and to aid in translocation of proteins across membranes (17, 49, 60, 131, 133). Binding and hydrolysis of ATP regulate the action of Hsp70. In the ATP-bound form Hsp70 can rapidly bind and release its substrate. Upon the hydrolysis of ATP Hsp70 becomes tightly associated with a substrate. Hsp70 can stimulate protein folding by binding to exposed hydrophobic sequences and preventing protein aggregation. The activity of Hsp70 is regulated by Hsp40 cochaperones. Hsp40 binds first to a substrate protein and then delivers it to Hsp70. In addition, Hsp40 stimulates the ATPase activity of Hsp70. Members of the Hsp40 cochaperone family preferentially bind hydrophobic polypeptides and can prevent aggregation of bound substrates.

Chaperonins are the conserved class of large, barrel-shaped complexes with the central cavity (39, 49). Chaperonins are also mediators of protein folding but their mechanism of action differs fundamentally from that of the Hsp70 system. However, in both cases binding and release of a substrate is regulated by ATP hydrolysis. Non-native substrate protein associates by hydrophobic interactions with multiple chaperonin subunits. This causes positioning of a substrate inside the central cavity of the complex where folding occurs. The time allowed for folding is determined by the rate of ATP hydrolysis. After hydrolysis of bound ATP, conformational changes in chaperonin structure allow release of a substrate.

Based on the analysis of migration of *in vitro* synthesized and radiolabelled ppαF in a sucrose gradient, it was proposed that the protein is assembled in two different cytosolic complexes: one with Hsp70, the second with the chaperonin TRIC (106). By analyzing *in vivo* expression of ppαF, another group has identified Ssa1p, the cytosolic member of the Hsp70 family, as the factor required for ppαF post-translational targeting. Beside Ssa1p, targeting of ppαF was dependent also on the yeast Hsp40 homologue Ydj1p (90). The requirement for cytosolic factors during translocation of ppαF can be by-passed *in vitro* by denaturing the protein in urea before incubation with membranes. After such treatment, translocation of ppαF could be reconstituted in a test tube with purified components in the absence of cytosolic proteins (22, 87). These results suggest that ppαF has to be in a specific conformational state in order to be post-translationally translocated into the ER. This state can be achieved either with the assistance of chaperones or after unfolding caused by denaturation.

Cross-linking experiments have shown that first contacts of ppαF with proteins of the ER membrane are established with the Sec62/63 complex (33, 45). This complex consists of Sec62p, Sec63p (both essential for viability), Sec71p and Sec72p (both non-essential proteins). Sec71p, Sec62p and Sec63p are integral membrane proteins, while Sec72p is peripherally associated with the cytoplasmic side of the membrane. Sec63p has the DnaJ domain located in the ER lumen. This domain mediates recruitment of the lumenal Hsp70 chaperone Bip to the sites of protein translocation. BiP is thought to be involved in the formation of a molecular ratchet that is important for the ATP-dependent

vectorial movement of a polypeptide into the ER lumen (87). Homologues of Sec62 and Sec63 were discovered in mammalians, but their function is currently not clear (92).

After initial interaction with the Sec62/63 complex, the signal sequence of ppαF is transferred to the Sec61 complex and can be found in the proximity of the transmembrane domains 2 and 7 of Sec61α (105). Prepro-alpha factor can bind to proteoliposomes containing components of the Sec61 translocon, but the actual translocation across the lipid bilayer requires presence of the lumenal Hsp70 family member Kar2p (Bip) and is dependent on ATP hydrolysis. Therefore, it can be concluded that the post-translational translocation of ppαF requires components of the ER membrane necessary for translocation of cotranslationally targeted substrates as well.

2.2.3.3. Post-translational targeting and insertion of tail-anchored proteins

Tail-anchored proteins (TA) can be generally defined by the presence of a single transmembrane domain at their carboxy terminus (15, 77, 143). This transmembrane domain usually has between 15 and 22 amino acids and is typically followed by a relatively short extracytosolic segment. Transmembrane domain of tail-anchored proteins is so close to the carboxy terminus that it emerges from the ribosome only upon termination of translation (Fig. 4). This hydrophobic domain is therefore not expected to interact with cytosolic factors, such as SRP. This feature distinguishes TA proteins from type II membrane proteins that are delivered to the ER by the cotranslational pathway. Considering that 30-40 carboxy terminal amino acids of a nascent chain are sequestered within the exit channel of the large ribosomal subunit (14) - a protein can be defined as being a member of the family of tail-anchored proteins if its transmembrane domain is followed by no more than around 20 residues.

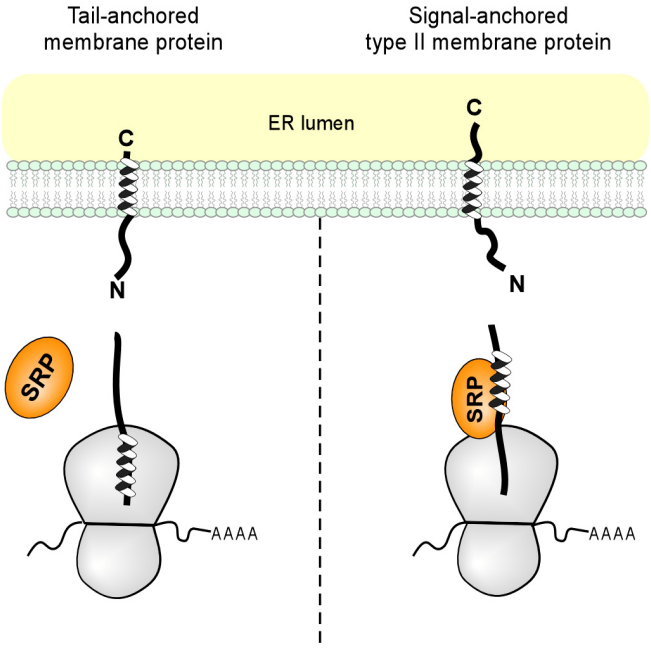

Fig. 4: Transmembrane domain (TM) of tail-anchored proteins is not accessible to cytosolic factors before termination of translation. At the end of translation, carboxy-terminally located TM segment of tail-anchored proteins is inside the ribosomal exit channel. It becomes exposed to cytosolic factors after release of a synthesized polypeptide (post-translationally). In contrast, TM domain of signal-anchored proteins is not located at the extreme carboxy terminus and becomes exposed to cytosolic factors during the elongation. After binding of SRP to the hydrophobic segment, ribosome-nascent chain-SRP complex is delivered to the ER by a cotranslational pathway.

TA proteins were found in the membranes of the mitochondria, ER, Golgi, vesicles of the secretory pathway and the plasma membrane (143). After being synthesized in the cytosol, TA proteins are delivered to the ER or mitochondria. TA proteins that reside in the Golgi, vesicles and plasma membrane are routed to these destinations after insertion into the ER. Two tail-anchored proteins (61, 68, 104) were found both in the mitochondrial outer membrane and the membrane of the ER.

The carboxy terminal transmembrane domain of TA proteins acts as a targeting signal which is neccessary and sufficient for the correct post-translational delivery. This observation is based on the results of experiments where fusion of the transmembrane domains of cytochrome b5 and Bcl2 to carboxy termini of the reporter proteins caused the reporters to be post-translationally targeted to the microsomal membrane *in vitro* (71, 100). Hydrophobicity rather than the specific sequence of amino acids within a transmembrane domain of TA proteins seems to be required for the post-translational targeting, at least *in vitro*. The tail-anchored protein synaptobrevin can be post-translationally inserted into microsomal membranes even when the carboxy terminal transmembrane region is replaced with minimum of 12 leucines (144).

It is not clear which cytosolic factors associate with tail-anchored proteins during targeting. Very recently, it has been reported that SRP can interact post-translationally with synaptobrevin and Sec61β but not with cytochrome b5 (1). Based on this, the authors suggested that different TA proteins can use alternative routes during the ER targeting step.

Conflicting reports have been presented regarding the ATP dependence of post-translational targeting and/or insertion of TA proteins. In one report, for example, cytochrome b5 was shown to insert in an ATP-independent manner (71) while another study showed that very low concentrations of ATP are indeed required (147). One of the reasons for such discrepancies may be the difference in assays used to estimate the membrane insertion. When bona fide translocation of the carboxy terminus of cytochrome b5 was assayed by N-glycosylation (147), instead of by sedimentation through the sucrose cushion (71), ATP dependence of the targeting/insertion proccess was revealed. However, this appears not to be the only example of such a dispute. Kutay et al. have shown that post-translational insertion of synaptobrevin is dependent on ATP hydrolysis (78). Abell and coworkers, on the other hand, have shown that GTP could promote post-translational insertion of synaptobrevin (1). This is in accordance with their finding that SRP and SRP receptor are required for the efficient ER targeting and insertion of this TA protein.

Insertion of tail-anchored proteins into the ER depends on at least one protein component of the membrane. Pure liposomes or rough microsomal membranes treated with trypsin do not support insertion of TA proteins (71, 78). The identity of membrane protein(s) involved, however, is not clear. Abell and co-workers have shown in a time-course experiment that during the early stages of targeting, synaptobrevin could be cross-linked to Sec61α, Sec61β, Sec62/63 and the subunit of the

signal peptidase complex SPC25 (2). Another report shows that proteoliposomes reconstituted from microsomal membranes immunodepleted of either Sec61 complex or SRP receptor, although incompetent for cotranslational insertion, were still capable of conducting post-translational insertion of synaptobrevin (78). Conclusions from this study are supported by the analysis of *in vivo* expression and ER targeting of another TA protein, cytochrome b5, in different yeast mutants defective in either Sec61, Sec62, Sec63 or Kar2/BiP (147). Neither of these ER components that are required for the cotranslational insertion or post-translational translocation of ppαF in yeast appear to be important for the post-translational insertion of cytochrome b5. These data leave open the possibility that post-translational insertion of tail-anchored proteins require yet undiscovered protein component(s) of the ER membrane.

2.3. Analysis of the post-translational targeting and insertion of a tail-anchored ER membrane protein

Mechanism of post-translational targeting and insertion of tail-anchored ER membrane proteins and the factors involved in this process are not well understood. This is reflected in the fact that contradictory results have been obtained during studies of the same protein or of similar types of proteins. One of the reasons for the lack of clear picture in the field of post-translational targeting of TA proteins might be the fact that very few substrates have been analyzed in detail so far. Majority of experiments have been conducted in *in vitro* systems, with only few reports based on genetic studies accomplished in yeast. The fact that different methodological approaches have been used in order to detect membrane insertion of TA proteins could also lead to the increase in the ammount of misinterpreted data and subsequent contradicting results.

The current knowledge of the post-translational targeting and ER insertion of TA proteins allows understanding of only the basic principles governing this process, which appears to be mediated by multiple pathways. Targeting and insertion of tail-anchored proteins into the ER membrane depends on a sufficiently long and hydrophobic segment of amino acids present *in cis* (143, 144, 147). It was often observed that the post-translational targeting and/or insertion of TA proteins depends on the ATP hydrolysis (78, 147). However, it is not clear whether ATP is required during the targeting phase, for the membrane insertion or both. Another important feature is the requirement for membrane proteins during the insertion of TA proteins into the lipid bilayer (71, 78). The identity of these membrane proteins has not been clearly determined.

Tail-anchored proteins used as substrates in studies of membrane targeting and insertion were often relatively long, with different hydrophilic segments located amino terminally from the transmembrane domain. These hydrophilic portions could potentialy engage in interactions with factors that are not important for the post-translational targeting to the ER. This makes it more difficult

to identify cytosolic and membrane associated factors that are specifically required during the targeting process.

In order to better understand mechanisms of post-translational targeting and insertion in mammalian systems, I have decided to analyze one of the smallest known tail-anchored proteins. This also allows to focus more clearly on a role that TM domain has in processes of ER targeting and insertion.

2.3.1. Ribosome Associated Membrane Protein 4 (RAMP4)

RAMP4 is an ER membrane protein that consists of 66 amino acids. Due to the presence of the carboxy terminally located transmembrane domain, RAMP4 can be classified as a member of the tail-anchored protein family (Fig. 5). Protease digestion of rough microsomes have shown that membrane integrated RAMP4 exposes its amino terminal hydrophilic portion on the cytoplasmic side and spans the membrane close to the carboxy terminal end (124). Because of the carboxy terminal location of the transmembrane domain, RAMP4 is expected to be targeted to the endoplasmic reticulum using a post-translational pathway.

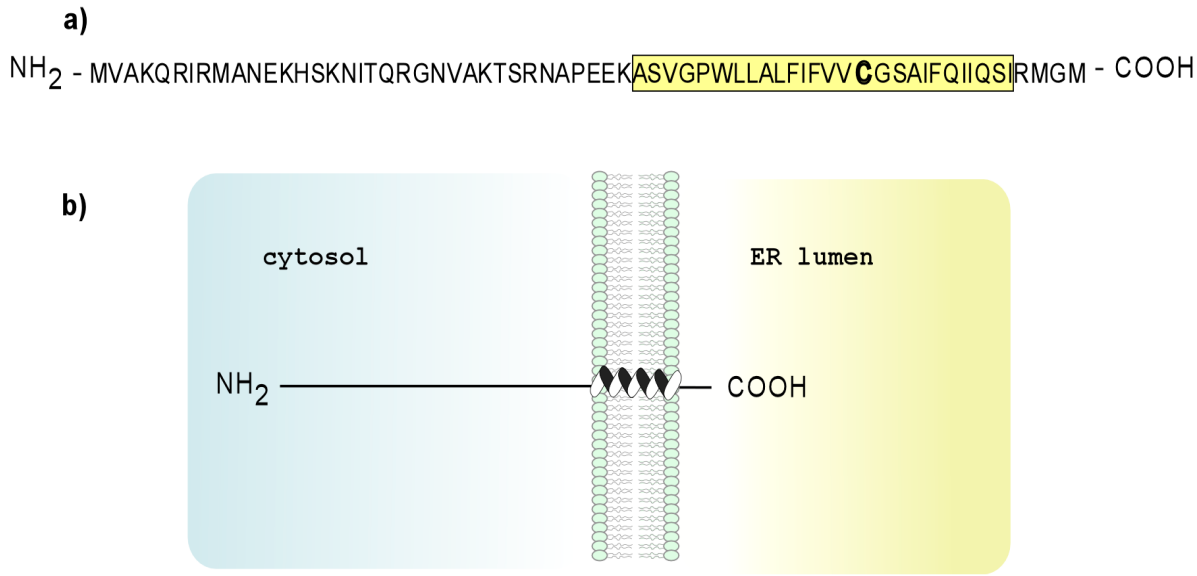

Fig. 5: Ribosome associated membrane protein 4 (RAMP4). (a) The amino acid sequence of mouse RAMP4 used in this study. Residues within the yellow box belong to the transmembrane domain, as predicted by TMPRED software. (b) The scheme of RAMP4 topology upon insertion into the ER membrane.

RAMP4 was identified as a member of the fraction containing ribosomes and membrane associated proteins after digitonin solubilization of the ER (44). RAMP4 was also found in a differential display screen as one of the overexpressed proteins in cultured rat astrocytes exposed to hypoxia (148). In cultured 293 cells subjected to ER stress, overexpression of RAMP4 suppressed aggregation and/or degradation of newly synthesized membrane proteins (148). Recently, ramp4 gene

was shown to be one of the genes induced by Xbp1, a transcriptional factor activated in the mammalian unfolded protein response (UPR) (79). These findings suggest that RAMP4 may have a role in stabilization of ER proteins in response to stress.

The aim of this study is to discover and analyze conditions and factors required for the ER targeting and insertion of RAMP4. To achieve this goal, an *in vitro* assay system will be established that allows monitoring of post-translational protein targeting and insertion into the ER. In addition, chemical crosslinking will be used to identify components interacting with newly synthesized RAMP4, as well as to characterize these interactions. The significance of membrane proteins in the process of RAMP4 insertion into the ER will be investigated using membranes treated with a protease or chemical compounds that modify proteins, such as N-ethylmaleimide.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

All standard chemicals were purchased from Sigma-Aldrich (Steinheim, Germany), Serva (Heidelberg, Germany), Merck (Darmstadt, Germany) or Gibco BRL (Eggenstein, Germany) unless otherwise indicated.

Radiochemicals methionine-S35 and Cell Labelling Mix were purchased from Amersham Pharmacia, Braunschweig.

3.1.2. Buffers, solutions and media

Solutions were made according to Sambrook et al. (119). Where necessary, sterilization was carried out by filtering through 0.22µ Millipore filter or by autoclaving at 121°C for 20 minutes.

10xDNA gel loading buffer	0.1% (w/v) Bromophenolblue 50% (v/v) glycerol
Laemmli buffer (4x, -20˚C)	200 mM TrisHCl, pH 6.8 400 mM DTT/ 10% (v/v) βME 4% (w/v) SDS 0.2% (w/v) Bromophenolblue 20% (v/v) glycerol
10xPBS	2g KCl 2.4g KH_2PO_4 80g NaCl 14.4g Na_2HPO_4, pH 7.0
1xPBST	1xPBS 0.02% (v/v) Tween 20
1000 x CLAP Mix	10g/l Aprotinin 10g/l Chymostatin 10g/l Leupeptin 10g/l Pepstatin
Lysis Buffer for HeLa cells (4^{o}C)	20 mM Hepes 100mM NaCl 5mM $MgCl_2$ 0.5mM EDTA 1% Triton X-100 1X Clap Mix
Homogenisation Buffer for HeLa cells (4^{o}C)	50mM Tris base

	25mM KCl 500 mM KOAc 1 mM $MgCl_2$ 250mM Sucrose
RM buffer for dog pancreatic microsomes (4°C)	50 mM Hepes 2mM $Mg(OAc)_2$ 50 mM KOAc 2mM DTT 250mM Sucrose
IP buffer "A"	10mM Tris-Cl pH 7.5 150mM NaCl 2mM EDTA 0.4% Triton X-100
IP buffer "B"	10mM Tris-Cl pH 7.5 500mM NaCl 2mM EDTA 0.2% NP-40
IP buffer "C"	10mM Tris-Cl pH 7.5
Anode I buffer for transfer to nitrocellulose membrane	30mM Tris base 20% Methanol
Anode II buffer for transfer to nitrocellulose membrane	300mM Tris base 20% Methanol
Cathode buffer for transfer to nitrocellulose membrane	25mM Tris base 40mM 6-Aminohexanoic acid 20% Methanol 0.01% SDS
PonceauS solution for staining proteins	1% Acetic acid 0.5% PonceauS
Comassie solution for staining proteins	Dissolve 7 tablets of SERVA Blue R (C.I. 42660) in 250 ml of 40% methanol / 10% acetic acid
SDS-PAGE buffer (5x)	25 mM Tris base 192 mM Glycine 0.5% (w/v) SDS

Media for bacterial and mammalian cells cultivation:

Bacteria were grown at 37°C in LB liquid media or on LB agar plates supplemented with 100 μg/ml of Ampicillin.

LB medium (autoclaved)	1% (w/v) Bacto tryptone 0.5% (w/v) Bacto yeast extract 170 mM NaCl adjusted to pH 7.6 with 5 N NaOH
LB agar (autoclaved)	1.5% (w/v) Bacto agar in LB medium

Dulbecco Modified Eagle's Medium (DMEM), Foetal Calf Serum, Penicillin/Streptomycin and Trypsin-EDTA were purchased from Gibco/Invitrogen, USA. All cells were grown at 37°C and 5% CO_2.

Transfection reagent used was Lipofectamine 2000 from Invitrogen, USA.

3.1.3. Bacterial strains and mammalian cell lines

Bacterial strains used in this study were DH5α and TOP10. DH5α genotype can be found at New England Biolabs (www.neb.com). TOP10 is the commercial strain available from Invitrogen (Karlsruhe, Germany).

Mammalian cell lines used in this study were HeLa, human cervical carcinoma cell line (ATCC, USA) and R2-15 hybridoma cell line producing the anti-opsin antibody. R2-15 cell line was provided by Prof. Dr. Stephen High, Manchester University, United Kingdom.

3.1.4. Enzymes

All enzymes were purchased from New England Biolabs (Beverly, MA, USA), Promega (Mannheim, Germany), Roche (Mannheim, Germany) or Amersham-Pharmacia (Uppsala, Sweeden), unless otherwise indicated.

SP6 RNA polymerase was produced and purified by K. Meese in the lab of B. Dobberstein.

3.1.5. Oligonucleotides

NAME	**SEQUENCE (5' - 3')**
GG_F1	GGCCCAAACTTCTACGTGCCTTTCTCCAACAAGACGGGCTGAAGTGACTGACCTTGA
pGG_R1	CATGCCCATCCTGATACTTTGAATAATCTGGAAAATTGCAGAGCCACAGACAA
RNC_Fwd1	GTGCTGCAAGGCGATTAAGTTG
RNC_Rew1	GCCCGTCTTGTTGGAGAAAGG
G3PDH_F	CATGAGAAGTATGACAACAGCCT
G3PDH_R	AGTCCTTCCACGATACCAAAGT
IVT_R_STOP	AGGCAGGCCGATTTACTCAATG
R4_CDS_F1	AACGAGAAGCACAGCAAGAACA
R4_CDS_R1	CAGCCCGTCTTGTTGGAGAAA
Rc_LnkF	AGCTTCCAGTGTGCTGGGCGGCCGCATCGATGTTAACCCTGCAGGT

Rc_LnkR	CTAGACCTGCAGGGTTAACATCGATGCGGCCGCCCAGCACACTGGA
CMV_GG_F1	GTGTGCTGGAATTCGCCCTTA
CMV_GG_R1	GAAAGATCTCAAGGTCAGTCAC

Oligo-dT used for cDNA synthesis was purchased from New England Biolabs.

