Signal Recognition Particle–dependent Membrane Insertion of Mouse Invariant Chain: A Membrane-spanning Protein with a Cytoplasmically Exposed Amino Terminus

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Abstract. Invariant (Ii) chain is a membrane-spanning protein that is found associated intracellularly with class II histocompatibility antigens.

In the endoplasmic reticulum Ii chain spans the membrane and exposes the NH_2 terminus on the cytoplasmic and the COOH terminus on the lumenal side. This orientation across the membrane is demonstrated directly with the monoclonal antibody In-1, which exclusively recognizes the NH_2 terminal cytoplasmically exposed part of Ii chain.

Membrane insertion of Ii chain requires signal recognition particle and docking protein. When tested in a wheat germ cell free system, signal recognition particle arrests translation of Ii chain. No signal sequence is cleaved from Ii chain upon membrane insertion.

Secretory and many membrane-spanning proteins are translocated across or inserted into the membrane of the endoplasmic reticulum (ER)[1] in a co-translational manner (for review see references 3, 21, 35, 50, and 51). Translocation requires a signal sequence on the nascent polypeptide chain and specific receptors, the signal recognition particle (SRP) and the docking protein (DP) or SRP receptor (1, 2, 6, 18, 19, 32, 33, 38, 46–49). SRP has been shown to interact with polysomes holding nascent preprolactin chains, and it has been proposed that SRP binds to the signal sequence (46). When probed in a wheat germ cell free system with prolactin or IgG light chain mRNAs, SRP arrests the peptide elongation after 70–80 amino acids have been polymerized (33, 47, 48). Elongation proceeds after SRP has bound to the DP, which is located in the ER membranes (18, 19, 33).

Cleavable signal sequences have also been found on proteins that span the membrane once and expose the carboxy (COOH) terminus on the cytoplasmic and the amino (NH_2) terminus on the extracytoplasmic side of the membrane. Examples of these types of proteins, which we call here type I membrane proteins, are the G protein of vesicular stomatitis virus (24, 30, 37) and class I and II histocompatibility antigens (12, 29). The PE_2 glycoproteins of Semliki Forest virus and Sindbis virus seem to have an uncleaved signal sequence (5, 16, 17). For the PE_2 protein of Sindbis virus, an SRP-mediated membrane insertion has been found (5). Translocation of the NH_2-terminal portion of type I membrane proteins is thought to proceed in a manner identical to that of secretory proteins (21, 35, 51). They become, however, arrested in the membrane by a hydrophobic stop transfer sequence, which is usually located close to the NH_2-terminal end of the protein (4, 13, 51).

No cleavable signal sequence has generally been found on proteins that span the membrane once and expose the NH_2 terminus on the cytoplasmic and the COOH terminus on the extracytoplasmic side. Among the investigated proteins are the influenza neuraminidase (8, 15), the rat and human asialoglycoprotein receptor (9, 20, 41), and the human transferrin receptor (39). These proteins we call type II membrane proteins. We are interested in the question of how these proteins become integrated into the membrane.

Invariant (Ii) chain (also called Iγ-chain) is thought to be a type II membrane-spanning glycoprotein (13, 31). It is found intracellularly assembled with mouse Ia and human HLA-DR (class II) histocompatibility antigens (23, 25, 27, 34, 43). Assembly of Ii chain with class II antigens occurs already in the ER (27, 43). During the intracellular transport, the oligomeric complex disassembles and only class II antigens appear on the cell surface. Ii chain seems to remain in a yet unidentified intracellular compartment (27, 43).

The complete sequences of human and mouse Ii chain have been determined (11, 31, 40, 42). DNA sequence analysis shows that Ii chain contains a single stretch of hydrophobic amino acids, and this is located between residues 31 and 56. Based on this finding, it has been proposed that Ii chain spans the membrane close to the NH_2 terminus and exposes the NH_2 terminus on the cytoplasmic side of the membrane (11, 40, 42). Here we provide direct experimental evidence that the mouse Ii chain exposes its NH_2 terminus on the cyto-

1. *Abbreviations used in this paper:* Con A, concanavalin A; DP, docking protein; ER, endoplasmic reticulum; Ii, invariant; SRP, signal recognition particle; TCA, trichloroacetic acid.

plasmic side of ER membranes and that Ii chain is inserted into the membrane in an SRP-dependent manner.

Materials and Methods

Materials

Concanavalin A (Con A)–Sepharose and protein A–Sepharose were obtained from Pharmacia, Uppsala, Sweden; guanidinium–hydrochloride was from Bethesda Research Laboratories, Bethesda, MD; [^{35}S]methionine and ENHANCE were from New England Nuclear, Boston, MA; 7-methyl-guanosine-5′-monophosphate and phenylmethylsulfonyl fluoride were from Sigma, München, FRG.; oligo (dT)-cellulose (type II) was from Collaborative Research, Inc., Waltham, MA; proteinase K was from Merck, Darmstadt, FRG.; RPMI 1640 was from Gibco, Bio-Cult Ltd., Paisley, Scotland; *Staphylococcus aureus* V8 protease was from Miles GmbH, Frankfurt, FRG.; tunicamycin was from Calbiochem, Giessen, FRG.

Monoclonal antibody In-1 has been described previously (26) and was a generous gift from Günther Hämmerling.

Methods

mRNA Purification and Size Fractionation. Total mRNA was isolated from the spleens of SL2 mice by a modified guanidinium hydrochloride method (10). Approximately 20 g of frozen spleens were homogenized in 200 ml 6 M guanidinium HCl/1 mM dithiothreitol/20 mM NaAc pH 7.0 (buffer 1), using a Sorvall Omni-mixer at full speed for pulses of 30-s each. The homogenate was centrifuged for 5 min at 5,000 *g* at 4°C. The resulting supernatant was adjusted to pH 5.0 with 4 N acetic acid and the RNA was precipitated by adding half a volume of ice-cold ethanol. Insoluble material was collected by centrifugation and dissolved in 100 ml of buffer 1, adjusted to pH 5, and reprecipitated as above. After centrifugation, the pellet was dissolved in 10 ml 50 mM Tris/HCl, pH 7.5, 120 mM NaCl, 5 mM EDTA, 1% SDS (buffer 2), and extracted twice with phenol/chloroform. Poly (A)$^+$ RNA was obtained by affinity chromatography on an oligo(dT)-cellulose column. mRNA was size fractionated by sucrose gradient centrifugation. Fractions enriched in mRNA that code for Ii chain were identified by cell free translation (see below) and immunoprecipitation (12).

Cell Free Protein Synthesis. Total or size fractionated mRNA was translated in a wheat germ cell free system (36). The system was supplemented with either 2 A_{280} U/ml of dog pancreas microsomes, pretreated with micrococcal nuclease and high salt, and/or 0.1 A_{280}/ml of gradient-fractionated SRP (48). Microsomes were prepared and treated as described previously (16, 32). SRP was prepared essentially as described (48) with the exception that Nikkol was omitted in the fractionation on DEAE Sepharose and in the sucrose gradients. Except when otherwise stated, cell free translation was done at 25°C. Proteins were analyzed by SDS PAGE (28) and bands were visualized by fluorography (7) using ENHANCE.

Cell Culture and Labeling of Cells. B-lymphoma cells (CH1.1.) were cultured in RPMI 1640 medium containing 8% fetal calf serum, 10 U penicillin/streptomycin, 10 μM mercaptoethanol. Cells were washed twice in methionine-free medium and incubated for 20 min at 37°C in the same medium. [^{35}S] Methionine was added to a final concentration of 400 μCi/ml, and the cells were incubated at 37°C for 60 min. Cells were washed and then solubilized in ice-cold 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 5 mM $MgCl_2$, 1% Triton X-100. Protease inhibitor phenylmethylsulfonyl fluoride was added to a final concentration of 20 μg/ml. Debris were removed by centrifugation at 4°C for 15 min in a microfuge, and the supernatant was used for immunoprecipitation and SDS PAGE. In some experiments glycosylation of asparagine residues was blocked by adding tunicamycin to the cell culture medium to a final concentration of 3 μg/ml. After incubation for 150 min at 37°C, cells were labeled as described above.

Protease Treatments. A lysate (0.5 ml) derived from 2 × 10^6 CH1.1. cells was treated with different amounts (5–50 μg) of *Staphylococcus aureus* V8 protease for 15 min at 30°C. Proteolysis was stopped by addition of trichloroacetic acid (TCA) to a final concentration of 10%. The TCA precipitate was washed twice with 10% TCA/50% acetone to remove the detergent and solubilized in 25 μl of sample buffer containing 3% SDS (3). After SDS PAGE, the proteins were transferred onto nitrocellulose filters (44).