All other oligonucleotides were purchased from Biospring, Frankfurt, Germany (www.biospring.de).

3.1.6. DNA standards for electrophoresis

A 100 bp DNA standard for estimation of molecular size was purchased from New England Biolabs. 1Kbp molecular size DNA standard was purchased from Gibco-BRL.

3.1.7. Plasmids

Plasmids	**Features**	**Reference**
pGEM4Z-RAMP4/5'3'UTR	Amp Sp6 T7 mouse RAMP4 with 5' and 3' UTRs	Joanne Young in Dobberstein lab
pGEM4Z-RAMP4/3'UTR	Amp Sp6 T7 mouse RAMP4 with 3' UTR	Joanne Young in Dobberstein lab
pGEM4Ii	Sp6, Invariant chain, Amp	(59)
pRc/CMV	Amp Neo CMV Sp6 T7	Invitrogen
pRc/CMV_Lnk1	Amp Neo CMV Sp6 T7	this thesis
pGEM4Z-RAMP4op/3'UTR	Amp Sp6 T7 mouse RAMP4 with 3' UTR and carboxy-terminal opsin tag	this thesis
pRc/CMV_R4op/3'UTR	RAMP4op CDS+3'UTR Amp Neo CMV Sp6 T7	this thesis
TOPO_R4op/CDS	RAMP4op CDS Amp Neo CMV T7	this thesis
TOPO_R4op/5'UTR	RAMP4op CDS+5'UTR Amp Neo CMV T7	this thesis
TOPO_R4op/3'UTR	RAMP4op CDS+3'UTR Amp Neo CMV T7	this thesis
TOPO_R4op/5'3'UTR	RAMP4op CDS+5'3'UTR Amp Neo CMV T7	this thesis

Sequencing of all constructed vectors was performed at Medigenomix, Martinsried, Munich, Germany (www.medigenomix.de).

3.1.8. Antibodies

3.1.8.1. Primary antibodies

Antibody	Properties	Reference
anti-Ii	rabbit, polyclonal	K. Meese (81)
anti-L23a	rabbit, polyclonal	M. Pool (107)
anti-RAMP4	rabbit, polyclonal	B. Martoglio Dobberstein lab
anti-opsin	mouse, monoclonal R2-15 hybridoma cells	Dr. Paul Hargrave (3)

3.1.8.2. Secondary antibodies

Antibody	Properties	Reference
anti-rabbit IgG	mouse, horseradish peroxidase-coupled	Sigma-Aldrich Steinheim Germany
anti-mouse IgG	goat, horseradish peroxidase-coupled	Sigma-Aldrich Steinheim Germany

3.1.9. Protein standards for electrophoresis

Radiolabelled ^{14}C molecular weight markers for low MW (2.35 - 30 kDa) and high MW range (14.3 - 200 kDa) were purchased from Amersham Pharmacia. Prestained broad range protein marker 6 - 175 kDa was purchased from New England Biolabs.

3.1.10. Kits

QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany)
QIAquick Gel Extraction-Kit (Qiagen, Hilden, Germany)
QIAquick PCR Purification-Kit (Qiagen, Hilden, Germany)
Nucleobond AX Plasmid-Purification-Kit (Machery-Nagel, Düren, Germany)
QIAGEN RNeasy mini kit for total RNA extraction
pcDNA3.1/V5-His TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany)
ExSite PCR-based site-directed mutagenesis kit (Stratagene, www.stratagene.com)
ECL Western-Blot Detection-Kit (Roche, Mannheim, Germany)
Rabbit reticulocytes lysate *in vitro* translation kit (Promega)

3.1.11. Computer programs

The text for this thesis was prepared using Microsoft Office for Macintosh version 2001. Figures were prepared with Adobe Photoshop 5 and Canvas 7.0.2. Autoradiography analyses were accomplished using MacBas 2 software for FUJI Bas 1000 phosphoimager. Sequence analyses of nucleic acids and proteins were performed using Lasergene software package and DNA Strider. Oligonucleotides were designed using Oligo 4 program.

3.2. Methods

3.2.1. DNA manipulation techniques

All standard molecular biology protocols used during cloning that are not given in detail in this chapter were described in Sambrook et al. (119). Extraction and purification of plasmid DNA from *E.coli* was accomplished using the aforementioned kits and according to manufacturer's instructions. Conditions for PCR reactions were as suggested in instruction manuals. Any changes in concentrations of PCR reagents or in program for DNA amplification were noted.

3.2.1.1. Constructions of plasmids

3.2.1.1.1. pGEM4Z-RAMP4op/3'UTR

A tag containing N terminus of bovine opsin was inserted into pGEM4Z-RAMP4/3'UTR using ExSite kit for PCR-based mutagenesis. PCR reaction contained:

- 64ng/µl pGEM4Z-RAMP4/3'UTR
- 1mM dNTP
- 0.6pmol/µl of primers GG_F1 and pGG_R1

Amplification of DNA was accomplished using the following program:

1. 94°C / 5min
2. 95°C/1 min
3. 50°C/2min
4. 72°C/6 min

Steps 2-4 repeated 2 times.

5. 93°C/1 min
6. 56°C/2 min

7. 72°C/5 min

Steps 5-7 were repeated 8 times.

3.2.1.1.2. TOPO_R4op/CDS

Cloning of this plasmid was performed using pcDNA3.1/V5-His TOPO TA cloning kit and according to manufacturers instructions. PCR reaction contained:

- 1.4ng/µl pGEM4Z-RAMP4op/3'UTR DNA
- 0.1mM dNTP
- 50 µM of primers CMV_GG_F1 and CMV_GG_R1

Amplification of DNA was performed according to the following program:

1. 94°C / 2min
2. 94°C/1 min
3. 53°C/1min 15sec
4. 72°C/1 min
5. 72°C/5 min

Steps 2 to 4 were repeated 30 times.

3.2.1.1.3. pRc/CMV_Lnk1

2 µl of each 50µM oligo RC_LnkF and Rc_LnkR were mixed with 96µl dH_2O and heated to 96°C in a water bath for 2 minutes. Annealing was accomplished by allowing the mixture to cool slowly to room temperature. Annealed linker was phosphorylated in the reaction containing 1mM ATP and 15U T4 polinucleotide kinase (Promega) for 1 hour at 37°C. Final concentration of annealed oligos in the reaction was 20ng/µl. Phosphorylated linker was ligated with the plasmid pRc/CMV digested with H*ind*III and X*ba*I.

3.2.1.1.4. pRc/CMV_R4op/3'UTR

Plasmid pGEM4Z-RAMP4op/3'UTR was digested with B*st*XI and P*st*I. The band of 2.1 Kb was isolated and purified. Plasmid pRc/CMV_Lnk1 was digested with B*st*XI and S*bf*I. The vector band containing replication origin and antibiotic resistance gene was isolated and ligated to the 2.1 Kb insert.

3.2.1.1.5. TOPO_R4op/5'UTR

pGEM4Z-RAMP4/5'3'UTR was digested with E*co*RI and P*pu*MI. The band of 400 bp was isolated and ligated with the vector band obtained after digestion of TOPO_R4op/CDS with the same pair of restriction enzymes.

3.2.1.1.6. TOPO_R4op/5'3'UTR

Plasmid TOPO_R4op/5'UTR was digested with A*pa*I and the vector band of 6 kb was purified. pRc/CMV_R4op/3'UTR was digested with A*pa*I and the band of 1.9 kbp was purified and ligated to previously isolated 6 kbp vector band.

3.2.1.1.7. TOPO_R4op/3'UTR

To obtain the band of 5.4 kb, plasmid TOPO_R4op/5'3'UTR was digested with B*st*XI and X*ba*I. This band was then ligated to the 2.1 kb insert obtained after digestion of pRc/CMV_R4op/3'UTR with the same pair of restriction enzymes.

3.2.2. *In vitro* transcription and purification of mRNA

5µg of linearized plasmid DNA containing Sp6 promotor was mixed with 5 µl NTP (stock 20mM ATP, UTP, CTP and 2mM GTP), 5 µl Cap Analog (NEB Cat.No. #1404, stock 4.5U/µl), 80U RNAsin inhibitor (Ambion), 10 µl of 5 x transcription buffer (200mM Tris-Cl pH 8, 100mM $MgCl_2$, 25mM DTT, 5 mM spermidine) and 2.5µl of Sp6 RNA polymerase. Water was added to adjust the final reaction volume to 50µl.

Reaction was incubated for 30 min. at 42°C. After this, 1µl of 100mM GTP was added and incubation was continued for another 1.5 hours. Reaction was centrifuged for 5min at 4°C on 13.000rpm. Unincorporated ribonucleotides were removed from the supernatant by centrifugation through pre-packed G-25 MicroSpin sephadex colums (Amersham Pharmacia). DNA was removed by treatment with 10U of RQ1 RNAse-free DNAse (Promega). RNA was purified using acidic phenol (pH 4.5)/chloroform/isoamyl alcohol extraction method described in Sambrook et al. (119). Purity and concentration of synthesized RNA was estimated after measuring absorbencies at 260nm and 280nm. RNA solution was aliquoted and stored at -80°C.

3.2.3. Protein synthesis

3.2.3.1. *In vitro* translation in the rabbit reticulocytes lysate

Synthesis of radiolabelled proteins was accomplished in rabbit reticulocytes lysate according to manufacturers instructions (Promega RRL kit for *in vitro* translation). Following components were assembled for 10µl reaction:

- 7µl RRL (centrifuged 10min/13000rpm/4°C before use to remove endogenous membranes)
- 0.2µl 1mM amino acid mix without Met
- 0.5µl ^{35}S (15 µCi/µl)
- 150 ng of mRNA template
- H_2O up to 10µl

Reaction was incubated for 30 minutes at 30°C and stopped by addition of puromycin to the final concentration of 2mM.

Post-translational insertion was accomplished by addition of RM to the final concentration of 25 OD280/ml and incubation for additional 30 minutes at 30°C. In reactions where newly synthesized protein was inserted cotranslationally, membranes were present during translation at concentration of 25 OD280/ml.

In reactions where cotranslational translocation of Ii into trypsin treated microsomal membranes was reconstituted, soluble recombinant SRP receptor was added to the final concentration of 100nm. Soluble SRP receptor was prepared by Oliver Schlenker in Irmi Sinning's lab, Biochemie Zentrum, Heidelberg, Germany.

3.2.3.1.1. Preparation of ribosome-nascent chain complexes (RNC) in RRL

RNCs were synthesized in RRL from a template mRNA lacking a STOP codon. Incubation time was 10 minutes at 30°C. After incubation, cycloheximide was added to the final concentration of 2mM and the reaction was incubated on ice for 5 minutes. Aggregates were removed by centrifugation in Eppendorf tabletop centrifuge for 5 minutes at 4°C and 15000 rpm. Supernatant was loaded onto the top of a sucrose cushion (25mM HEPES-KOH pH 7.6, 500mM KOAc, 5mM $Mg(OAc)_2$, 2mM DTT, 1mM cycloheximide and 500mM sucrose). RNCs were pelleted for 40 minutes at 4°C and 100.000 rpm in TLA 100.2 rotor. Pelleted RNCs were resuspended either in RRL compensation buffer (20mM Hepes-KOH pH 7.6, 80mM KOAc, 0.5mM $Mg(OAc)_2$, 1mM DTT) or appropriate source of cytosolic factors.

3.2.3.2. *In vitro* translation in wheat germ

3.2.3.2.1. Preparation of wheat germ extract for in vitro *translation*

Sephadex G-25 was autoclaved in buffer C (40mM Hepes-KOH pH 7.6, 50mM KOAc, 1mM $Mg(OAc)_2$, 0.1% β-mercaptoethanol) and allowed to cool to 4°C. Swollen matrix was used to pack a Pharmacia column (1.2 x 40mm) in cold room. Column was equilibrated with 2 volumes of ice-cold buffer C.

2g of fresh what germ were put in a mortar containing liquid nitrogen and grinded in cold room until finely powdered. Powder was transferred to another mortar and grinded on ice in buffer H (40mM Hepes-KOH pH 7.6, 100mM KOAc, 1mM $Mg(OAc)_2$, 2mM $CaCl_2$, 1mM DTT) until a thick paste was obtained. This homogenate was transferred to a 15ml polypropylene tube on ice and centrifuged in Sorvall SS-34 rotor for 10 minutes at 4°C and 14000 rpm (23000g). Supernatant was collected in another polypropylene tube and centrifuged again as before. Recovered supernatant was collected into a new polypropylene tube (S23 wheat germ extract).

Volume of the collected extract was measured and the whole extract was passed through a packed G-25 gel filtration column by gravity flow. Elution was achieved using buffer C with fresh β-mercaptoethanol. The turbid material migrating in front of a yellow pigment fraction was collected in aliquots of 2ml. First two aliquots were discarded. Next three aliquots were pooled together, transferred in a 15ml polypropylene tube and incubated on ice for 10 minutes. Aggregates were removed by centrifugation for 10 minutes at 4°C and 14.000 rpm. Supernatant was distributed into 200µl aliquots, flash-frozen in liquid nitrogen and stored at -80°C.

3.2.3.2.2. Standard protocol for translation in wheat germ extract

Before use, aliquot of wheat germ extract was thawed and centrifuged 5 minutes at 13000 rpm in tabletop Eppendorf centrifuge precooled to 4°C. *In vitro* translation reaction was assembled as follows:

- 4 µl wheat germ extract
- 0.8 µl Energy mix (250µM amino acid mix -Met, 12.5mM ATP, 250µM GTP, 41mg/ml creatinine phosphate, 10mg/ml creatine phosphokinase, 25mM Hepes-KOH pH 7.6)
- 0.4 µl 25 x translation salts (500mM Hepes-KOH pH 7.6, 1M KOAc, 50mM $Mg(OAC)_2$)
- 0.4 µl ^{35}S-Met 15µCi/µl
- 150 µg mRNA
- 0.2 µl RNAsin inhibitor 40 U/µl

Reaction was incubated for 30 minutes at 25°C and stopped by addition of puromycin to the final concentration of 2mM.

Post-translational insertion was accomplished by addition of RM to the final concentration of 25 OD280/ml and incubation for additional 30 minutes at 30°C. In reactions where newly synthesized protein was inserted cotranslationally, membranes were present during translation at concentration of 25 OD280/ml.

3.2.3.2.3. Preparation of ribosome-nascent chain complexes in wheat germ extract

Ribosome bound nascent chains were synthesized in wheat germ extract using template mRNA lacking a STOP codon. Incubation period for translation was 10 minutes. Pelleted RNCs were prepared using the same procedure as for RNC preparation in RRL.

3.2.4. Depletion of nucleotides

Removal of nucleotides was accomplished by gel filtration using prepacked G-25 columns MicroSpin from Amersham Biosciences. Before use, a column was pre-equilibrated with RRL compensation buffer (20mM Hepes-KOH pH 7.6, 80mM KOAc, 0.5mM $Mg(OAc)_2$, 1mM DTT). Between 30 and 50 µl sample volume was applied onto a column and centrifuged 2 minutes at 2600 rpm (735g) in a tabletop centrifuge.

3.2.5. Protein precipitation

3.2.5.1. Ammonium sulfate precipitation

Two volumes of saturated ammonium sulfate were added into a reaction containing proteins to be precipitated. Precipitation was carried for 30 minutes on ice. Precipitated proteins were pelleted by centrifugation for 5 minutes / 8000 rpm at 4°C. The pellet was resuspended in 20 or 40µl of dH_2O and 2 volumes of ice-cold 96% ethanol (40 or 80 µl). As before, mixture was incubated 30 minutes on ice and centrifuged. Protein pellet was dried for 5 minutes at 37°C, resuspended in 1 x Laemmli buffer for SDS PAGE and heated 5 minutes with shaking at 75°C before loading on a gel.

3.2.5.2. TCA precipitation

A solution containing proteins to be precipitated was mixed with equal volume of 20% ice-cold TCA and incubated 30 minutes on ice. If a sample contained detergent, the mixture of 20% TCA and 80% acetone was used. After centrifugation for 5 minutes / 8000 rpm at 4°C, pellet was briefly washed in ice-cold acetone and air-dried. Before loading on a gel, protein pellet was resuspended in 1 x Laemmli buffer.

3.2.6. Protein electrophoresis

Proteins were separated on 15% or 10-17% denaturing polyacrylamide gels (SDS PAGE). Electrophoresis was conducted using the constant current of 60 mA for one hour and 20 minutes.

3.2.6.1. Coomassie staining

A staining solution was prepared by dissolving Coomassie Blue R-250 in water:methanol:glacial acetic acid mixture (5:5:2 by vol). Gel was incubated overnight at room temperature in the staining solution. Destaining was accomplished by washing in ddH_2O until desired contrast between bands was observed.

3.2.6.2. Silver staining

Immediately after electrophoresis, a gel was put in 5% acetic acid / 50% methanol(1:1 v/v) for 30 minutes. A gel slab was rinsed with ddH_2O (2 changes, 2 minutes per change) and left in water overnight on a shaking platform. A gel was then treated with 0.02% sodium thiosulfate for 1-2 minutes and again rinsed with ddH_2O (2 changes, 30 seconds per change). Incubation was continued in 0.1% (w/v) $AgNO_3$ for 30 minutes. As before, gel was washed in ddH_2O (2 changes, 30 seconds per change). To develop a gel, a solution of 0.01% formaldehyde (v/v) in 2% sodium carbonate (w/v) was added. Developing solution was replaced with fresh portion after becoming yellowish. Upon reaching a sufficient degree of staining, quenching of the reaction was accomplished by discarding the developing solution and adding 1% acetic acid. A gel was washed with 1% acetic acid several times and stored in plastic bag at 4°C.

3.2.7. Western blotting

To transfer proteins from a gel onto the nitrocellulose membrane PROTRAN from Schleicher & Schuell, a semidry electrotransfer blotting apparatus was used. 3MM paper was cut into 9 pieces of appropriate size. First three pieces were soaked in Anode II buffer and positioned on lower electrode of an electrotransfer unit (plus pole). Bubbles were removed before next 3 papers were soaked in Anode I and put on top. Nitrocellulose membrane was also soaked in Anode I buffer and put on top of the stack of 3MM papers. Gel was put on top of the membrane and air bubbles were removed with wet fingers. Last 3 papers were soaked in Cathode buffer and put on top of the stack. Electrotransfer was conducted for one hour under constant current calculated according to the formula:

membrane surface (cm^2) x 0.8 +10 = current (mA)

After transfer, nitrocellulose membrane was washed 3-5 minutes in the solution of 0.2% PonceauS in 2% acetic acid. Membrane was then washed in water to removed excess of PonceauS. Blocking was accomplished by washing the membrane in 7% skim milk in 1 x PBST for 30 minutes. Nitrocellulose membrane was then incubated for 1 hour with shaking in a solution of primary antibodies made in 7% milk/1 x PBST. Unbound antibodies were removed by 5 washings in 1 x PBST (without milk, each washing 5 minutes). A solution of peroxidase-coupled secondary antibodies in 7% skim milk/1 x PBST was then added and incubated for additional one hour with shaking. As before, unbound antibodies were removed with 5 washings in 1 x PBST, 5 minutes each washing step. A blot was developed and bands visualized using Roche ECL Chemiluniscence kit for peroxidase detection and according to manufacturer's instructions.

3.2.8. Denaturing immunoprecipitation

A protein sample was dissolved in 1%SDS / 10mM Tris-Cl pH 7.5 and heated for 5 minutes at 55°C. Volume of a sample was adjusted to 500µl with IP buffer "A". To pre-clear a sample, 10 µl of protein A-sepharose beads were added and incubated for 1 hour at 4°C with shaking. Beads were sedimented by centrifugation at 13000 rpm for 30 seconds. An antibody was added to the supernatant and incubation was carried out for 2 hours at 4°C with shaking. Aggregates were removed by centrifugation for 5 minutes at 13000 rpm. 10 µl of protein A-sepharose beads were added to the supernatant and incubated for 1 hour at 4°C with shaking. Beads with bound protein were sedimented and washed 2 times in IP buffer "A", two times in IP buffer "B" and 2 times in IP buffer "C".

After the last washing step, approximately 30 µl of liquid was left on top of the beads. 15 µl of 3 x concentrated Laemmli sample buffer was added. A sample was heated for 5 minutes at 85°C with

moderate shaking. Before loading onto a gel, sample was centrifuged for 2 minutes at 13000 rpm in a tabletop centrifuge.

3.2.9. Preparation and coupling of antibodies to CNBr-sepharose

3.2.9.1. Purification of anti-opsin antibodies

Hybridoma cells producing anti-opsin antibodies were grown in 250ml cell culture flasks for 7 days in DMEM medium supplemented with fetal calf serum, penicillin and streptomycin. Cells were pelleted by centrifugation at 3000g for 10 minutes. 2 liters of a supernatant containing secreted antibody was collected and proteins were precipitated according to the protocol used for antibody purification presented by Harlow and Lane (48). After this, purification of the anti-opsin antibody was achieved by affinity chromatography using a sepharose matrix coupled to protein A (48). Purified antibody was stored at 4°C in 1 x PBS in the presence of 0.02% sodium azide.

3.2.9.2. Coupling of anti-opsin antibodies to CNBr-sepharose

Coupling of purified anti-opsin antibodies was accomplished according to instructions given in the manual for CNBr-activated sepharose 4B from Amersham Pharmacia.

2.5ml of protein A-purified anti-opsin antibody (approximately 3.5 mg of the protein) was passed over a G-25 gel filtration column equilibrated with a coupling buffer (200mM $NaHCO_3$, 500mM NaCl, pH adjusted to 8.3).

0.3g of dried CNBr-sepharose was dissolved in 6ml of 1mM HCl and washed 5 times with 8ml of 1mM HCl. Final washing step was with 8 ml of the coupling buffer.