In vitro synthesized proteins were digested with protease by adding 1 vol of 2% Triton X-100, 100 mM Tris HCl, pH 7.5, 300 mM NaCl, and *Staphylococcus aureus* V8 protease to a final concentration of 50 μg/ml and incubating the sample for 15 min at 30°C. Proteolysis was stopped by addition of phenylmethylsulfonyl fluoride (20 μg/ml). For proteolysis of immunoadsorbed Ii chain, In-1 antibody and protein A–Sepharose were added to either a CH1.1. cell lysate or a cell free lysate (12). The mixture was incubated for 60 min and the Sepharose beads with bound IgG and Ii chains washed and resuspended in 20 μl of 10 mM Tris-HCl pH 7.5 and 50 μg/ml of *Staphylococcus aureus* V8 protease. After incubation for 15 min at 30°C, protein fragments were immediately analyzed by SDS PAGE.

Immunoblotting. Proteins were electrophoretically transferred from SDS polyacrylamide gels onto a nitrocellulose filter (44). The nitrocellulose filter was washed twice for 20 min in phosphate-buffered saline (PBS) containing 10% newborn calf serum, then treated for 90 min with cell culture supernatant containing In-1 antibody, washed 4× in PBS/newborn calf serum and once in PBS/newborn calf serum containing 0.05% Triton X-100. Bound antibody was detected with rabbit anti-rat IgG coupled to horseradish peroxidase and stained with diaminobenzidine (44).

Binding of Proteins to Con A–Sepharose. After translation of spleen cell mRNA in a 25-μl wheat germ cell free system in the presence of rough microsomes, membranes were pelleted by centrifugation for 20 min at 10,000 rpm at 4°C. They were then solubilized in 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 4 μg/ml of phenylmethylsulfonyl fluoride and then 50 μl of Con A–Sepharose was added (a slurry containing beads to solubilization buffer in a 1:1 ratio). The mixture was incubated for 60 min at room temperature. Beads were then washed three times with 100 mM acetate at pH 6, 150 mM NaCl, 1 mM MnCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. To the pelleted beads sample buffer for SDS PAGE was added and proteins analyzed as described (28).

Results

To characterize the disposition of Ii chain across the membrane of the ER, we translated mRNA from mouse spleen cells in a wheat germ cell free system in the presence of microsomal membranes derived from dog pancreas. In such a system membrane proteins become inserted into the membrane asymmetrically in the same way as in the cell and the orientation across the membrane can be determined by protease treatment (24, 37). Protease will cleave only that part of a membrane protein that is exposed on the cytoplasmic side, whereas the lumenally disposed part is protected by the membrane. The orientation of the protein across the membrane can then be deduced, if the means exist for detecting the COOH or NH_2 termini of the protein. Since we could make use of a monoclonal antibody specific for Ii chain, we tested whether the antibody recognizes exclusively either the lumenally or the cytoplasmically disposed portion of Ii chain. Depending on the location of the antigenic determinant of In-1 we would be able to discriminate between the two possible orientations of Ii chain across the membrane.

Mouse Ii Chain Spans the Membrane

When spleen mRNA is translated in the wheat germ cell free system and labeled proteins are immunoprecipitated with monoclonal In-1 antibody, a 25-kD form of Ii chain is selected (Ii′) (Fig. 1, lane *1*). When translation is performed in the presence of microsomal membranes, a 31-kD protein is synthesized and this has the same molecular weight as the authentic Ii chain synthesized in vivo (Fig. 1, lane *2*). The latter form is glycosylated, whereas the former is not (see below). Both forms have been described previously (40). Due to the binding to protein A or Con A, the heavy chains of IgG are also precipitated (Fig. 1, lanes *1*, *2*, *3*, *5*, and *6*). IgG is a typical secretory protein that is translocated across microsomal membranes and therefore can be used to assess the intactness of the microsomal membranes. After digestion with proteinase K no Ii chain-related protein can be precipitated with In-1 antibody (Fig. 1, lane *3*). Heavy chain of IgG,