An antibody solution was added to CNBr-sepharose and mixed for 2 hours at room temperature on a rotating wheel. Beads were sedimented and washed 2 times with the coupling buffer. Unreacted groups were blocked by incubation with 8ml of 100mM Tris-Cl pH 8, overnight at 4°C. Beads were then washed with 0.5 mM NaCl in acetone buffer pH 4, followed by washing in the coupling buffer. Alternate washings with acetone buffer and coupling buffer were repeated 4 times more.

Beads were finally washed with 1 x PBS and left at 4°C in 1 x PBS supplemented with 0.02% sodium azide.

3.2.10. Affinity purification of RAMP4op

Anti-opsin sepharose beads were washed in the binding buffer (20mM Tris-Cl pH 7.6, 250mM KOAc). Reticulocytes lysate containing synthesized RAMP4op was centrifuged for 5 minutes at 13000 rpm in a tabletop centrifuge to remove aggregates. The supernatant was mixed with the anti-opsin sepharose beads and the final volume was adjusted to 300 µl with the binding buffer. Incubation was conducted overnight at 4°C on a rotating wheel. Beads were then washed 2 times with 700 µl of the washing buffer (20mM Tris-Cl pH 7.6, 500mM KOAc) or washing buffer supplemented with Triton X-100 to the final concentration of 0.1% (v/v). To elute bound proteins, 40µl of 100mM glycine pH 2.5 was added and the beads were incubated at room temperature for 10 minutes with moderate shaking. The beads were then centrifuged for 30 seconds at 13000 rpm and the supernatant containing eluted proteins was neutralized with 4 µl (1/10 of the total sample volume) of Tris-Cl pH 8.

3.2.11. Gel filtration chromatography of proteins from the HeLa cytosol

Separation of HeLa cytosolic proteins according to their MW was achieved using a column XK26 from Pharmacia pre-packed with Superdex 200 gel filtration matrix. All solutions were made from distilled, autoclaved water, filtered through 0.22µ Millipore filter, degassed and precooled to 4°C. Before use, a column was equilibrated with the elution buffer (150mM KOAc, 1mM $Mg(OAc)_2$, 1mM EGTA, 1mM DTT, 20mM Hepes-KOH pH 7.6).

HeLa cytosol was centrifuged for 30 minutes at 120000g and then filtered through PVDF 0.22µ filter MILLEX-GV (Cat.No. SLGV033RS). 1 ml of the filtered HeLa cytosol (10 mg/ml) was loaded on top of the column connected to FPLC unit. Elution was conducted at the flow rate of 1ml/min. 57 fractions were collected of 4 ml volume each. Fractions 20-22 were pooled together and labeled as "A", fractions 23-25 as "B", fraction 26-28 as "C", fractions 29-31 as "D", fractions 32-34 as "E" and 35-37 as "F". Total proteins in 200 µl from each of 6 pooled fractions were precipitated with TCA, separated on 10-17% gradient SDS PAGE and stained with silver. Remaining volumes of pooled fractions were concentrated 4 times using Centriprep 5 concentrator (Amicon) according to instructions of the manufacturer. Concentrated samples were flash frozen and stored at -80°C.

3.2.12. Preparation of rough microsomal membranes

3.2.12.1. Preparation of dog pancreas rough microsomal membranes (RM)

Rough microsomal membranes were prepared according to the protocol of Walter and Blobel (141). All procedures and centrifugation steps were carried out at 4°C.

After removing skin and blood vessels, pancreas was cut into small pieces and placed in 150ml of homogenization buffer (250mM sucrose, 100mM Hepes-KOH pH 7.6, 50mM KOAc, 6mM $Mg(OAc)_2$, 1mM EDTA pH 8, freshly added DTT to the concentration of 1mM and 10µg/ml PMSF). Pancreas pieces were then transferred into a tissue press and the buffer was discarded. Upon pressing the tissue, material was collected into a beaker containing 120ml of homogenization buffer. Homogenization was carried out using Potter-Elvejhem homogenizer. Homogenate was centrifuged in SS34 rotor at 2900 rpm (1000g) for 10 minutes. Supernatant was collected in a new beaker and pellets were re-extracted as before with a fresh batch of homogenization buffer. All supernatants were pooled and centrifuged at 9500 rpm (10000g) for 10 minutes in SS34 rotor. Supernatant was collected taking care to avoid white layer on top of the pellet.

Pooled supernatants were loaded onto a gradient composed of 3 sucrose cushions. Volume of each cushion was 10ml and the composition was as follows:

2M sucrose cushion:

- 80ml 2.5M sucrose
- 5ml 1M Hepes-KOH pH 7.6
- 1.25ml 4M KOAc
- 0.6ml 1M $Mg(OAc)_2$
- 0.2ml EDTA
- add water to 100ml

1.75M sucrose cushion:

- 70ml of 2.5M sucrose
- 5ml 1M Hepes-KOH pH 7.6
- 1.25ml 4M KOAc
- 0.6ml 1M $Mg(OAc)_2$
- 0.2ml EDTA
- add water to 100ml

1.5M sucrose cushion:

- 60ml of 2.5M sucrose
- 5ml 1M Hepes-KOH pH 7.6
- 1.25ml 4M KOAc
- 0.6ml 1M $Mg(OAc)_2$
- 0.2ml EDTA
- add water to 100ml

Centrifugation was carried out in Ti45 rotor at 42000 rpm for 16 hours. Rough microsomal membranes were collected from the interface between 1.75M and 2M sucrose cushions. Pooled RMs were diluted with RM buffer (without sucrose) to the final volume of 300ml and centrifuged again in Ti45 rotor at 42000 rpm for 1 hour. Pelleted RMs were resuspended in 30ml of RM buffer. RMs were aliquoted, flash frozen in liquid nitrogen and stored at -80°C.

3.2.12.2. Preparation of puromycine-high salt washed membranes (PKRM)

5 ml of RM was prepared in the final concentration of 50 OD280/ml. 2.5 ml of 3 x concentrated buffer "A" was added and the incubation was carried out for 15 minutes at room temperature. 22.5 ml of buffer "B" was added, solution was mixed and distributed between 4 tubes for the SW40 rotor. After overlaying with 5ml of buffer "C", centrifugation was carried out in SW40 rotor at 38000 rpm (260000g) for 16 hours at 4°C. PKRMs were collected from the interface between 0.5M and 1.7M sucrose, diluted 4 times with RM buffer (without sucrose) and pelleted in SW60 rotor at 100000g for 1 hour at 4°C. Pelleted PKRMs were resuspended in RM buffer to obtain the final concentration of 50 OD280/ml.

3 x buffer "A":

- 150mM Hepes-KOH pH 7.6
- 1.5M KOAc
- 250mM sucrose
- 6mM puromycin
- 1mM DTT
- 2mM $Mg(OAc)_2$
- 3mM PMSF

buffer "B":

- 50mM Hepes-KOH pH 7.6
- 0.5M KOAc
- 2.2M sucrose
- 1mM DTT
- 2mM $Mg(OAc)_2$
- 1mM PMSF

buffer "C":

- 50mM Hepes-KOH pH 7.6
- 0.5M KOAc
- 0.5M sucrose
- 1mM DTT
- 2mM $Mg(OAc)_2$
- 1mM PMSF

3.2.12.3. Preparation of trypsin-treated PKRM (PKRM-T)

Desired amount of trypsin was added to 1ml of PKRM (50 OD280/ml). This mixture was incubated for 60 minutes on ice. Reaction was stopped by addition of RM buffer containing 1mM

PMSF, 10 μg/ml aprotinin and 0.75M KOAc. Mixture was then incubated on ice for another 15 minutes. Membranes were pelleted by centrifugation for 1 hour in SW60 rotor at 121000g and 4°C. Pelleted membranes were resuspended in 1ml of RM buffer, aliquoted, flash-frozen in the liquid nitrogen and stored at -80°C.

Mock-treated membranes were prepared in the same way, except water was used instead of trypsin.

3.2.12.4. Preparation of NEM-treated PKRM (PKRM-NEM)

N-ethylmaleimide was added to 1ml of PKRM (50 OD280/ml) to the final concentration of 5mM. Incubation was carried out for 15 minutes at 25°C. Reaction was stopped by addition of DTT to the final concentration of 20mM. After addition of DTT, mixture was incubated for further 5 minutes at 25°C. Membranes were pelleted by centrifugation for 1 hour in SW60 rotor at 121000g and 4°C. Membrane pellet was resuspended and washed once in the RM buffer. After the second centrifugation step (same as before), membrane pellet was resuspended in 1 ml of RM buffer, aliquoted, flash-frozen in liquid nitrogen and stored at -80°C.

Mock-treated membranes were prepared in the same way, except water was used instead of NEM.

3.2.13. Sucrose density gradient centrifugation

To make 10-20% sucrose density gradient, two solutions with the following composition were prepared.

- 80mM KOAc
- 1mM $Mg(OAc)_2$
- 1mM DTT
- 50mM Hepes-KOH pH 7.5

In addition to this, ATP or ADP was added to the final concentration of 2mM. Finally, sucrose was added to the final concentration of 10% (first tube) or 20% (second tube). Gradient was prepared in a polypropylene tube for SW60 rotor using Nycomed Pharma Gradient Master gradient maker.

Reticulocytes lysate containing newly synthesized RAMP4op was layered on top of a gradient. Centrifugation was carried out using the SW60 rotor, 15 hours at 45000 rpm (270000g). Fractions 1-11 were collected using ISCO density gradient fractionator. Fraction number 12 was obtained by resuspending the pellet at the tube bottom in a buffer containing 10mM Tris-Cl pH 7.5, 80mM KOAc,

1mM $Mg(OAc)_2$. Total proteins from all fractions were precipitated with ammonium sulfate and RAMP4op was immunoprecipitated using anti-opsin antibodies.

Marker proteins of known molecular weight were: chymotrypsin A (25kDa), albumin (67 kDa) and lysozyme (13 kDa). These proteins were separated in sucrose density gradients prepared in the same way as described above. Marker proteins were resuspended in 80mM KOAc, 1mM $Mg(OAc)_2$, 1mM DTT and 10mM Tris-Cl pH 7.5 to the final concentration of 1 mg/ml each. 500 ng of each protein was loaded on top of a sucrose gradient. After centrifugation and fractionation, marker proteins were resolved on a gel and stained with Coomassie color.

3.2.14. HeLa cells manipulation

3.2.14.1. Transfection

For transfection of HeLa cells, Lipofectamine 2000 (Invitrogen) reagent was used.

HeLa cells were grown in 6-cm cell culture dishes to 70% confluency, and washed 2 times with 1 x PBS. 4 ml of DMEM medium without fetal calf serum and antibiotics (DMEM -/-) was then added to cells.

In one sterile 1.5 ml eppendorf tube 500 µl of DMEM -/- was mixed with DNA. In a second eppendorf tube 500 µl of DMEM -/- was mixed with 20 µl of Lipofectamine 2000. Contents of both tubes were then pooled together and incubated at room temperature for 20 minutes. After that, the mixture of DNA and Lipofectamine was added to a dish with HeLa cells. Cells were incubated at 37°C / 5% CO_2 for 3 hours, washed 2 times with 1 x PBS and cultivated in DMEM with FCS and streptomycin/penicillin for further 18 hours.

3.2.14.2. Cell lysis

Upon cultivation, cells grown to confluency on 6cm cell culture dishes were collected using rubber scrapper and transferred together with medium into a 15 ml Falcon tube. Cells were then pelleted by centrifugation for 3 minutes at 1500 rpm in Heraeus megafuge 1.0R and washed twice with 1 x PBS. After the second wash, cells were transferred into 1.5 ml eppendorf tube and resuspended in 100 µl of lysis buffer (20mM Hepes-KOH pH 7.5, 100mM NaCl, 5mM $MgCl_2$, 1% Triton X-100). Incubation was carried out on ice for 15 minutes with shaking every 5 minutes.

Obtained cell lysate was centrifuged for 15 minutes at 13000 rpm / 4°C to pellet cellular debris and nuclei. Supernatant was aliquoted in 2 x 50 µl , flash-frozen in liquid nitrogen and stored at -80°C.

3.2.14.3. Isolation of mRNA and RT-PCR

HeLa cells were transfected as previously described and grown to approximately 70% confluency on 6cm cell culture dishes. After pelleting cells, extraction of RNA was accomplished using QIAGEN RNAeasy kit, according to instructions of the manufacturer. Pure RNA was stored at -80°C.

For the synthesis of cDNA, following components were assembled in an eppendorf tube:

- 6.5 µl First Strand buffer
- 3 µl DTT (100mM)
- 1.5 µl random hexamers (200 ng/µl)
- 1.5 µl dNTPs (10 mM)
- 1.5 µl Superscript reverse transcriptase (200 U/µl)
- 16.5 µl RNA (1 µg total)

Before addition into the reaction, RNA was heated for 5 minutes at 85°C and then immediately cooled on ice for 2 minutes.

Synthesis of cDNA was carried out for 90 minutes at 42°C. Reaction was stopped by addition of 2 µl 0.5 M EDTA. Reverse transcriptase was inactivated by heating at 70°C for 5 minutes. Synthesized cDNA was stored at -20°C.

PCR reaction for the detection of G3PDH was assembled as follows:

- 2 µl 10 x PCR buffer (w/o Mg)
- 0.6 µl $MgCl_2$
- 1 µl dNTP (10mM)
- 1 µl G3PDH_F primer (10 µM)
- 1 µl G3PDH_R primer (10 µM)
- 13.2 µl H_2O
- 1 µl template cDNA
- 0.2 µl Taq polymerase (5 U/µl)

Amplification of DNA was accomplished using the following program:

1. 95°C for 5 minutes
2. 95°C for 1 minute
3. 60°C for 1 minute
4. 72°C for 1 minute
5. 72°C for 10 minutes

Steps 2-4 were repeated for 16 cycles.

PCR reaction for the detection of RAMP4op was assembled as follows:

- 2.5 µl 10 x PCR buffer (with Mg)
- 0.5 µl dNTP (10 mM)
- 0.25 µl R4_CDS_F1 (100 pmol/µl)
- 0.25 µl R4_CDS_R1 (100 pmol/µl)
- 1.5 µl Taq polymerase (5 U/µl)
- 18 µl H_2O
- 2 µl template cDNA

Amplification of DNA was accomplished using the following program:

1. 95°C for 5 minutes
2. 95°C for 45 seconds
3. 55°C for 1 minute
4. 70°C for 1 minute
5. 70°C for 7 minutes

Steps 2-4 were repeated for 16 cycles.

3.2.14.4. Preparation of cytosolic extracts from HeLa cells and frog oocytes

Packed HeLa cells were purchased from CILBIOTECH s.a., Mons, Belgium (www.cilbiotech.be). 15 ml of pelleted HeLa cells were resuspended in equal volume of a homogenization buffer (20mM Hepes-KOH pH 7.5, 1mM $Mg(OAc)_2$, 1mM EGTA, 1mM DTT, 0.5

mM PMSF) and incubated for 15 minutes at 4°C to allow swelling. Cells were homogenized with 30 strokes in Dounce homogenizer. Efficiency of homogenization was estimated using phase contrast microscopy.

1/10 V of equilibration buffer (2.5M sucrose, 800mM KOAc) was then added and nuclei and mitochondria were pelleted by centrifugation in a 50 ml Falcon tube for 15 minutes at 5000 rpm. Supernatant was centrifuged for 20 minutes at 25000g to sediment the ER. ER membranes were resuspended in RM+ buffer, aliquoted, flash-frozen in liquid nitrogen and stored at -80°C.

Ribosomes were pelleted from the second supernatant by centrifugation in Ti50.2 rotor for 2 hours at 275000g. Supernatant obtained after this centrifugation step contains soluble cytosolic components. This supernatant was aliquoted, flash frozen in liquid nitrogen and stored at -80°C.

Xenopus laevis oocytes interphase cytosolic extract was given to us by Oliver Grüss, ZMBH, Heidelberg.

3.2.15. Chemical cross-linking

Before cross-linking, small molecules were removed by gel filtration using Pharmacia Microspin columns pre-packed with Sephadex G-25. All crosslinkers were purchased from Pierce company and dissolved in DMSO. Final concentrations of cross-linkers in reaction mixtures are indicated in figure legends. Cross-linking was conducted for 15 minutes at room temperature. Reaction was stopped by addition of glycine and DTT to the final concentration of 10 mM each and incubation on ice for further 5 minutes.

4. RESULTS

4.1. An assay for post-translational targeting and insertion of RAMP4op into dog pancreas microsomes

In order to investigate membrane targeting and insertion of RAMP4, this protein was synthesized in the rabbit reticulocytes lysate (RRL) *in vitro* translation system supplemented with rough microsomal membranes (RM) from a dog pancreas. Reticulocytes lysate was used as the source of ribosomes, translation factors and energy. Besides RAMP4 mRNA, the reaction contained optimal concentrations of magnesium and potassium, as well as 35S-labelled methionine. This allowed detection of newly synthesized RAMP4 by autoradiography. Rough microsomal membranes prepared from dog pancreas were added to test the membrane insertion competence of radioactively labeled RAMP4. To allow post-translational targeting and insertion of RAMP4, RMs were added into the reaction after termination of translation by puromycin. Puromycin is a tRNA analogue that is incorporated into a growing polypeptide chain during elongation. This causes the release of a nascent chain and dissociation of ribosomal subunits.

Due to the presence of the hydrophobic segment at its carboxy terminus, insertion of RAMP4 into the ER occurs in type II orientation. This was confirmed by showing that the amino terminal cytosolic segment of membrane inserted RAMP4 is accessible to an exogenously added protease (124). In order to study ER targeting and membrane insertion of RAMP4, it was necessary to clearly discriminate between cytosolic and membrane inserted forms of RAMP4. Therefore, an assay was required that could reliably detect both membrane insertion and acquired topology of RAMP4 in the ER membrane.

A direct way for monitoring the insertion of membrane proteins into the ER is based on detection of protein modifications that can occur exclusively in the ER lumen. One such modification is the addition of the preformed dolichol-linked oligosaccharide to an asparagine located within a specific amino acid context in a polypeptide chain (21, 30). Addition of the preformed oligosaccharide increases molecular weight of a protein for about 2.5 kDa. This causes a shift in migration between non-glycosylated and glycosylated protein forms in a denaturing polyacrylamide gel. In this way, a clear distinction between cytosolic, non-glycosylated and membrane inserted, glycosylated protein can be made. Because wild type RAMP4 does not contain N-glycosylation signal, the tag of 13 amino acids originating from the N-terminus of bovine opsin and containing such a signal was added to the carboxy terminus of RAMP4 (Fig. 6). The tagged version of RAMP4 is named RAMP4op. N-glycosylation can occur only on the segment of a polypeptide chain located within the ER lumen. Therefore, the tag added to the carboxy terminus allows monitoring of the translocation of the

RAMP4op carboxy terminal segment across the ER membrane. From this, the topology of the membrane inserted RAMP4op can be deduced. As shown in Fig. 6, the opsin tag added to RAMP4 also contains an epitope recognized by the monoclonal α-opsin antibody R2-15 which is used in this study for specific detection of RAMP4op and for immunopurification.

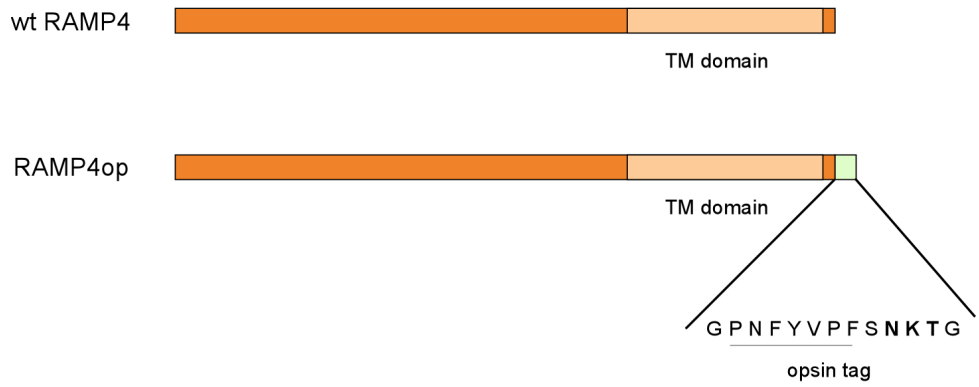

Fig. 6: Schematic representation of wild type RAMP4 and RAMP4op. RAMP4op contains additional 13 amino acids of the bovine opsin tag at the carboxy terminus. The tag provides the N-glycosylation site (bold letters) and the epitope recognized by the monoclonal antibody R2-15 (underlined sequence). TM domain - transmembrane domain.

Newly synthesized, radioactively labeled RAMP4op was immunoprecipitated using the antibody R2-15. As a positive control used to test insertional competence of RM, a protein known to be exclusively cotranslationally inserted into the ER, invariant chain (Ii) was *in vitro* translated in the RRL system. Due to the presence of two N-glycosylation sites, membrane insertion of invariant chain can easily be detected by the appearance of slower migrating bands after SDS PAGE and autoradiography.

In the *in vitro* translation reaction programmed with mRNA encoding Ii (Fig. 7a, lane 1) a protein band of about 26 kDa could be detected. This band was not present in the control reaction where no mRNA was added (Fig 7a, lane 10). Estimated molecular weight (MW) of the protein in this band corresponds to the expected MW of Ii and it could be immunoprecipitated using anti-Ii antibody (Fig 7a, lane 2). Treatment with EndoH, an enzyme that can remove oligosaccharides added during N-glycosylation in the ER, did not influence migration of the *in vitro* synthesized Ii (Fig 7a, lane 3). When the synthesis of Ii was carried out in the presence of RM an additional, higher molecular weight band that could be immunoprecipitated using anti-Ii antibody appeared (Fig 7a, lane 5). Treatment with EndoH caused disappearance of this band and increase in the amount of non-glycosylated Ii migrating at about 26 kDa (Fig 7a, lane 6). Upon the addition of RMs post-translationally, only non-glycosylated Ii could be detected (Fig 7a, lanes 8 and 9). This confirms that Ii can be inserted into RM exclusively by the cotranslational mechanism.

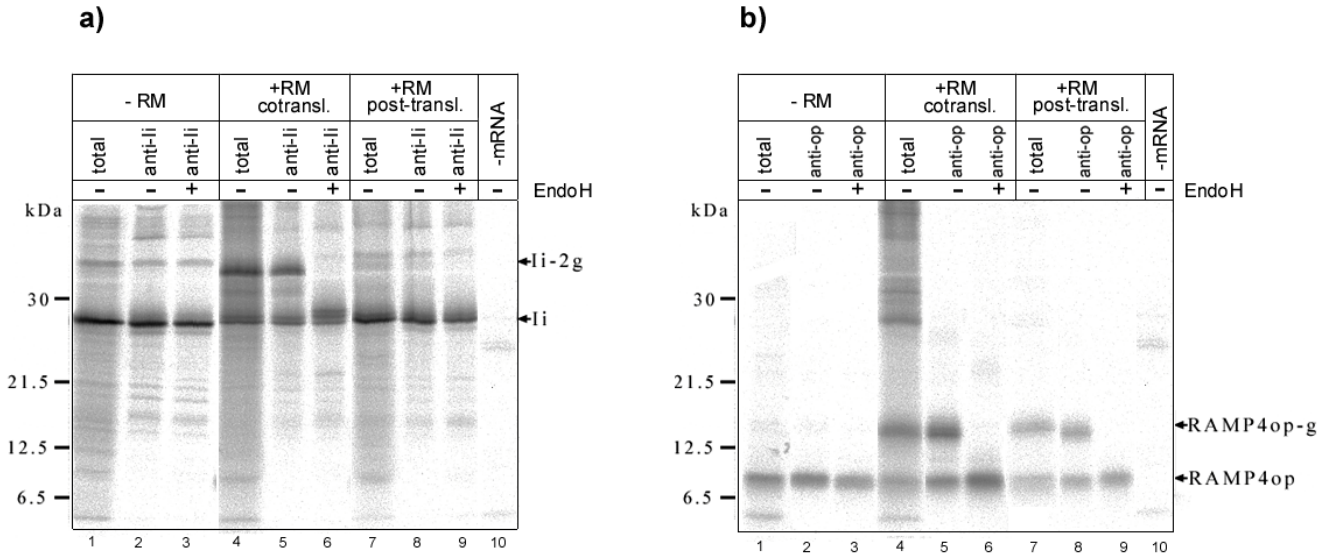

Fig. 7: *In vitro* translation and membrane insertion of Invariant chain (Ii) and RAMP4op. Proteins were synthesized in the RRL, in the absence or presence of rough microsomal membranes (RM). In post-translational reactions, RMs were added after release of nascent chains from ribosomes by treatment with puromycin. Where indicated, an aliquot was taken and subjected to denaturing immunoprecipitation using either anti-Ii or anti-opsin antibodies. (a) *in vitro* translation of Ii, (b) *in vitro* translation of RAMP4op. Bands are labeled as: RAMP4op : non-glycosylated RAMP4op; RAMP4op-g : glycosylated RAMP4op.

After *in vitro* translation of the mRNA encoding RAMP4op, a band of about 8 kDa could be detected (Fig 7b, lane 1). This estimated molecular weight corresponds to the calculated MW of non-glycosylated RAMP4op. The protein contained in this band could be immunoprecipitated using the anti-opsin antibody R2-15 (Fig 7b, lane2). Treatment with EndoH had no effect on migration of this band in a denaturing gel (Fig 7b, lane 3). When the translation of RAMP4op mRNA was conducted in the presence of RM, higher molecular weight band of about 14 kDa that could be immunoprecipitated with an anti-opsin antibody appeared (Fig 7b, lane 5). Treatment with EndoH lead to disappearance of this band followed by simultaneous increase in the amount of non-glycosylated RAMP4op (Fig 7b, lane 6). This suggests that the protein with higher MW that could be precipitated with the anti-opsin antibody represents the glycosylated form of RAMP4op. In the reaction where rough microsomal membranes were added post-translationally, both non-glycosylated and EndoH sensitive, glycosylated RAMP4op could be detected in a gel (Fig 7b, lanes 8 and 9).

Taken together, these results show that RAMP4op synthesized *in vitro* in the RRL can be efficiently inserted into RM added post-translationally. Under these conditions, membrane inserted RAMP4op can be efficiently N-glycosylated after the membrane insertion.

4.2. Investigation of the influence of RAMP4op mRNA 5' and 3' untranslated regions on the targeting to the ER

During cotranslational targeting, ribosomes synthesizing nascent chains are delivered to the endoplasmic reticulum by SRP. SRP acts as a targeting factor specific for proteins with signal sequences or signal-anchor sequences. During post-translational targeting to the ER, a protein that can be synthesized anywhere in the cytosol is targeted to the ER after being released from cytosolic ribosomes. Post-translational targeting of membrane proteins probably occurs with the help of a factor (or factors) with chaperone-like properties that is capable of maintaining an insertionally competent folding state of its substrate. Alternatively, an mRNA encoding membrane protein could be localized to the vicinity of the ER where protein synthesis then occurs. Upon termination of translation, such locally translated membrane protein would be inserted into the ER membrane using post-translational insertion mechanism. Previous reports on RNA localization have pointed out the significance of untranslated mRNA regions in the process of RNA targeting to specific locations within a cell (73).

In order to analyze eventual contribution of an mRNA to RAMP4op ER localization, four constructs encoding RAMP4op with different combinations of mRNA untranslated regions (UTR) were made. As can be seen in Fig. 8, these constructs contained either both UTRs (wt RAMP4op), coding sequence with 5'UTR (RAMP4opΔ3'UTR), coding sequence with 3'UTR (RAMP4opΔ5'UTR) or coding sequence alone (RAMP4opΔ5'3'UTR). These four different constructs were used to program an *in vitro* translation reaction in the RRL.

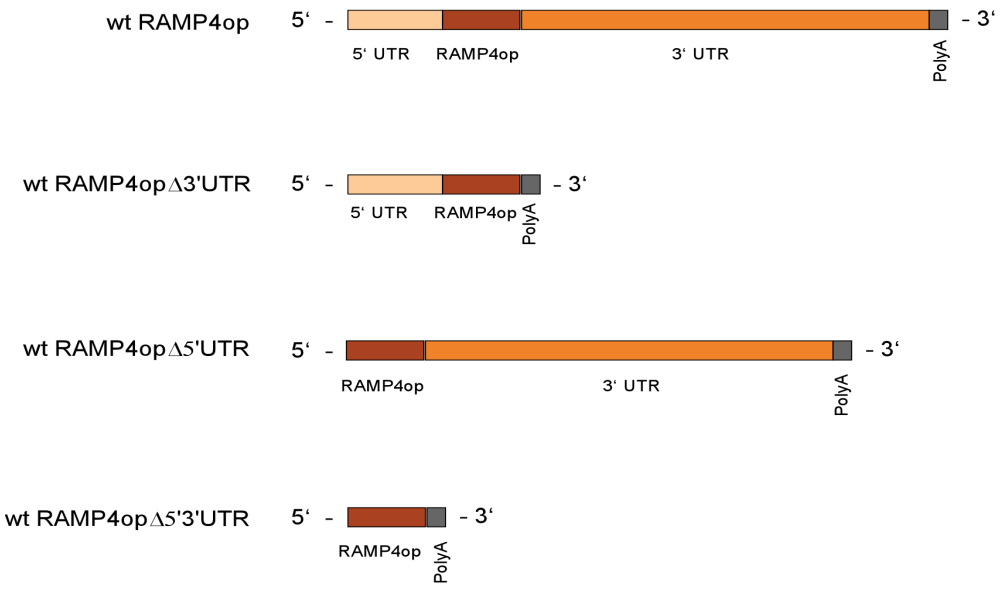

Fig. 8: Outline of mRNAs transcribed from RAMP4op constructs with different combinations of untranslated regions. PolyA: poly adenine tail; 5'UTR: 5' untranslated region; 3'UTR: 3' untranslated region; RAMP4op: coding sequence for RAMP4op.

Different amounts of RAMP4op were synthesized after *in vitro* translations of RAMP4op mRNAs containing different UTR combinations. The highest protein levels were seen in reactions programmed with mRNAs lacking either 5‘UTR (Fig. 9, lane 2) or both UTRs (Fig. 9, lane 4). The lowest amount of RAMP4op was detected after the *in vitro* translation of the full length mRNA (Fig. 9, lane 1). Upon addition of RMs post-translationally to any of four reactions programmed with different mRNAs, the additional band of the glycosylated RAMP4op appeared on a gel (Fig. 9, lanes 7-10). Similar to the reaction without membranes, the highest amount of RAMP4op could be detected in samples programmed with mRNAs lacking either 5‘UTR or both UTRs (lanes 8 and 10, respectively). As before, *in vitro* translation of the full length RAMP4op mRNA produced the lowest protein levels (lane 7). In our *in vitro* assay, the efficiency of N-glycosylation is taken as a measure of the efficiency of ER membrane insertion. In order to estimate efficiencies of N-glycosylation of RAMP4op synthesized from different constructs, intensities of non-glycosylated and glycosylated RAMP4op bands on a gel obtained after autoradiography were quantified. Comparison of the efficiencies of RAMP4op N-glycosylation showed no significant differences in the ratio between glycosylated and non-glycosylated forms of RAMP4op (lanes 7-10, calculated percent of glycosylation).

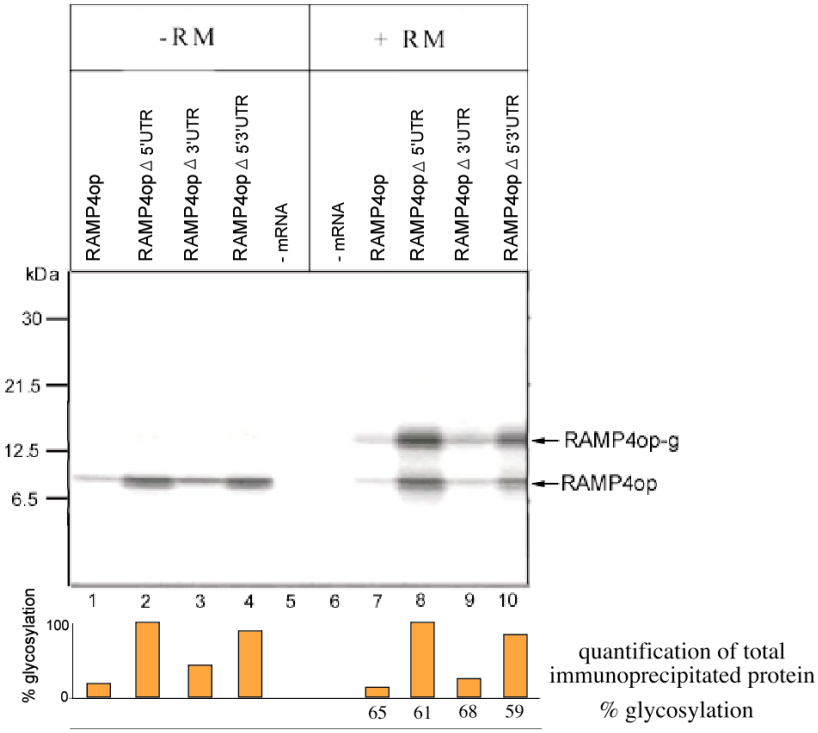

Fig. 9: *In vitro* translation of the authentic RAMP4op mRNA or mRNAs lacking either 5’UTR, 3’UTR or both UTRs. One set of reactions was incubated in the absence of membranes (“-RM”), while to the reactions marked with “+RM” rough microsomal membranes were added post-translationally. RAMP4op was immunoprecipitated using the anti-opsin antibody. Amounts of non-glycosylated and glycosylated RAMP4op were determined by quantification of intensities of protein bands visualized after SDS PAGE and autoradiography. Numbers below the histogram represent percentage of glycosylation calculated from the ratio between non-glycosylated and glycosylated RAMP4op.

To analyze eventual contribution of UTRs to RAMP4op targeting *in vivo*, four constructs with different UTR combinations were transiently expressed in HeLa cells. The anti-RAMP4 antibody was used to visualise both RAMP4op expressed from transfected constructs and endogenous RAMP4. Western blot analysis is shown in Fig. 10a. In lane 1, showing sample from mock transfected cells, no RAMP4op could be seen. The protein detected in the range of about 7-8 kDa is probably endogenous RAMP4. Band of about 47 kDa present in all lanes in the gel is a protein that cross-reacts with the anti-RAMP4 antibody. Lysates from cells transfected with any of four RAMP4op constructs show presence of the band corresponding in size to glycosylated RAMP4op (Fig. 10a, lanes 2-5). However, total amount of the protein precipitated by the antibody differ between samples from cells transfected with different constructs. The highest amount of RAMP4op was detected after transfection with the construct lacking both UTRs (lane 2). Somewhat lower RAMP4op levels were detected after transfection with the full-length mRNA–encoding plasmid (lane 5). The lowest amount of RAMP4op was detected after immunoprecipitation from cells transfected with constructs lacking either 3‘UTR (lane 3) or 5‘UTR (lane 4). Regardless of a RAMP4op construct used for the expression, virtually all of RAMP4op was found to be N-glycosylated and therefore membrane inserted. This means that presence or absence of untranslated regions of RAMP4op mRNA do not affect membrane targeting, insertion or glycosylation of RAMP4op.

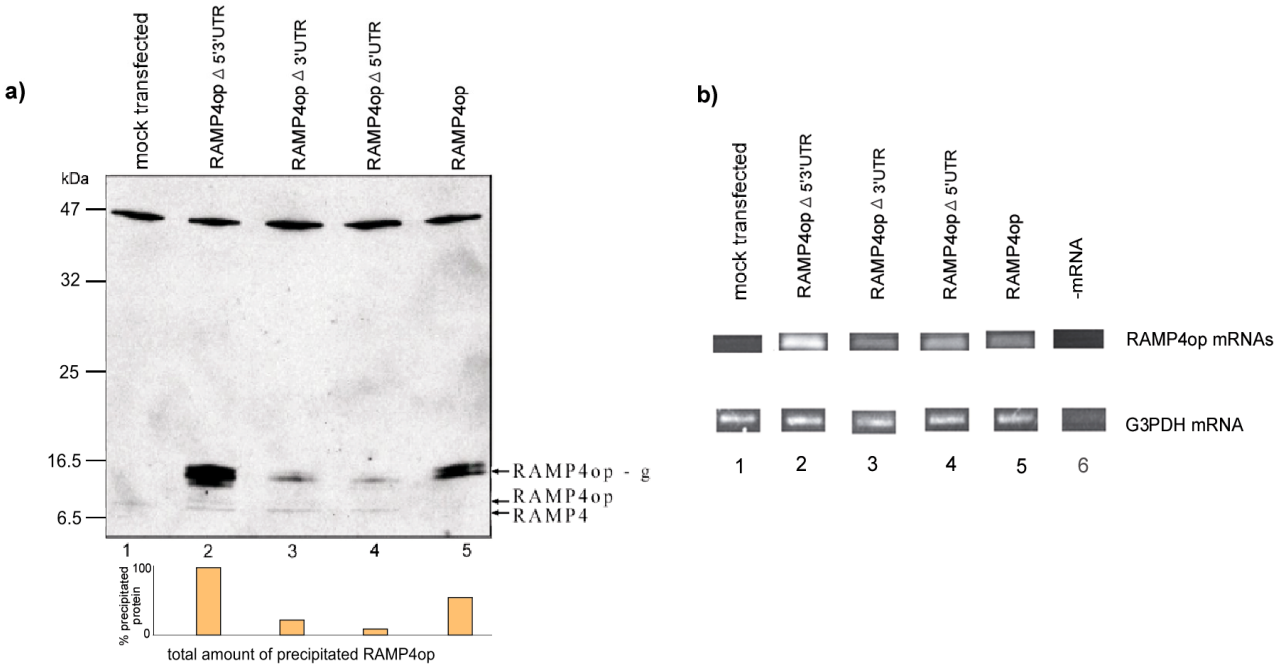

Fig. 10: *In vivo* expression of the constructs containing coding region of RAMP4op mRNA with different combinations of untranslated regions. Plasmids encoding different RAMP4op constructs were transfected in HeLa cells. Cells were lysed after 30 hours of growth. (a) Total proteins were resolved on 12.5% polyacrylamide SDS gel and transferred to a nitrocellulose membrane for Western Blotting. Endogenous RAMP4 and RAMP4op expressed from transfected constructs were visualized using anti-RAMP4 antibody. (b) RT PCR quantification of the amount of RAMP4op mRNA transcribed after transfections with RAMP4op constructs encoding different combinations of UTRs. Total RNA was extracted from one half of each lysate and cDNA was synthesized using the oligo-dT primer. Detection of cDNA molecules originating from different RAMP4op mRNAs was accomplished using the specific pair of primers in a PCR reaction. As the input control, amount of the mRNA encoding house-keeping enzyme glycero-3-phosphate dehydrogenase (G3PDH) in each sample was estimated using the same procedure.

Presence or absence of 5' or 3' UTRs in mRNA can influence messenger stability as well as the efficiency of protein synthesis (41). Furthermore, the amount of mRNA transcribed from different constructs encoding RAMP4op could be different. All these factors can contribute to observed differences in the amounts of RAMP4op synthesized *in vivo* from constructs encoding different UTR combinations.

To assess the contribution of mRNA levels to the expression levels of RAMP4op, RT-PCR was used as a semi-quantitative technique for estimation of mRNA amounts. Total RNA extracted from lysates of HeLa cells transfected with different RAMP4op constructs was used for cDNA synthesis and PCR amplification of RAMP4op-encoding sequences. In order to estimate the amounts of RAMP4op mRNAs present in the starting material, PCR amplification was accomplished using small number of cycles to avoid reaching the plateau of an amplification reaction. The same primer pair that could amplify the coding sequence of RAMP4op, but not endogenous RAMP4, was used in all reactions.

As shown in Fig. 10b, the highest amount of the mRNA encoding RAMP4op could be detected after expression from the construct lacking both UTRs (lane 2). This correlates with the highest amount of RAMP4op synthesized from this construct (Fig. 10a, lane 2). Lower RAMP4op mRNA levels could be detected after transfection with the construct carrying both UTRs or constructs carrying either 5' or 3' UTR. No major difference in the amount of mRNA could be seen between these three constructs (Fig. 10b, lanes 3-5). It appears therefore that the highest amount of RAMP4op protein detected after expression from the construct lacking both UTRs can be attributed to high amount of the messenger RNA transcribed from this construct.

4.3. Requirements for the post-translational targeting of RAMP4op to the ER membrane

Previous reports have shown that post-translational targeting to and/or insertion into the RM can be dependent on ATP or GTP hydrolysis (1, 78, 98). To determine whether this is also the case for membrane insertion of RAMP4op, the post-translational targeting/insertion assay was performed in the absence or presence of nucleotides. When no nucleotides were present in the reaction, only non-glycosylated RAMP4op could be detected (Fig. 11, lane 2). Addition of ATP after removal of nucleotides by gel filtration re-establishes post-translational insertion of RAMP4op. This is shown by the presence of glycosylated RAMP4op in Fig. 11, lane 3. Neither addition of ADP nor of non-hydrolyzable ATP analogue, AMP-PNP, could promote post-translational membrane insertion of RAMP4op. As shown in Fig. 11, lanes 4 and 5, no glycosylated RAMP4op could be seen under these

conditions. This suggests that ATP hydrolysis is necessary for the efficient post-translational targeting and/or insertion of RAMP4op.

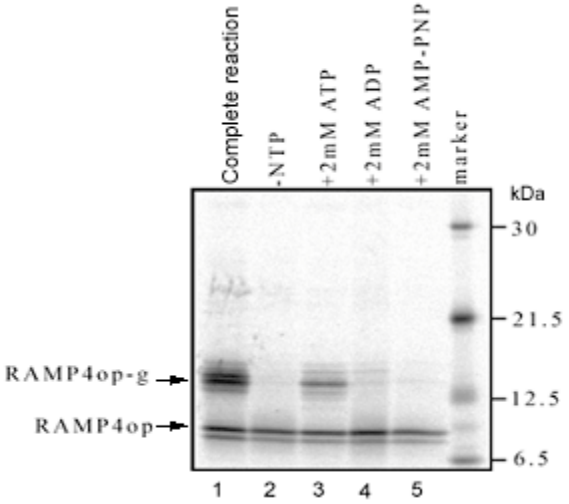

Fig. 11: Nucleotide dependence of the post-translational membrane insertion of RAMP4op. RAMP4op was synthesized in the rabbit reticulocytes lysate, translation was stopped with puromycin and small molecules were removed by Sephadex G-25 gel filtration. Specified nucleotides were re-added and the mixture was incubated with RM.

In order to determine the concentration of ATP required for the post-translational insertion of RAMP4op, aliquots of an *in vitro* translation reaction depleted of nucleotides were incubated with increasing amounts of ATP before addition of RMs. Glycosylated RAMP4op could only be observed in the presence of 2mM ATP (Fig. 12, lanes 8-10). Addition of GTP or CTP (up to 2mM final concentration) failed to promote post-translational insertion of RAMP4op (Fig. 12, lanes 4-7 and lane 11).

These results suggest that the post-translational targeting and/or membrane insertion of RAMP4op is dependent on ATP hydrolysis. GTP and CTP cannot substitute for ATP in this process.

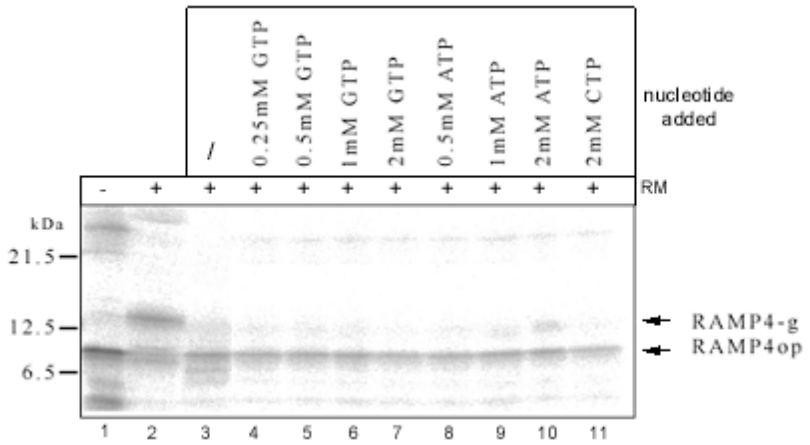

Fig. 12: Concentrations of nucleotides required for the post-translational insertion of RAMP4op. *In vitro* translation was conducted as given in the legend for Fig. 5. After puromycin treatment, nucleotides were removed by gel filtration. Different concentrations of GTP, ATP or CTP were added to aliquots of the reaction prior to incubation with RM.

4.4. Maintenance of RAMP4op targeting and insertional competence

After release from ribosomes, newly synthesized proteins become exposed to the cytosolic environment. Under these conditions proteins with hydrophobic segments, for example membrane proteins such as RAMP4op, could become misfolded or aggregated. Due to this RAMP4op may loose its competence for post-translational targeting and membrane insertion. To determine how long newly synthesized RAMP4op can be maintained in a targeting and insertionally competent state, an *in vitro* translation reaction was incubated in the absence of membranes for different amounts of time after termination of protein synthesis. To test whether ribosomes can influence ER targeting of RAMP4op, reaction mixture was depleted of ribosomes by ultracentrifugation. Aliquots were taken at 0, 30 and 60 minutes after the termination of translation and incubated with RM for another 30 minutes at 30°C to allow for post-translational insertion to occur. RAMP4op was than immunoprecipitated and characterized by SDS PAGE and autoradiography.

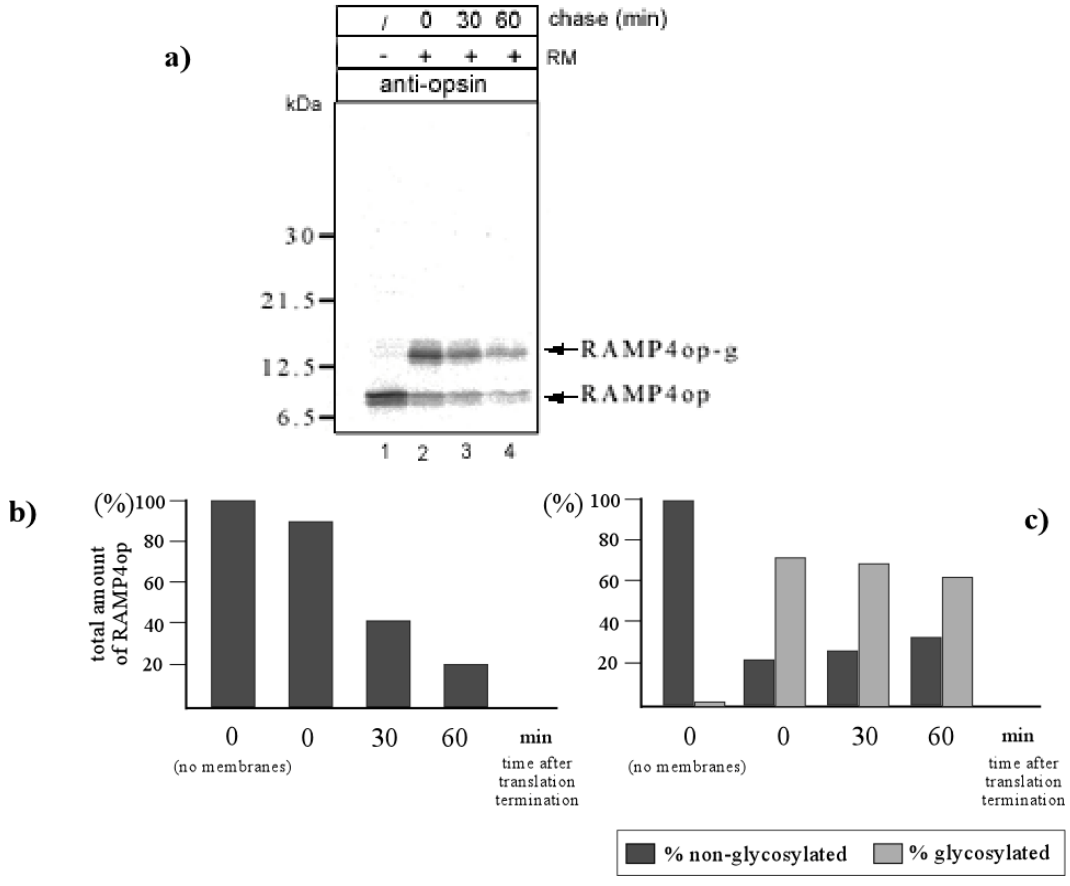

Fig. 13: Estimation of the RAMP4op insertional competence after its release from the ribosome. (a) RAMP4op was synthesized in the RRL, in the absence of RMs. Reaction was stopped with puromycin and incubated for one hour at 32°C. At specified time points, aliquots were taken and mixed with RMs to allow for post-translational insertion. Immunoprecipitation of RAMP4op was accomplished using the anti-opsin antibody. Quantification of the amount of immunoprecipitated RAMP4op was accomplished after SDS PAGE and autoradiography. (b) Quantification of the total amounts of RAMP4op precipitated by the antibody. (c) Quantification of non-glycosylated and glycosylated RAMP4op precipitated by the antibody.

In the absence of RMs only non-glycosylated RAMP4op could be detected (Fig. 13a, lane 1). Samples to which RMs were added show in addition presence of the higher molecular weight band corresponding to the glycosylated form of RAMP4op (Fig. 13a, lanes 2-4).

Quantification of glycosylated and non-glycosylated RAMP4op showed that the total amount of RAMP4op recovered was decreasing during the post-translational incubation period (Fig. 13b). After one hour of incubation about one third of RAMP4op present at the beginning of reaction (time point 0) could be detected. The ratio of glycosylated to non-glycosylated RAMP4op, however, did not change drastically during prolonged incubation (Fig. 13c). Upon addition of RMs to the aliquot taken at the beginning of incubation, around 70% of RAMP4op became glycosylated. When RMs were added to the aliquot of the reaction incubated for one hour in the absence of membranes, still more than 60% of RAMP4op could be glycosylated and therefore membrane inserted. This suggests that RAMP4op, once synthesized and released from ribosomes, can be maintained in a targeting and insertionally competent state for prolonged period of time.

4.5. Association of RAMP4op with cytosolic factors after release from ribosomes

To investigate oligomeric state of cytosolic RAMP4op in the absence of ER membranes, a sucrose density gradient analysis was performed. To see whether oligomerization or aggregation of cytosolic RAMP4op depends on the presence of ATP or ADP, two sucrose density gradients containing either of these nucleotides were used for the analysis.

RAMP4op co-migrated in sucrose gradients with a marker protein of 67 kDa (Fig. 14). In the presence of ATP, RAMP4op migrated to a slightly lower molecular weight than in the presence of ADP (Fig. 14, compare left and right gel). These data show that cytosolic RAMP4op can be found in a defined complex of about 70 kDa when no ER membranes are present. As RAMP4op itself is a small protein of about 7 kDa, this suggest that it may interact with one or more cytosolic proteins having total molecular weight of about 60 kDa. Since in the presence of ATP, RAMP4op migrates in a sucrose density gradient to a slightly higher MW, it appears that the oligomeric state of cytosolic RAMP4op may depend on the ATP/ADP ratio.

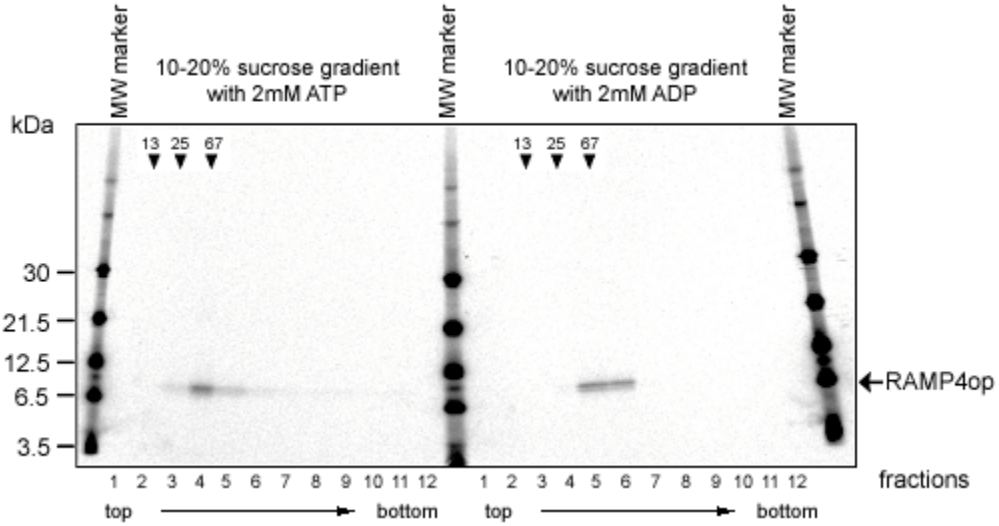

Fig. 14: Sucrose density gradient analysis of cytosolic RAMP4op in the absence of RMs. Radiolabelled RAMP4op was synthesized in the RRL in the absence of RM. One half of the reaction was loaded on top of a 10-20% sucrose density gradient containing 2mM ATP, while another half was loaded on top of a similar gradient containing 2mM ADP. After centrifugation, proteins from collected fractions were precipitated and separated on 10-17% gradient denaturing polyacrylamide gel and analyzed by autoradiography. Filled triangles and numbers above the gel indicate molecular weights and migration positions within gradients of the proteins used as molecular markers. Numbers below the gel indicate fractions collected from the gradient (1-top of a gradient, 12-pellet fraction).

4.6. Probing the molecular environment of newly synthesized RAMP4op by chemical cross-linking

In order to detect potential RAMP4op interacting partners, a chemical cross-linking approach was used. As can be seen from the sequence of RAMP4op shown in Fig. 15, residues with amino groups are located in the amino terminal and carboxy terminal hydrophilic parts of the molecule. Therefore, use of cross-linkers specific for NH_2-groups (DSS, MBS) would allow detection of proteins interacting with hydrophilic segments of RAMP4op. The single cysteine residue positioned within the TM domain offers a convenient way to detect proteins interacting with the hydrophobic segment of RAMP4op. For this purpose, the cross-linker specific for SH groups (BMH) was used.

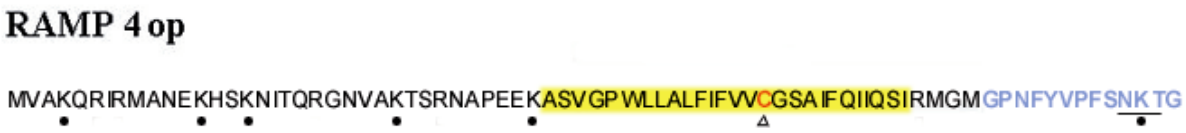

Fig. 15: Amino acid sequence of RAMP4op with residues containing either NH_2 or SH groups in their side chains marked. Filled squares: lysines containing the cross-linkable amino group; open triangle: the single cysteine with the SH-group; yellow segment: transmembrane domain; blue letters: sequence of the added opsin tag; underlined residues: consensus N-glycosylation site

Cross-linkers were added to the reaction after termination of RAMP4op synthesis in the RRL and in the absence of RMs. After crosslinking, RAMP4op was immunoprecipitated using the anti-opsin antibody and characterized by SDS PAGE and autoradiography. In all reactions where crosslinkers were added, the band of about 46 kDa could be detected, beside the non-crosslinked RAMP4op migrating at about 7 kDa (Fig. 16, lanes 3-6). In addition, protein bands of lower intensity and higher molecular weight were seen in the reaction where high concentration of MBS was used to induce formation of cross-linked products (Fig. 16, lane 6). Both the prominent 46 kDa band as well as the less intense higher molecular weight bands could not be detected in the control reaction where no cross-linker was added (Fig. 16, lane 2). Considering the MW of RAMP4op (≈ 7 kDa), it can be calculated that the detected 46 kDa band contains RAMP4op-crosslinked to a protein with molecular weight of about 40 kDa. From now on, this protein is referred to as "p40".

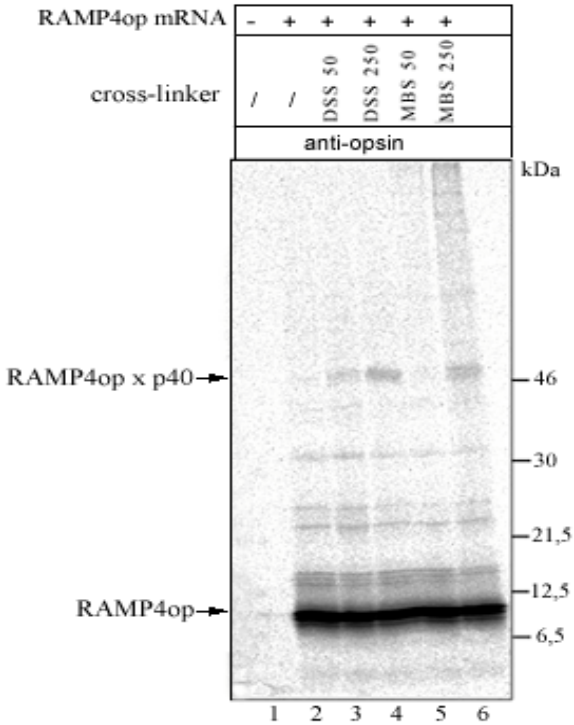

Fig. 16: Cross-linking of newly synthesized RAMP4op in the RRL. After termination of translation, aliquots of the reaction were incubated without cross-linker (lanes 1 and 2) or with different concentrations of DSS or MBS, as indicated. The anti-opsin antibody was used to immunoprecipitate RAMP4op.

To determine whether p40 is a ribosomal protein, ribosomes were sedimented by centrifugation prior to addition of a cross-linker. Both supernatant and pellet fractions were tested for the presence of ribosomes using antibody against the large ribosomal subunit protein L23a. This ribosomal component was found exclusively in the pellet fraction after ultracentrifugation (Fig. 17b), confirming quantitative sedimentation of ribosomes. As shown in Fig. 17a, the protein of about 40 kDa could be cross-linked to RAMP4op only in the supernatant containing ribosome-free cytosol (lane 4). No crosslink between RAMP4op and p40 could be observed in the ribosome-enriched pellet fraction (lane 2). This shows that p40 is not a ribosomal protein.

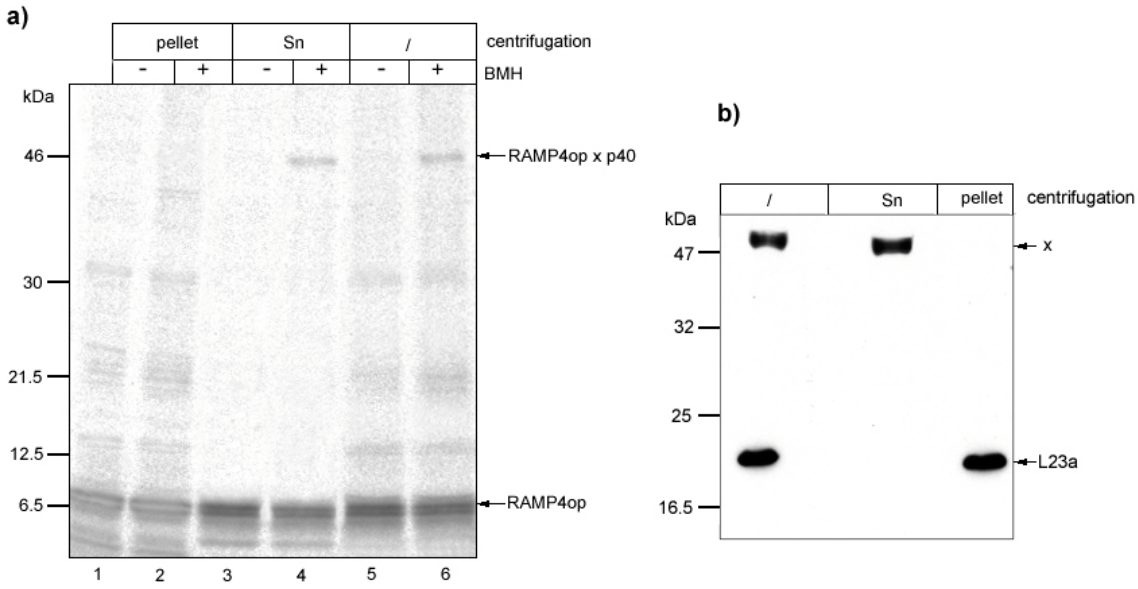

Fig. 17: Cross-linking of RAMP4op in the presence of absence of ribosomes. (a) RAMP4op was synthesized in the RRL, in the absence of membranes. One half of the reaction was centrifuged to pellet ribosomes before cross-linking with BMH (lanes 1-4). Second aliquot was directly incubated with DMSO (lane 5) or BMH (lane 6); (b) An aliquot of RRL was subjected to ultracentrifugation in order to sediment ribosomes. Proteins from the starting material ("/"), supernatant ("**Sn**") and pellet were resolved on SDS PAGE and analyzed by Western blotting for the presence of the protein L23a, a component of the large ribosomal subunit. A cytosolic protein that cross-reacts with the antibody against L23a is denoted with "**x**".

To investigate the influence of nucleotides on the efficiency of cross-linking between RAMP4op and p40, small molecules were removed by gel filtration before addition of DSS. As can be seen from Fig. 18a, lane 2, nucleotide removal did not inhibit formation of the RAMP4op-p40 crosslink. Addition of either ATP or ADP do not influence the efficiency of cross-linking between RAMP4op and p40 (Fig. 18a, lanes 3 and 4).

In order to detect interacting partners that associate with the hydrophobic segment of RAMP4op, the cross-linker that can form a covalent bond between two SH groups (BMH) was used.

Under these conditions, the cross-linked product similar in size to the 46 kDa protein obtained after DSS cross-linking was detected (Fig. 18a, lane 5). This means that the 40 kDa protein could be detected in the proximity of RAMP4op after cross-linking by either BMH or DSS. However, more efficient cross-linking between RAMP4op and p40 was observed when BMH was used to induce formation of a covalent bond between the two proteins.

To analyze whether cross-linking to p40 also occurs with wild type RAMP4, this protein was synthesized *in vitro* in the RRL and cross-linked in the absence or presence of nucleotides. SDS PAGE analysis of the obtained crosslinked products showed presence of the band of about 46 kDa in size (Fig. 18b, lane 2). This means that wild type RAMP4 which lacks the opsin tag can also be cross-linked to p40. Cross-linking between wild type RAMP4 and p40 was not influenced by the removal or re-addition of ATP/ADP (Fig. 18b, lanes 2-4). As before, the highest amount of the 46 kDa RAMP4-p40 crosslink was seen in the reaction where BMH was used to induce cross-linking (Fig. 18b, lane 5)

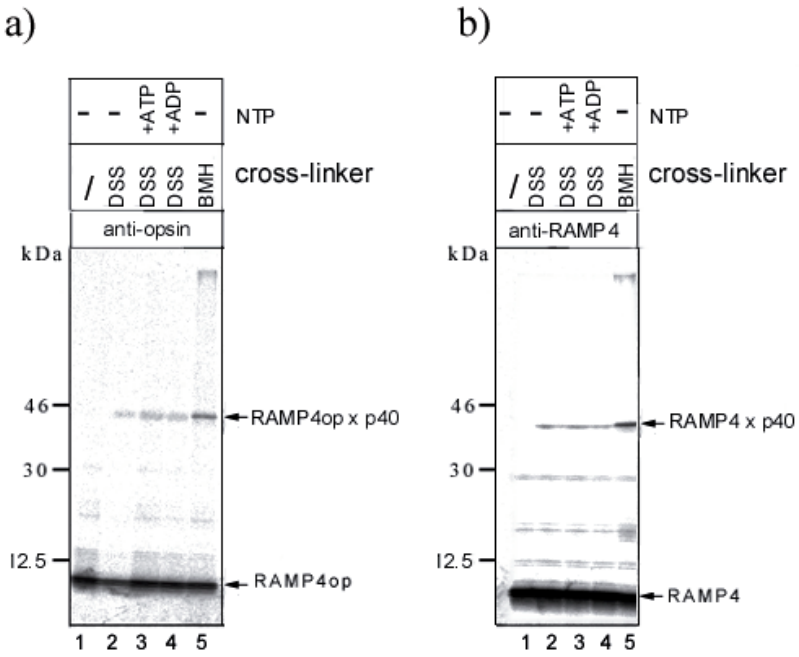

Fig. 18: Analysis of nucleotide dependence of the cross-linking between RAMP4op or RAMP4 and p40. Opsin-tagged RAMP4op (a) or authentic RAMP4 (b) were synthesized in the RRL, in the absence of RM. Small molecules and NTPs were removed using gel filtration. Where indicated, ATP or ADP were re-added to achieve the final concentration of 2mM. Cross-linking was accomplished using either DSS or BMH.

Taken together, these data show that p40 can be cross-linked to the transmembrane domain of both RAMP4 and RAMP4op in a nucleotide-independent manner.

4.7. Characterization of the interaction between RAMP4op and p40

In order to analyze the contribution of ionic bonding to the postulated interaction between RAMP4op and p40, RAMP4op synthesized in RRL was cross-linked in the presence of different KOAc concentrations. As shown in Fig. 19, cross-linking of RAMP4op to p40 was not inhibited by increasing salt concentrations (up to 1 M KOAc) (Fig. 19, lanes 2-5). To see whether presence of Mg^{++} is critical for the RAMP4op-p40 interaction, the chelating agent EDTA was added to a reaction containing *in vitro* synthesized RAMP4op and before addition of a cross-linker. This treatment increased the efficiency of RAMP4op-p40 cross-linking (Fig. 19, lane 6).

As shown before, RAMP4op could be cross-linked to p40 via the single cysteine residue present within its TM domain (Fig. 18a, lane 5). This suggests that hydrophobic interactions may play a role in establishment of the RAMP4op-p40 interaction. To test this possibility, cross-linking was conducted in the presence of different concentrations of the non-ionic detergent Triton X-100 (0.02 - 0.1% v/v). As shown in Fig. 20, even the lowest concentration of this detergent (0.02%) completely abolished cross-linking between RAMP4op and p40.

These findings show that interactions which maintain p40 in the vicinity of RAMP4op are mainly based on hydrophobic forces. Ionic interactions appear not to be important for the establishment of the RAMP4op x p40 crosslink. Chelating Mg^{++} by EDTA appears not to have an effect on the efficiency of RAMP4op-p40 cross-linking.

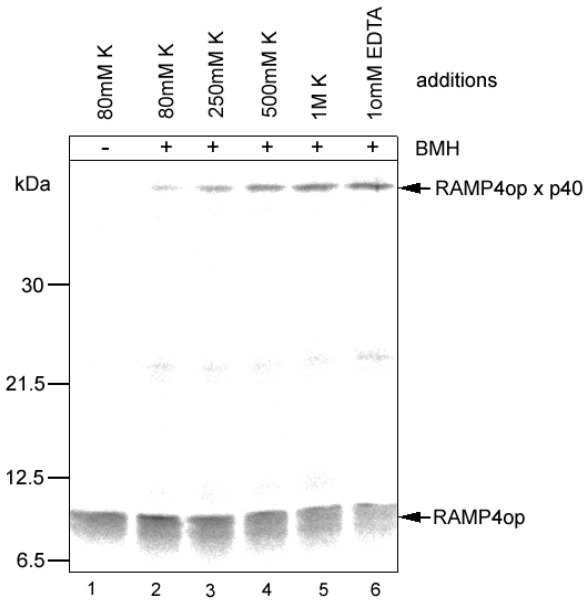

Fig. 19: Influence of salt concentration on RAMP4op x p40 cross-linking. RAMP4op was synthesized in the RRL. After removal of small molecules by gel filtration, KOAc (K) or EDTA were added to aliquots of the reaction to achieve the final concentrations as indicated on top of the figure. Proteins were characterized by SDS PAGE and autoradiography.

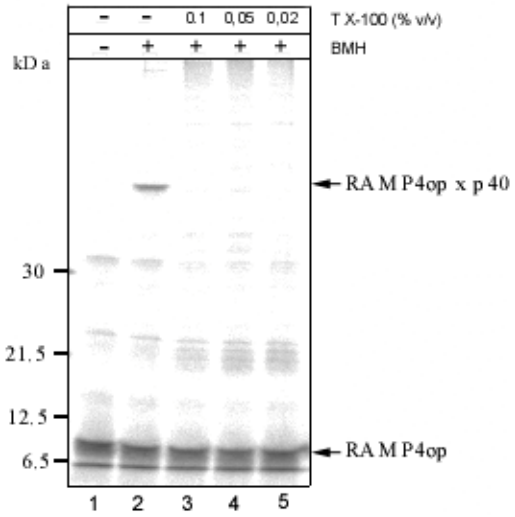

Fig. 20: Influence of Triton X-100 on RAMP4op x p40 cross-linking. RAMP4op was synthesized in RRL. Triton X-100 was added to aliquots of the reaction to achieve the final concentrations as indicated. Cross-linking was accomplished using BMH. Proteins were characterized by SDS PAGE and autoradiography.

4.8. Requirements for membrane insertion of RAMP4op

Results presented so far show that delivery of newly synthesized RAMP4op to the ER membrane can occur post-translationally. To analyze whether the insertion of RAMP4op is dependent on the presence of ER membrane proteins, I have used proteolytically treated RM in the RAMP4op insertion assay. Furthermore, I have investigated whether membrane-anchored SRP receptor is involved in the post-translational insertion of RAMP4op.

RM used in the assay were first washed in a buffer containing high salt concentration (0.5M K) and puromycin. This washing step removes ribosomes and electrostatically bound peripheral membrane proteins, leaving the surface of RM more accessible to a protease. RM prepared in this way were labeled as "PKRM". PKRMs were then incubated with trypsin in order to digest cytosolic domains of integral membrane proteins. This treatment was performed under conditions of a high salt concentration to prevent possible membrane re-binding of protein fragments removed by proteolysis. Trypsin-treated PKRM were labeled as "PKRM-T".

RAMP4op was efficiently inserted into PKRM post-translationally, as shown by the presence of the glycosylated RAMP4op band in Fig. 21, lane 10. This also shows that electrostatically bound peripheral ER membrane proteins removed during washing in the presence of 0.5M K are not essential for the post-translational insertion of RAMP4op.

In the next experiment, efficiency of membrane insertion into PKRM treated with low concentrations of trypsin (1 and 2 μg/ml) was compared between post-translationally targeted RAMP4op and cotranslationally targeted Invariant chain (Ii). As can be seen in Fig. 21, lanes 13 and 15, PKRM-T1 and PKRM-T2 remained competent for post-translational insertion of RAMP4op. The efficiency of RAMP4op glycosylation after the insertion into these PKRM-Ts was similar to the efficiency of RAMP4op glycosylation after insertion into mock-treated PKRMs (lane 11).

PKRMs treated with low trypsin concentrations used in our experiment have been previously shown to be incompetent for cotranslational ER insertion or translocation (110, 128). This is because mild trypsin proteolysis of ER membranes inactivates membrane-bound SRP receptor (SR). Cotranslationally inserted membrane protein invariant chain that becomes N-glycosylated after membrane insertion was used to verify this. When Ii was *in vitro* translated in the presence of either PKRM-T1 or PKRM-T2 much lower amounts of the glycosylated and membrane inserted protein were detected than when Ii synthesis was conducted in the presence of mock treated PKRM (Fig 21, lanes 3, 5 and 7). Upon addition of the soluble form of SRP receptor to the reaction mixture, efficient cotranslational insertion of Ii into PKRM-Ts was re-established (Fig. 21, lanes 6 and 8). This confirms that the treatment with low concentrations of trypsin had inactivated SRP receptor causing the inhibition of cotranslational insertion. Treatment of PKRMs with low concentrations of trypsin can therefore be used to test the dependence of post-translational insertion on the presence of functional SR. Since RAMP4op can be efficiently glycosylated in the assay containing PKRM-T1 and PKRM-T2, it can be concluded that SR is not required for the post-translational membrane insertion of RAMP4op. Furthermore, addition of the soluble recombinant form of SR had no effect on the insertion efficiency of RAMP4op (Fig. 21, lanes 14 and 16).

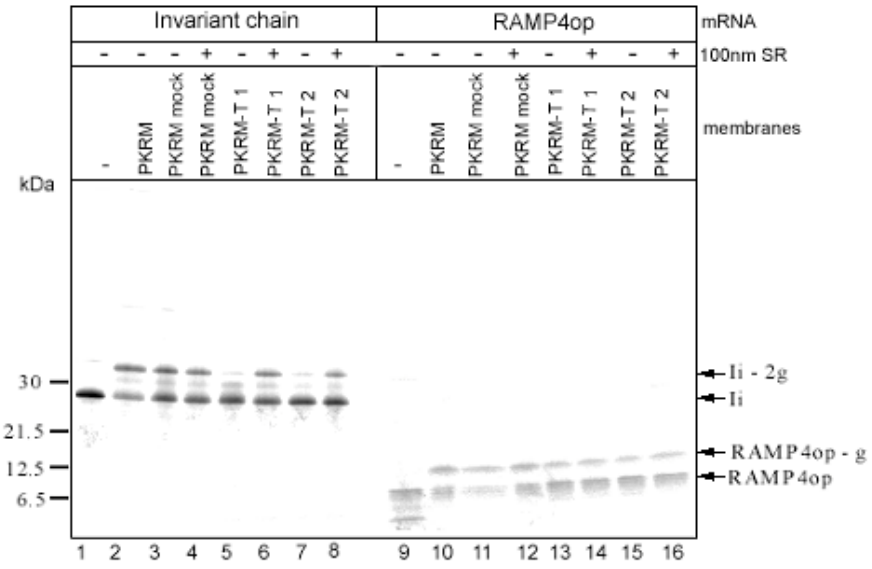

Fig. 21: Membrane insertion of RAMP4op and Ii into trypsin-treated RM. RAMP4op and invariant chain (Ii) were synthesized in the RRL. Puromycin/high salt washed membranes (PKRM), mock treated (PKRM mock) or membranes treated with 1 or 2 μg/ml trypsin (PKRM-T 1 and PKRM-T 2) were present during synthesis of Ii. RAMP4op was incubated with these membranes post-translationally. Where indicated, 100nm of soluble SR was added to the reaction. Proteins were analyzed by SDS-PAGE and autoradiography.

To investigate whether trypsin present in higher concentrations could inactivate ER component(s) required for the post-translational insertion of RAMP4op, radiolabelled RAMP4op was incubated with PKRMs treated with either 20 or 50 μg/ml of trypsin (PKRM-T20 and PKRM-T50).

As shown in Fig. 22, the amount of membrane inserted and glycosylated RAMP4op was reduced in comparison with the amount detected in the reaction containing mock treated PKRM (Fig. 22, lanes 6, 4 and 5).

Taken together, presented results show that at least one trypsin-sensitive ER membrane protein is required for the post-translational insertion of RAMP4op. SRP receptor is not required for the efficient post-translational insertion of RAMP4op. The amounts of trypsin required to block post-translational insertion of RAMP4op are higher than the amounts needed to inhibit cotranslational insertion of Invariant chain.

N-ethylmaleimide (NEM) is an alkylating agent that can modify SH groups of proteins. It has been previously shown that activity of SRP and SRP receptor can be affected by NEM (91, 142). To investigate whether an ER membrane protein with SH groups is also required for the post-translational insertion of RAMP4op, PKRMs were treated with NEM and tested in the post-translational insertion assay. As shown in Fig. 22, lane 7, NEM treatment of PKRMs severely reduced the competence of PKRMs to mediate post-translational insertion of RAMP4op.

This shows that post-translational insertion of RAMP4op into RM depends on at least one membrane protein sensitive to modification by NEM.

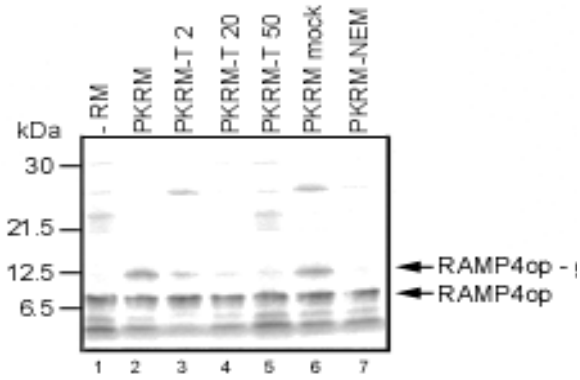

Fig. 22: RAMP4op insertion into PKRM treated with higher concentrations of trypsin or with NEM. RAMP4op was radiolabelled during synthesis in RRL. Mock-treated (PKRM mock), trypsin treated (2, 20 or 50 μg/ml – PKRM-T 2, 20, 50) or NEM treated (10mM NEM - PKRM-NEM) membranes were added post-translationally. After 30 minutes of incubation, total proteins were precipitated with ammonium sulfate and analyzed by autoradiography.

4.8.1. Cross-linking of newly synthesized RAMP4op after addition of ER membranes

To discover potential interacting partners of RAMP4op when RMs were present in a reaction, cross-linking was induced after incubation of *in vitro* synthesized RAMP4op with RM. This experimental approach allows detection of both cytosolic and RM-associated RAMP4op cross-linking partners.

In the absence of RM, newly synthesized RAMP4op could be cross-linked to p40 (Fig. 23, lane 2). When RMs were present in the reaction only bands corresponding to non-glycosylated and membrane inserted, glycosylated RAMP4op could be observed (Fig. 23, lanes 3 and 4). No cross-

linked products, including p40 x RAMP4op, could be detected. It appears therefore that incubation of newly synthesized RAMP4op with RM effectively displaced RAMP4op from its association with p40. Under investigated conditions, RAMP4op becomes efficiently inserted into RM where no cross-linking to p40, or other interacting partners could be observed.

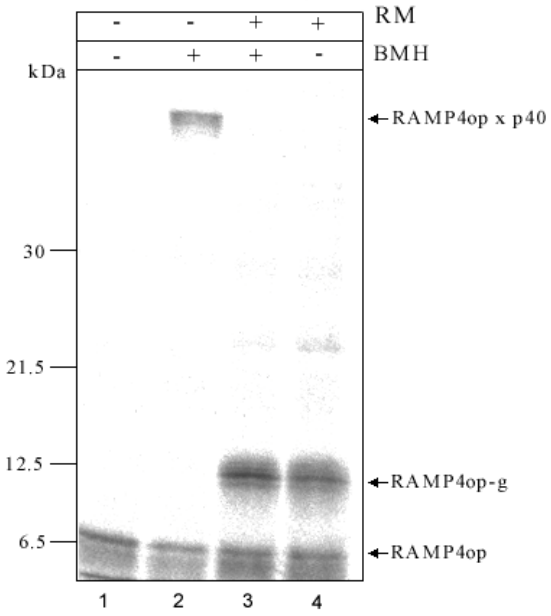

Fig. 23: Cross-linking between RAMP4op and p40 in the presence or absence of RM. RAMP4op was synthesized in RRL. Cross-linking was induced by the addition of BMH either immediately after termination of translation (lane 2), or after the incubation with RM (lane 3). Total proteins were precipitated and analyzed by SDS PAGE and autoradiography.

4.8.2. Membrane association of the RAMP4op - p40 complex

A pathway for post-translational targeting and membrane insertion of RAMP4op can be viewed as a process consisting of a sequence of stages. RAMP4op is first synthesized on cytosolic ribosomes. Upon termination of translation, RAMP4op is targeted to the ER membrane, most likely in a complex with other cytosolic factors. Upon contacting the ER membrane, RAMP4op undergoes process of membrane insertion for which it requires assistance of other ER membrane proteins. Membrane insertion of RAMP4op may be a receptor mediated process during which p40-RAMP4op complex interacts with specific ER membrane components.

In order to detect intermediate complexes that may be assembled after RAMP4op targeting to the ER, but before membrane insertion, I have employed cross-linking under conditions that would not allow insertion of RAMP4op into RM. Using this approach it may be possible to detect a RAMP4op-containing cytosolic targeting complex upon its docking onto the surface of the ER membrane. The conditions applied included depletion of nucleotides, re-addition of the non-hydrolysable ATP analogue AMP-PNP and NEM treatment of PKRMs. After incubation of newly synthesized RAMP4op with either PKRM or PKRM-NEM, cytosolic and membrane-containing fractions were separated by centrifugation. Cross-linking in both fractions was accomplished by addition of BMH.

Nucleotide depletion almost completely abolished post-translational insertion of RAMP4op into PKRM as only minimal amounts of glycosylated RAMP4op could be detected in the membrane pellet (Fig. 24, lane 5). In the absence of nucleotides, majority of RAMP4op is non-glycosylated and found soluble in the cytosol (Fig. 24, lane 4). Re-addition of AMP-PNP could not reconstitute post-translational insertion of RAMP4op into PKRM. Under these conditions, RAMP4op is also found exclusively in the non-glycosylated form and in the supernatant fraction containing cytosolic material (Fig. 24, lane 6). As shown before, post-translational insertion of RAMP4op into PKRM-NEM is blocked, leaving non-glycosylated RAMP4op in the cytosol (Fig. 24, lane 9). The same was observed when PKRM-NEM were added to reactions where nucleotides were depleted or AMP-PNP was added after the depletion (Fig. 24, lanes 11-12 and 13-14, respectively).

Under all tested conditions, the crosslink between RAMP4op and p40 was detected. Regardless of a treatment used to disrupt membrane insertion of RAMP4op, the RAMP4op-p40 cross-linked product was found exclusively in the cytosolic fractions (Fig. 24, lanes 4, 6, 9, 11 and 13). Efficiency of cross-linking between RAMP4op and p40 in the cytosol was lowest in the reaction where insertionally competent PKRMs were used in the assay (Fig. 24, lane 2).

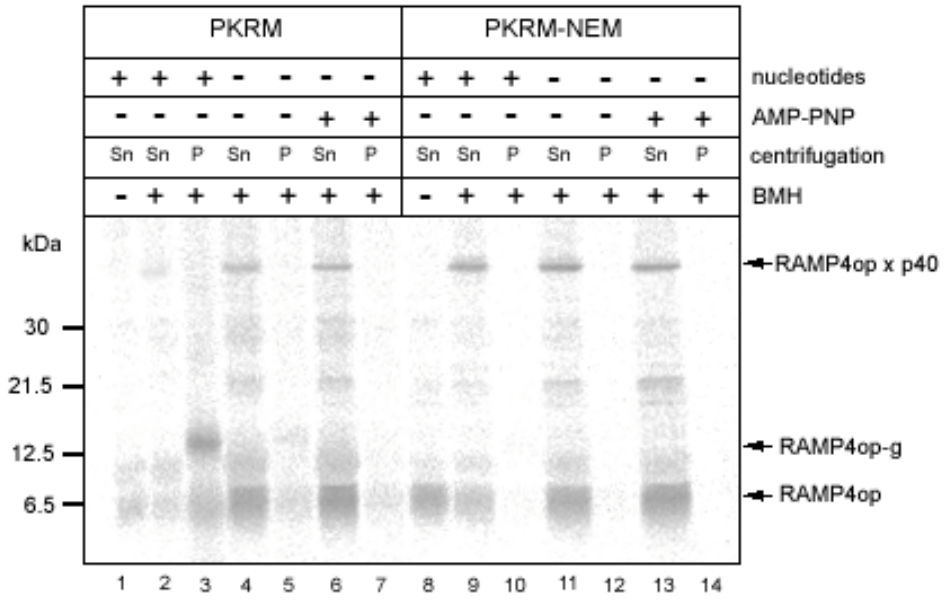

Fig. 24: Fate of the RAMP4op-p40 complex under conditions of inhibited membrane insertion. RAMP4op was synthesized in the RRL. Where indicated, aliquots of the starting reaction were depleted of nucleotides by gel filtration, supplemented with AMP-PNP and incubated with either PKRM or PKRM-NEM. After incubation, membranes were separated from the soluble material by sedimentation and resuspended in RRL compensation buffer. Small molecules were removed prior to addition of BMH to both membrane pellet and supernatant containing cytosolic proteins. Total proteins were precipitated and analysed by SDS PAGE and autoradiography.

4.9. Towards the identification of p40, the RAMP4op interacting partner

Two experimental strategies were designed in order to determine the identity of the RAMP4op interacting partner p40. Both ultimately rely on mass spectroscopy (MS) analysis as a method for determination of p40 amino acid sequence. One approach is based on purification of p40 from a fractionated cytosol. The second approach involves immunopurification of RAMP4op-p40 complex from a large scale *in vitro* translation reaction conducted in the rabbit reticulocytes lysate.

4.9.1. Purification of p40 from the fractionated cytosol

In order to determine and isolate a cytosolic fraction enriched in p40, an assay for detection of p40 in various cytosolic extracts was established.

Newly synthesized RAMP4op released from ribosomes readily interacts with p40. To be able to test different cytosolic fractions in the assay, it was necessary to uncouple RAMP4op ribosomal release from its interaction with p40. This was achieved by arresting RAMP4op synthesis at the step of termination using RAMP4op mRNA lacking a STOP codon. In this way, RAMP4op remained attached to ribosome as a nascent chain. After synthesis, the RAMP4op-containing ribosome-nascent chains (RNC) were separated from remaining cytosolic components by sedimentation. Ribosome-nascent chains prepared using this procedure were incubated with different sources of cytosolic factors in the presence of puromycin to induce release of RAMP4op from ribosomes. Cross-linking was then used to detect the RAMP4op-p40 interaction.

4.9.1.1. Synthesis of RAMP4op ribosome-nascent chain complexes

In order to produce RAMP4op mRNA lacking a STOP codon, PCR amplification was used to synthesize linear DNA containing the coding sequence for RAMP4op without a STOP codon. The 3' end of the mRNA transcribed from this template DNA ends with the last codon of RAMP4op (Fig. 25). Due to lack of a STOP codon, RAMP4op synthesized from this mRNA remains bound to ribosomes after incorporation of the last amino acid into the nascent chain.

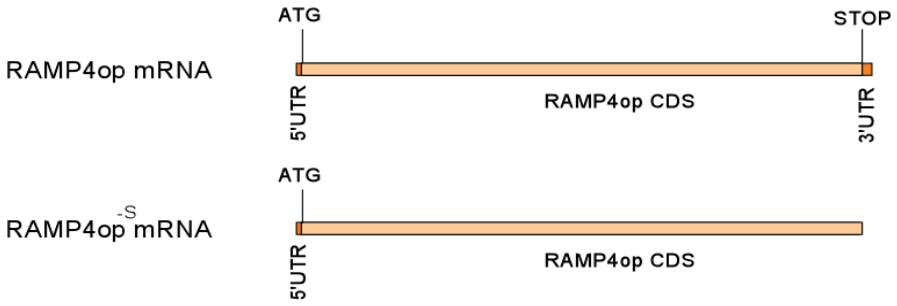

Fig. 25: Schematic outline of RAMP4op-encoding mRNAs that differ in the presence or absence of a STOP codon and the 3' UTR.

RAMP4op mRNA lacking a STOP codon, as well as the authentic RAMP4op mRNA were used as templates in *in vitro* translation reactions in RRL. After sedimentation of ribosomes, majority of RAMP4op synthesized from both mRNA templates was found in the supernatant (Fig. 26, lanes 2-3 and 5-6). This shows that RAMP4op synthesized from both mRNA templates did not remain attached to ribosomes.

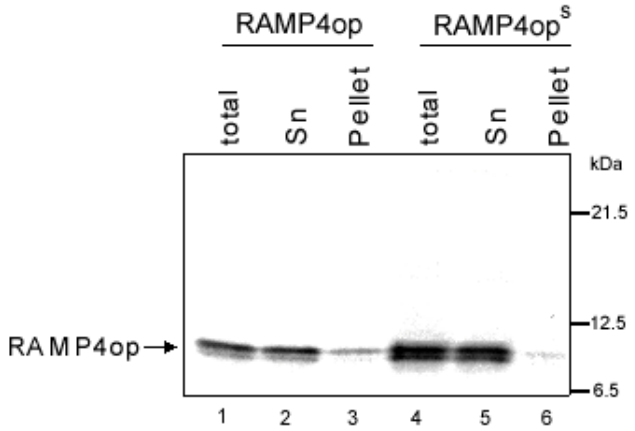

Fig. 26: Translation from RAMP4op mRNA with or without a STOP codon in the RRL. RAMP4op was synthesized in the RRL from either authentic mRNA (RAMP4op) or mRNA lacking a STOP codon (RAMP4op $^{-S}$). One aliquot was directly precipitated, and the other was centrifuged to pellet ribosomes. Proteins were analyzed by SDS PAGE and autoradiography.

Since we were unable to obtain RAMP4op nascent chains attached to ribosomes in the RRL system, mRNAs encoding RAMP4op with and without a STOP codon were translated in the wheat germ extract. As before, ribosomes were sedimented and the amount of RAMP4op present in the pellet and in the supernatant was visualized by SDS PAGE. As can be seen in Fig. 27a, lanes 2 and 3, the majority of RAMP4op synthesized from the authentic mRNA remains in the supernatant after centrifugation. In contrast, majority of RAMP4op synthesized from the mRNA lacking a STOP codon was found in the ribosomal pellet (Fig. 27a, lanes 5 and 6). This may be due to either formation of RNCs or because of increased aggregation of synthesized RAMP4op. To discriminate between these possibilities, puromycin was added after translation to induce release of the RAMP4op nascent chains

from ribosomes. Released RAMP4op is expected to be found in the supernatant fraction after centrifugation. Puromycin treatment, on the other hand, should not have an effect on formation of aggregates which would be found in the pellet fraction. As shown on Fig. 27b, lane 3, in the absence of puromycin, majority of radiolabelled RAMP4op was found in the pellet. In the presence of puromycin, RAMP4op was detected exclusively in the supernatant fraction (Fig. 27b, lane 5).

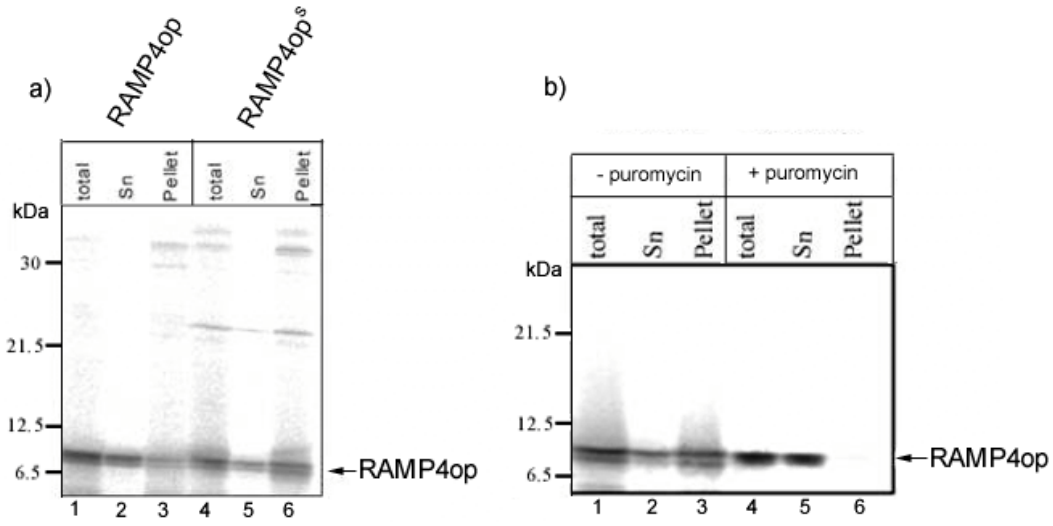

Fig. 27: Translation of RAMP4op mRNA with or without a STOP codon in the wheat germ extract. (a) RAMP4op was synthesized from either authentic mRNA (RAMP4op) or mRNA lacking a STOP codon (RAMP4op^{-S}). One aliquot was directly precipitated, and the other was centrifuged to pellet ribosomes. (b) RAMP4op synthesized from RAMP4op^{-S} mRNA was mock-treated or incubated with puromycin. After that, ribosomes were pelleted by ultracentrifugation. Proteins were precipitated and analyzed by SDS PAGE and autoradiography.

Taken together, these results show that RAMP4op-containing RNCs can be effectively produced from the mRNA lacking a STOP codon in the wheat germ system for *in vitro* protein synthesis.

4.9.1.2. Using RAMP4op RNC to test different cytosolic preparations for the presence of p40

In order to identify sources from which p40 can be purified, RNCs were generated in the wheat germ system programmed with RAMP4op^{-S} mRNA. After centrifugation, pelleted RNCs were resuspended in cytosolic preparations from different mammalian sources and incubated in the presence of puromycin. Cross-linking between RAMP4op and p40 was induced by the addition of BMH. Analysis of cytosolic preparations of different origin by this approach allows testing for the presence of p40 homologues in different cells or tissues.

After resuspension of RAMP4op RNCs in buffer alone, no crosslink to p40 could be observed (Fig. 28, lane 1). The cross-linked product of 46 kDa was detected in the reaction where ribosome-depleted reticulocytes lysate was added to pelleted RAMP4op RNCs (lane 2). The crosslink product between p40 and RAMP4op could also be detected in reactions supplemented with either HeLa cytosol or frog oocyte interphase extract (lanes 4 and 5, respectively). The efficiency of cross-linking between RAMP4op and p40 in frog oocyte extract is weaker than in RRL or HeLa cytosol. Upon addition of the wheat germ extract, no p40 x RAMP4op crosslink product could be detected (lane 3). In conclusion, using the RAMP4op RNC assay, reticulocytes lysate, HeLa cytosol and frog oocyte interphase extract were detected as sources of p40 that could be cross-linked to RAMP4op.

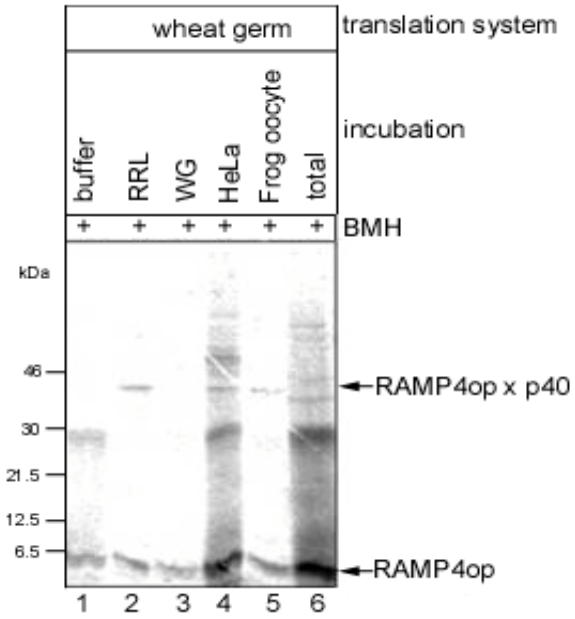

Fig. 28: Use of RAMP4op RNCs to analyze cytosols of different origins for the presence of p40. Pelleted RAMP4op RNCs were resuspended in a compensation buffer or different cytosolic preparations as indicated and incubated in the presence of puromycin. BMH was added to induce cross-linking. Proteins were precipitated and characterized by SDS PAGE and autoradiography.

4.9.1.3. Detection of p40 in the fractionated HeLa cytosol

The HeLa cytosol was chosen for further purification of p40 because it is an abundant source of mammalian cytosolic factors that can be prepared easily and in large amounts required for purposes of protein purification and identification. As the first step in a purification scheme, a size exclusion chromatography was conducted using Superdex 200 matrix to separate proteins on the basis of molecular weight. Obtained fractions were tested for the presence of p40 using RAMP4op RNC cross-linking assay.

As shown in Fig. 29, the cross-linked product between RAMP4op and p40 could only be detected in the reaction where fraction F was used as the source of cytosolic factors (Fig. 29, lane 14). Analysis of total proteins on a silver stained denaturing gel showed that fraction F was enriched in proteins having molecular weights between 14 and 60 kDa (Fig. 30, lane 6).

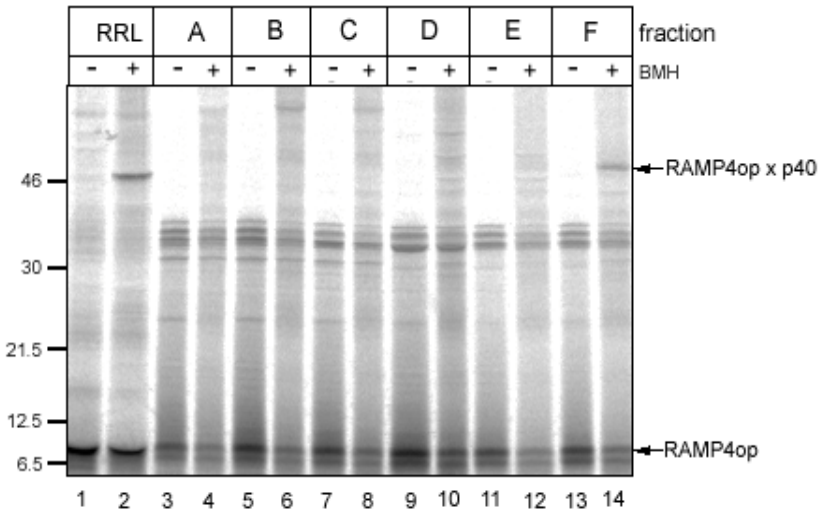

Fig. 29: Cross-linking of RAMP4op to p40 in fractionated HeLa cytosol. RAMP4op RNCs were made by translating the RAMP4op^{-s} mRNA. RNCs were pelleted by ultracentrifugation and resuspended in either RRL or fractions of the HeLa cytosol obtained after size exclusion chromatography (fractions A-F). Release of RAMP4op nascent chains from ribosomes was achieved by the addition of puromycin. Cross-linking was induced by the addition of BMH. Total proteins were precipitated and analyzed by SDS PAGE and autoradiography.

In order to further purify p40, the HeLa cytosolic fraction F was submitted to an ion exchange chromatography. Regardless of a matrix used for the chromatography analysis (anion exchanger or cation exchanger), p40 could not be detected using the RNC cross-linking assay, neither in the flow-through nor in fractions eluted from the column.

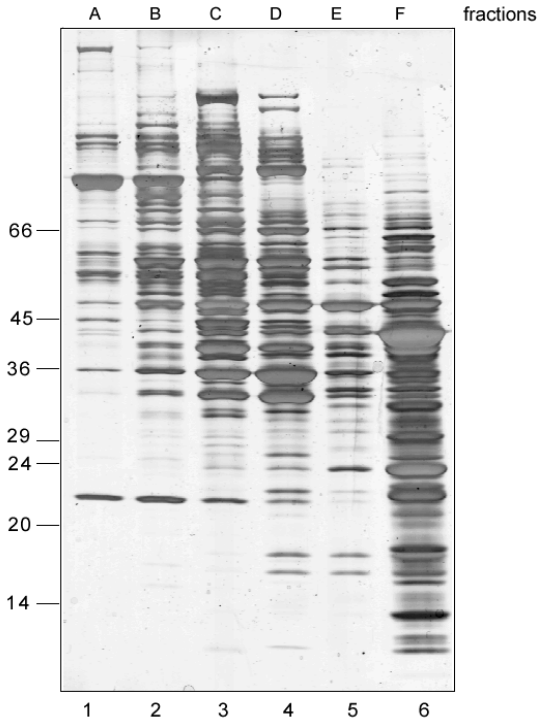

Fig. 30: Silver staining of proteins in the fractions of the HeLa cytosol prepared by gel filtration. HeLa cytosolic proteins were separated according to their size on a gel filtration column containing Superdex 200 matrix. An aliquot from each fraction was taken for silver staining of total proteins after their separation on SDS PAGE.

4.9.2. Immunopurification of the RAMP4op - p40 complex after large scale in vitro translation in RRL

A second approach designed to obtain purified p40 in the amounts sufficient for mass spectroscopy analysis was based on the immunopurification of RAMP4op-p40 complex from the reticulocytes lysate. For this purpose, I have used the anti-opsin antibody coupled to CNBr-sepharose beads (anti-opsin sepharose). Newly synthesized, radioactively labeled RAMP4op was incubated with the anti-opsin sepharose. The beads were then washed with a buffer containing high salt concentration (0.5M KOAc) to remove background. For disruption of the antibody-antigen interaction and elution of the bound protein, an acidic (pH 2.5) solution of 100 mM glycine was used.

As shown in Fig. 31, lane 3, radiolabelled RAMP4op could be bound to and eluted from the anti-opsin sepharose using the glycine buffer. When control beads which were not coupled to the anti-opsin antibody were used in the binding reaction, only a very small portion of radiolabelled RAMP4op could be bound to and eluted from the column (Fig. 31, lane 5).

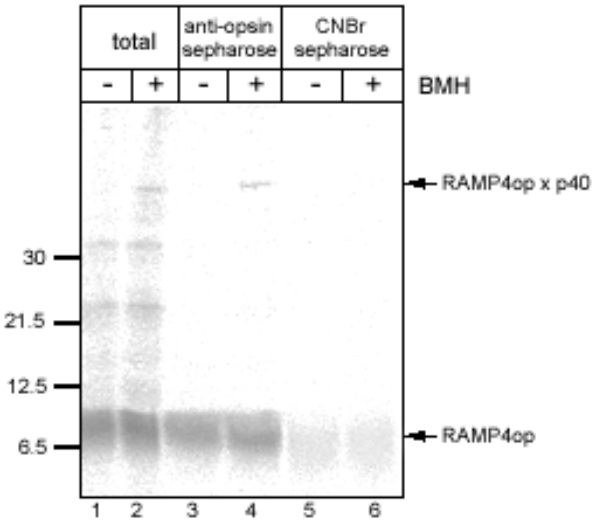

Fig. 31: Purification of RAMP4op and p40 on anti-opsin sepharose beads. RAMP4op was synthesized in RRL. Cross-linking was induced in one half of the reaction by the addition of BMH. Both reactions containing cross-linked and non cross-linked RAMP4op were either directly precipitated (lanes 1 and 2) or incubated with the anti-opsin coupled sepharose (lanes 3 and 4), or non coupled sepharose (lanes 5 and 6). After washing of columns with a buffer containing 0.5M K, elution of proteins was achieved using an acidic glycine buffer (pH 2.5). Total proteins were precipitated and analyzed by SDS PAGE and autoradiography.

In order to test whether cross-linked and therefore covalently stabilized RAMP4op-p40 complex could bind to the anti-opsin sepharose beads, cross-linking was induced prior to incubation with the anti-opsin sepharose. After the elution using the glycine buffer, both non-crosslinked as well as p40-crosslinked RAMP4op could be detected on the gel (Fig. 31, lane 4). No RAMP4op-p40 crosslink could be detected after the binding and elution from the beads that were not coupled to the anti-opsin antibody (Fig. 31, lane 6).

To analyze if the native, non cross-linked complex between RAMP4op and p40 could be bound by anti-opsin sepharose beads, RRL with synthesized RAMP4op that has not been cross-linked to p40 was incubated with the affinity matrix. As before, washing of beads was accomplished using a buffer

with high salt concentration (0.5M K). In addition, one binding reaction was washed with 0.1% Triton X-100 (v/v). Since it was shown before that Triton X-100 can disrupt RAMP4op-p40 interaction, washing with this detergent was aimed at testing whether p40 could be removed from the complex with RAMP4op after the binding to the anti-opsin sepharose. Elution of bound RAMP4op and any associated proteins was accomplished using the acidic glycine buffer (pH 2.5). Cross-linking to RAMP4op was used in order to detect presence of p40 in eluted fractions.

As shown in Fig. 32, radiolabelled RAMP4op was eluted from anti-opsin sepharose beads washed with either high salt buffer or high salt/detergent containing buffer (lanes 2 and 4, respectively). Therefore, the non-ionic detergent Triton X-100 cannot disrupt interactions that keep RAMP4op bound to the anti-opsin sepharose. The RAMP4op-interacting cytosolic protein p40 was detected by cross-linking only in material eluted from the anti-opsin sepharose washed with a buffer containing high salt concentration (Fig. 32, lane 3), but not from the anti-opsin sepharose washed with the detergent (Fig. 32, lane 5). This shows that the washing step with Triton X-100 removed p40 from its association with RAMP4op bound to the affinity matrix.

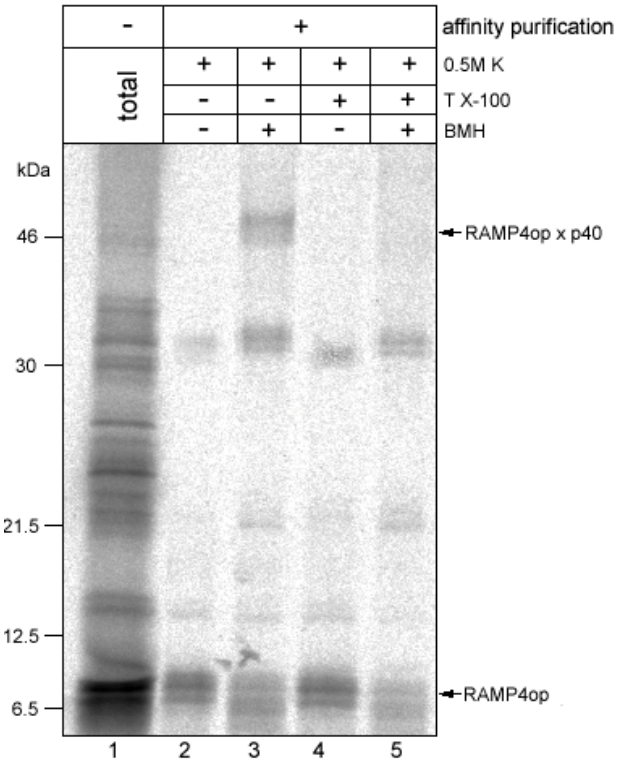

Fig. 32: Binding of the RAMP4op-p40 complex to the anti-opsin sepharose. RAMP4op was synthesized in RRL. Total proteins from one aliquot were directly precipitated ("total"). The remaining reaction volume was incubated with the anti-opsin sepharose. Washing of the column was accomplished using either high salt (0.5M K) buffer (lanes 2 and 3) or high salt buffer containing 0.1% Triton X-100 (lanes 4 and 5). Bound proteins were eluted using the glycine buffer at pH 2.5. After neutralization, cross-linking was induced by the addition of BMH.

Taken together, these results show that RAMP4op synthesized in RRL can be efficiently bound to the anti-opsin sepharose beads. Bound RAMP4op can be eluted with an acidic glycine buffer. The RAMP4op-p40 complex, either in the native state or after stabilization by cross-linking, could also be bound to and eluted from the anti-opsin sepharose using an acidic glycine buffer. Apparently, p40 is

not destabilized or denatured by low pH to the extent that prevents its association with RAMP4op. However, p40 is removed from the complex with RAMP4op after washing of beads with a non-ionic detergent.

5. DISCUSSION

5.1. The assay for analysis of membrane targeting and insertion of RAMP4op

To study targeting and insertion of RAMP4op, I have used the assay based on the rabbit reticulocytes lysate *in vitro* translation system. Newly synthesized and radiolabelled RAMP4op was incubated with RM under conditions that allow post-translational, but not co-translational insertion into the membrane. This was achieved by adding RM after the termination of translation. Membrane insertion of RAMP4op was monitored on a gel by the appearance of the N-glycosylated protein form. Since the N-glycosylation site in RAMP4op is located at the carboxy terminus, appearance of glycosylated RAMP4op was also used to confirm its type II orientation in the membrane, characteristic for tail-anchored proteins.

Other groups have used sedimentation through a sucrose cushion in order to separate membrane inserted from soluble forms of the TA proteins Bcl-2 and synaptobrevin (61, 71, 72). However, by using this technique proteins that associate peripherally with the ER membrane cannot be discriminated from proteins that are inserted into the ER membrane. To circumvent this problem, rough microsomal membranes with inserted TA proteins can be washed in alkaline buffers containing Na-carbonate prior to sedimentation. This approach was used in studies of membrane insertion of TA proteins synaptobrevin, cytochrome b5 and microsomal aldehyde dehydrogenase (1, 2, 86, 129, 138). During centrifugation, protein aggregates can also be pelleted together with ER membranes. To avoid this, groups of Rapoport and von Heijne have used flotation of membranes in alkaline sucrose gradients for the analysis of synaptobrevin insertion into the ER (78, 144).

N-glycosylation has been also used as a reliable method for detection of TA proteins inserted into the endoplasmic reticulum (1, 103, 147). Main advantage of this approach is that it is based on a biological process that naturally occurs in the lumen of the ER (21, 30). Since addition of the oligosaccharide increases MW of a protein, N-glycosylation can be detected easily by the appearance of a band with slower migration in a denaturing gel. Oligosaccharides added during N-glycosylation can be specifically removed by the enzyme EndoH.

Using the assay based on N-glycosylation, I have shown that newly synthesized RAMP4op can be efficiently inserted into RM in a post-translational manner. Since this assay cannot detect membrane inserted but non-glycosylated proteins, the true amount of RAMP4op inserted into RM is likely to be even higher than the amount calculated purely on the basis of N-glycosylation.

5.2. Nucleotide requirements for the post-translational targeting and insertion of RAMP4op

Post-translational targeting and/or insertion of RAMP4op into the ER depends on ATP hydrolysis. Concentration of 2mM ATP was found to be sufficient in reconstituting post-translational insertion of RAMP4op after depletion of nucleotides by gel filtration. GTP present in the same concentration could not reconstitute post-translational targeting and insertion of RAMP4op.

It was recently reported that another small TA protein Sec61β, upon *in vitro* synthesis in RRL, can be post-translationally inserted into the mammalian ER in a GTP-dependent manner (1). In the same study it was found that synaptobrevin 2 (Syb2), the TA protein with the longer cytosolic segment, is post-translationally targeted to and inserted into RM also in a process requiring GTP. Both Sec61β and Syb2 could be cross-linked in the absence of ribosomes to SRP54, the 54kDa subunit of SRP (1). Based on these findings, Abell et al. concluded that Sec61β and Syb2 represent TA proteins that are using GTP- and SRP-dependent post-translational targeting pathway in order to reach and insert into the ER membrane. However, these authors have not investigated possible involvement of ATP in the post-translational membrane targeting and insertion of Sec61β and Syb2. In the report presented by Kutay et al., membrane targeting/insertion of Syb2 was shown to be dependent on ATP hydrolysis but could occur, although very inefficiently, also in the presence of GTP (77). TA proteins cytochrome b5 and Nyv1p were shown to require ATP hydrolysis for post-translational targeting and/or insertion into the ER (71, 72, 129).

Post-translational translocation of the yeast secretory protein prepro-alpha factor (ppαF) that contains the cleavable signal sequence is dependent on ATP (129). This has been shown after synthesis of the protein in the RRL system supplemented with yeast microsomal membranes post-translationally. Partial depletion of ATP to the level that cannot support ppαF translocation, still allows post-translational ER insertion of the TA protein Nyv1p, although with decreased efficiency (129). This difference in nucleotide concentrations requirements was interpreted as an indication for the presence of different pathways involved in post-translational ER targeting/insertion of TA proteins and secretory proteins.

Analysis of ATP concentrations required for post-translational targeting/insertion of TA proteins have been conducted only for Syb2 upon its synthesis in RRL (72, 78, 147). Range of ATP concentrations reported by different authors varied between 0.2μM - 10mM. One of the reasons for such a wide range of concentrations might lie in the fact that in different studies different methods were used to deplete nucleotides. Secondly, precise determination of an ATP concentration is hampered by the fact that ATP is constantly hydrolyzed in an *in vitro* system for protein translation. In addition, these systems contain an energy regeneration mechanism which can replenish used ATP.

Since we could not identify conditions under which RAMP4op becomes associated but not inserted into the ER membrane, targeting and insertion could not be investigated separately. Therefore, it is not clear at which stage ATP hydrolysis is required. Considering what is currently known about post-translational targeting and insertion/translocation of proteins, ATP may be required for at least one of the following processes: (1) the interaction of RAMP4op with chaperones during the transit through the cytosol; This interaction may be important for the maintenance of RAMP4op insertional competence. (2) ATP can also be hydrolyzed by membrane associated factors involved in insertion of proteins. For example, in *E. coli* SecA ATPase acts as a molecular motor that mediates insertion/translocation of proteins into the bacterial membrane (25, 76). In the lumen of the ER, ATP-hydrolyzing chaperone BiP is involved in translocation of proteins after their insertion into the Sec61 translocon (87). Both SecA and BiP are required for translocation/insertion of proteins with larger lumenal domains. Since RAMP4op does not contain a large ER lumenal domain, it is unlikely that ATP hydrolysis is required during its membrane insertion for the action of BiP or a SecA-like protein.

5.3. Interactions of RAMP4op with cytosolic factors during the targeting to the ER

Upon being released from ribosomes, cytosolic RAMP4op can be maintained in an insertionally competent conformation for more than one hour in the absence of RM. All of radiolabelled cytosolic RAMP4op is present within the defined complex of about 40-80 kDa in size. Using chemical cross-linking approach, a cytosolic protein of 40 kDa (p40) was found to be in proximity of the RAMP4op TM domain. Since RAMP4op can be cross-linked to a protein of about 40 kDa present in extracts from human, dog and frog cells, it appears that p40 is widely distributed among different cells and organisms. The interaction between RAMP4op and p40 depends on hydrophobic forces because incubation with a non-ionic detergent prevents formation of the crosslinked product. Disruption of ionic interactions by increasing salt concentrations (up to 1M KOAc) has no effect on the association of p40 with RAMP4op. Presence or absence of ATP does not influence the interaction between p40 and RAMP4op.

Two alternative explanations can be invoked in order to explain functional significance of RAMP4op-p40 interaction. One possibility is that p40 becomes associated with RAMP4op upon entry into a degradation pathway. Alternatively, it is possible that RAMP4op becomes associated with p40 during the targeting to the ER. The latter possibility is strongly supported by the fact that addition of RM before crosslinking prevents formation of the RAMP4op-p40 cross-linked product. It therefore appears that a factor (or factors) associated with RM can displace p40 from its complex with RAMP4op. Since this complex is re-assembled in the cytosol upon removal of RM, the interaction

with p40 may be important for maintenance of an insertionally competent conformation of RAMP4op in the absence of RM.

Cross-linking approach has been also previously used to study cytosolic interactions established during the post-translational targeting of the TA protein Sec61β upon its synthesis in RRL (1). One of cytosolic proteins to which Sec61β could be cross-linked was identified as the GTPase SRP54. As post-translational targeting of Sec61β depends on GTP, it is possible that the interaction with SRP54 represents one stage along the targeting pathway of Sec61β. SRP54 could be also cross-linked to the TA protein synaptobrevin 2 (1). It should be noted however that besides SRP54 several other cytosolic proteins could be cross-linked to newly synthesized Sec61β or Syb2 (1). Among them, the prominent crosslink was established with a protein having an apparent MW of about 40kDa. Therefore, it is possible that additional cytosolic factors, beside SRP54, may be involved in post-translational targeting of Sec61β and Syb2.

Post-translational targeting and/or translocation of the yeast secretory protein prepro-α-factor is dependent on the cytosolic yeast Hsp40 co-chaperone called Ydj1p. This has been shown *in vivo* by the absence of ER translocation of ppαF in the yeast strain carrying non-functional Ydj1p (17). Cotranslational targeting and translocation of secretory proteins Kar2p (BiP) and carboxypeptidase Y in this yeast strain was not affected. Efficient translocation of ppαF into the ER in the Ydj1p-mutant yeast strain could be restored upon the expression of *E.coli* Hsp40 homologue DnaJ (17).

Prepro-α factor synthesized in RRL and released from ribosomes interacts with cytosolic Hsp70, as shown by crosslinking analysis (106). Post-translational translocation of purified ppαF into yeast RM is stimulated by Ssa1p (yeast cytosolic Hsp70) and Ydj1p (99). In the same study it was shown that Ssa1p and Ydj1p prevent aggregation of purified ppαF. Wheat germ-synthesized and urea-denatured ppαF could be translocated into yeast RM without the assistance of Hsp70/Hsp40 system (99). It appears therefore that Hsp70/Hsp40 chaperones are required to prevent aggregation and maintain the translocationally competent state of ppαF in the cytosol and/or during translocation into the ER. Crosslinking experiments showed that newly synthesized ppαF could also be found in proximity of many other cytosolic proteins synthesized in RRL. Among them, members of the TRiC/CCT Hsp60 chaperonin complex were identified (106).

Members of the Hsp40 and Hsp70 families were shown to be required for ATP-dependent post-translational targeting of presequence-containing proteins to mitochondria in yeast and mammals (17, 133) and to chloroplasts in plants (60). Post-translational targeting of proteins destined for peroxisomes also depends on the functional Hsp70/Hsp40 cytosolic chaperone system (131). In *E. coli* strain lacking SecB chaperone, secretion of SecB-dependent maltose binding protein (MBP) and LamB is facilitated by bacterial Hsp70 (DnaK) and Hsp40 (DnaJ) (145). In wild type *E. coli* DnaK and DnaJ participate in secretion of three SecB-independent proteins (alkaline phosphatase, ribose-

binding protein and β-lactamase) (146). Based on these facts, it can be concluded that in wide range of organisms Hsp70/Hsp40 chaperone system is involved in post-translational targeting of proteins to different cellular subcompartments, including the ER.

Hsp70 chaperones act through cycles of substrate binding and release (39, 49). A substrate is first recognized and bound by an Hsp40 co-chaperone. Hsp40 mediates delivery of a bound protein to Hsp70. Both Hsp70 and Hsp40 can bind to hydrophobic segments of substrate proteins (34, 39). Upon hydrolysis of ATP, Hsp70 becomes tightly associated with its substrate which can be released only upon the exchange of ADP for ATP. Released substrate can be re-bound by Hsp40 and the whole cycle is repeated. These cycles of binding to and releasing from Hsp70 facilitate folding of a substrate into the native conformation. In addition, proteins bound to Hsp70 are protected from aggregation. While in a complex with Hsp70, unfolded proteins can be maintained in an unfolded conformation (39). Hsp70 chaperone system is assumed to be important for maintenance of the insertionally/translocationally competent folding state of proteins that are post-translationally targeted to the ER, mitochondria, chloroplasts or bacterial plasma membrane (17, 60, 90, 99, 133, 146, 151).

Involvement of the Hsp70/Hsp40 chaperone system in the ER targeting of RAMP4op is suggested on the basis of the following observations:

- Post-translational ER targeting and/or insertion of RAMP4op occurs in an ATP-dependent manner.
- The 40 kDa cytosolic protein is found to interact with the TM domain of RAMP4op in the absence of RM.

The 40 kDa cytosolic interacting partner of RAMP4op, p40, could be the member of the Hsp40 co-chaperone family. ATP can be used by a member of the Hsp70 chaperone family involved in targeting and/or membrane insertion of RAMP4op.

The defining structural feature of all Hsp40 proteins is the presence of J-domain which is important for the interaction between Hsp40 and Hsp70 (42). J-domain also regulates functional status of Hsp70 by stimulating its ATPase activity. Many Hsp40 family members can bind non-native protein substrates in order to deliver them to Hsp70 chaperones (39, 49). Hsp40 proteins can be localized to a specific subcellular compartment. For example, ER membrane anchored Sec63 is involved in recruitment of the lumenal Hsp70 chaperone BiP to sites of protein translocation (23). In yeast, Pam18/Tim14 is anchored to the inner leaflet of the mitochondrial inner membrane where it is involved, together with Hsp70 Ssc1, in import of proteins into mitochondria (97). Hsp40 family members can also be involved in targeting of proteins to specific subcellular locations. In yeast cells lacking cytosolic Hsp40 Djp1, peroxisomal matrix proteins are mislocalized and remain in the cytosol. In Δ*djp1* strain targeting of mitochondrial and ER proteins to these organelles occur with a same

efficiency as in wild type *S. cerevisiae* (58). This suggests involvement of Djp1 in the post-translational targeting of peroxisomal matrix proteins to this organelle.

Combined approaches using genomic, biochemical and bioinformatic tools have lead to discovery of many Hsp40 homologues in eukaryotic and bacterial cells (18, 140). However, only three human cytosolic Hsp40 homologues have been functionally characterized in more details. These are Hdj1, Hdj2 and neuronal-specific Hsj1p (19, 20, 102). Hdj1 is localized mainly in the cytosol and partially in the nucleus and is essential for cell viability. It associates with ribosomes and is engaged in folding of nascent polypeptides. The structure of Hdj2 closely resembles structures of bacterial DnaJ and yeast Ydj1p (24). Both Hdj2 and Ydj1p are farnesylated and found partially on the ER (16, 67). Although functional significance of this modification of Hdj2 is unknown, *in vivo* studies of the yeast mutant expressing non-farnesylated Ydj1p revealed temperature-sensitive defects in post-translational import of ppαF into the ER (17).

If p40 is indeed the member of the Hsp40 co-chaperone family, its interaction with RAMP4op may be necessary for delivery of RAMP4op to Hsp70. Based on our findings, two distinct steps during targeting/insertion of RAMP4op can be discriminated. One is the nucleotide-independent interaction between RAMP4op and p40 which occurs in the cytosol. The second step is dependent on ATP hydrolysis, possibly required for the interaction between RAMP4op and Hsp70. This interaction may be important for prevention of RAMP4op aggregation in the cytosol. The RAMP4op-Hsp70 association can also be required for maintenance of the insertionally competent conformation of RAMP4op.

Our efforts to immunoprecipitate the RAMP4op-p40 crosslink product using antibodies against Hdj1 or Hdj2 were unsuccessful. To identify p40 by determining its amino acid sequence using mass spectroscopy, larger amounts of this protein have to be obtained in the sufficiently pure form. Two possible approaches can be applied in order to achieve purification of p40 from a large amount of RRL. One way would be to stabilize the RAMP4op-p40 complex by cross-linking before binding to the affinity column. After washing and elution, a purified eluted fraction should contain non-crosslinked RAMP4op, as well as the RAMP4op-p40 cross-linked product that can be further purified on a gel. Second approach is based on binding of the native RAMP4op-p40 complex to the affinity matrix. Elution of p40 would be accomplished by washing the beads with a buffer containing the non-ionic detergent Triton X-100.

5.4. Characterization of requirements for membrane insertion of RAMP4op

By using trypsin-treated RM in the insertion assay, it has been shown that the post-translational insertion of RAMP4op depends on at least one trypsin-sensitive membrane protein. This protein is probably an integral membrane protein because RAMP4op can be efficiently inserted into PKRM from which peripheral membrane proteins were removed by washing in a buffer with high concentration of salt. Since N-ethylmaleimide treatment of RM decreases the efficiency of RAMP4op insertion, at least one cysteine residue present within an integral membrane protein has to be involved in the process of post-translational insertion of RAMP4op.

Post-translational insertion of the TA proteins Syb2 and Nyv1p synthesized *in vitro* in RRL was also shown to be dependent on a trypsin-sensitive component of RM (78, 129). The only TA protein for which it was shown *in vitro* that it could be inserted into pure liposomes is cytochrome b5 (CytB5) (4, 71, 132). However, it is unlikely that protein-independent insertion of CytB5 occurs *in vivo* as well since this protein, which normally resides in the ER and mitochondria would then be found in many different cellular membranes.

Post-translational insertion of RAMP4op could still occur into RM treated with lower amounts trypsin. It is known from previously published reports that trypsin in low concentrations inhibits the pathway for cotranslational insertion/translocation of proteins by releasing the cytoplasmic domain of membrane-integrated SRP receptor (SR) (110, 128). I have confirmed this by reconstituting cotranslational insertion using the soluble recombinant form of SR. Since both proteolytic degradation of SR using trypsin in low concentrations and subsequent addition of soluble SR do not affect membrane insertion of RAMP4op, it can be concluded that functional SR is not required for the post-translational insertion of RAMP4op.

Abell et al. have reported that post-translational insertion of the TA protein Sec61β depends on SR, Sec61α and Sec62/63 (1, 2). Conflicting reports exist regarding membrane requirements for the post-translational insertion of the TA protein Syb2. Abell et al. have shown that SR, Sec61α and Sec62/63 are required for the ER insertion of this protein (1, 2), while Rapoport group has reported that Syb2, after being synthesized in RRL, can be efficiently inserted into proteoliposomes lacking both SR and Sec61α (78). Post-translational insertion of other analyzed TA proteins appear to be independent of the Sec61 translocon and factors associated with it. Cytochrome b5 and Nyv1p expressed in yeast mutants defective in either Sec61, Sec62, Sec63 or Bip could be efficiently inserted into the ER (129, 147). In any of these mutants, however, post-translational translocation of the signal sequence containing secretory protein ppαF was either completely or partially inhibited. This suggests that in yeast post-translationally targeted TA and secretory proteins become inserted/translocated into the ER using different translocation systems. It is known that yeast *Saccharomyces cerevisiae* has the

second translocon complex in the membrane of the ER, composed of Ssh1 ("Sec61 homologue"), Sbh2 and Sss1 subunits (35). It has been shown that post-translational ER insertion of the TA protein Nyv1p also occurs in the double yeast mutant Sec61/Ssh1, suggesting that neither of the two yeast translocons are required for Nyv1p insertion.

Currently, the only known protein conducting channel for translocation/insertion into the mammalian ER membrane is the Sec61 translocon (135). Based on the analysis of post-translational insertion into trypsin-treated PKRM presented in this thesis, it cannot be deduced whether the Sec61 translocon is involved in the ER insertion of RAMP4op. It is known that trypsin in higher concentrations can degrade the carboxy terminal part and cytosolic loops 6 and 8 of Sec61α inhibiting in that way cotranslational translocation (110, 128). Whether this degradation of Sec61α also causes disruption of the post-translational insertion of RAMP4op is not known. This issue is hard to resolve due to the fact that trypsin, in addition to proteolysis of Sec61α, affects other membrane proteins as well (e.g. Sec61β, TRAM) (128).

5.5. Conclusions

RAMP4op is a membrane protein which is targeted to the ER after its synthesis has been accomplished on cytosolic ribosomes. Proteins with hydrophobic transmembrane domains, such as RAMP4op, are prone to misfolding and aggregation upon exposure to the cytosolic environment. However, newly synthesized cytosolic RAMP4op is found within a soluble complex of about 60-90 kDa in size. It is possible that the complex formation is important for the maintenance of RAMP4op in the soluble, insertionally competent state. The cytosolic protein of about 40 kDa (p40) has been identified as an interacting partner of RAMP4op. This interaction is hydrophobic in nature and is established with the transmembrane domain of RAMP4op. Therefore, p40 is likely to be involved in prevention of RAMP4op aggregation and/or maintenance of an insertionally competent conformation during the targeting to the membrane.

It is known that folding of newly synthesized proteins in the cytosol occur with the assistance of chaperones (39). Members of the Hsp70/Hsp40 chaperone system were shown to be involved in post-translational targeting of yeast secretory proteins containing hydrophobic signal sequences to the ER. In addition, Hsp70 chaperones are required for post-translational targeting of mitochondrial, peroxisomal and chloroplast proteins to these organelles. It is therefore possible that the Hsp70 chaperone system is involved in targeting of RAMP4op to the ER membrane. This suggestion is supported not only by the estimated size of the RAMP4op cytosolic complex, but also by the fact that the post-translational targeting/insertion of RAMP4op requires ATP hydrolysis. In this scenario, p40

would be the candidate for an Hsp40 co-chaperone responsible for the delivery of RAMP4op to an Hsp70 chaperone. In order to better understand functional significance of the RAMP4op-p40 interaction in the process of the post-translational ER targeting of RAMP4op, p40 has to be identified.

6. ABBREVIATIONS

AD(T)P	Adenosin di (tri)phosphate
Bip	Heavy chain binding protein
bp	Base pair
cDNA	Complementary DNA
CNBr	Cyanogen bromide
CytB5	Cytochrome B5
DMEM	Dulbecco' Modified Eagle Medium
DMSO	DimethylSulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxy nucleotide triphospahte
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylendiaminetetraacetic acid
Endo H	Endoglycosydase H
ER	Endoplasmic reticulum
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine-5'-triphosphatase
Hsp	Heat shock protein
Ii	Invariant Chain
Kbp	Kilobase pair
kDa	Kilo-Daltons
KOAc	Potassium acetate
KRM	High salt washed rough microsomal membranes
M	Molar
mM	Milimolar
mRNA	Messenger ribonucleic acid
NEB	New England Biolabs
NEM	N-ethylmaleimide
PAGE	Polyacrylamid gel elctrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Phosphatidylinositol
PKRM	Puromycin high salt washed rough microsomal membranes
PKRM-T	PKRM trypsin treated
ppαF	Prepro-alpha factor
RAMP4	Ribosome Associated Membrane Protein 4
RAMP4op	Ribosome Associated Membrane Protein 4 - opsin tagged
RM	Rough Microsomes
RNA	Ribonucleic acid
RNC	Ribosome-nascent chain
RRL	Rabbit Reticulocytes Lysate
S. cerevisiae	Saccharomyces cerevisiae
SDS	Sodiumdodecylsulfate
SDS PAGE	SDS polyacrylamide gel electrophoresis
SR	SRP receptor
SRP	Signal Recognition Particle
Syb2	Synaptobrevin 2
TA	Tail-anchored
TCA	Trichloro Acetic acid
TM	Transmembrane
TRAP	Translocon-associated protein complex
Tris	Tris-(Hydroxymethyl)-Aminomethane
UTR	Untranslated region
WB	Western Blotting

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