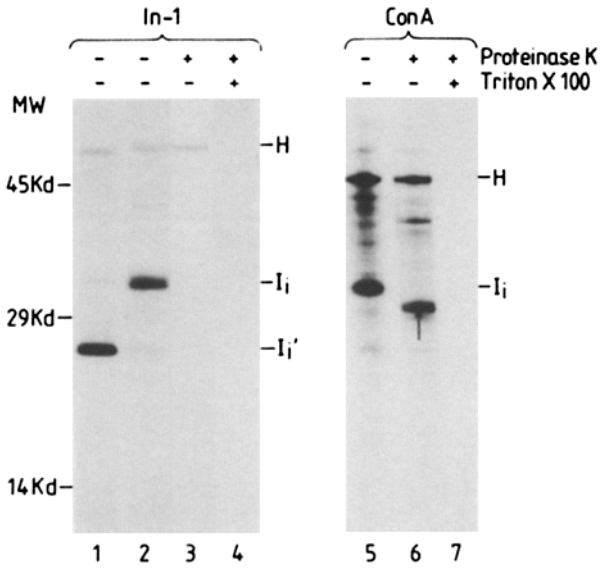

Figure 1. Protease digestion of Ii chain from intact and detergent-solubilized microsomal vesicles. mRNA from mouse spleen cells was translated in a wheat germ cell free system in the absence (lane *1*) or presence of dog pancreas microsomal membranes (lanes 2–7). After translation, proteinase K, 0.5 mg/ml final concentration (lanes *3* and *6*), or proteinase K and 0.5% Triton X-100 (lanes *4* and *7*) were added and the mixture incubated for 15 min at 30°C. Ii chain was immunoprecipitated using In-1 antibody and protein A–Sepharose (lanes *1–4*). Glycoproteins were isolated by binding to Con A–Sepharose (lanes *5–7*). Antigens were separated by SDS PAGE and visualized by fluorography. *Ii*, membrane inserted and glycosylated invariant chain, *Ii'*, unglycosylated form of Ii chain. *H*, heavy chain of IgG that binds to protein A–Sepharose and Con A–Sepharose.

however, is readily precipitable, demonstrating that a protection against protease digestion has been obtained for proteins located in the lumen of microsomal vesicles. When Triton X-100 is used together with proteinase K, the lumenally located heavy chain of IgG is digested, indicating that protection is dependent on the intact membrane barrier (Fig. 1, lanes *4* and *7*).

The inability for In-1 antibody to precipitate Ii chain after protease digestion of intact microsomes could indicate that the antibody recognizes the part of the polypeptide chain that is exposed on the cytoplasmic side of the membrane. To detect processed Ii chain, we used Con A–Sepharose. Ii chain is known to be glycosylated and should therefore bind to Con A (43). It furthermore is the major ^{35}S-labeled glycoprotein synthesized by spleen cells and should therefore be readily detectable among the spleen cell glycoproteins. Fig. 1, lane *6* shows that this is indeed the case and that proteinase K treatment of microsomal vesicles reduces the size of Ii chain by ~3 kD. This reduction in size indicates that mouse Ii chain, as its human counterpart, spans the membrane and exposes ~30 amino acid residues on the cytoplasmic side of the membrane. This result also demonstrates that the cytoplasmic portion is essential for the recognition of Ii chain by In-1 antibody.

In-1 Antibody Recognizes the NH_2-Terminal Portion of Ii Chain

To determine the recognition site for In-1 on Ii chain, proteolytic fragments of Ii chain were generated using *Staphylococcus aureus* V8 protease.

Mouse CH1.1. cells, which express relatively large amounts of Ii chain, were labeled for 1 h with [^{35}S]methionine and Ii chains were precipitated with In-1 antibody. Immunoprecipitated polypeptides were characterized by SDS PAGE, either directly (Fig. 2, lanes *1* and *4*) or after treatment with 5 μg/ml (Fig. 2, lane *2*) or 50 μg/ml (Fig. 2, lane *3*) V8 protease. As expected, Ii chain and a 41-kD protein were precipitated with the In-1 antibody (26, 52, 53). The 41-kD protein is an Ii gene product generated most likely by differential splicing (52). With increasing concentrations of the V8 protease, Ii chain was digested into three major polypeptides, labeled A, B, and C (Fig. 2). Polypeptide A has a molecular weight of ~29 kD, B of ~20 kD, and C of ~10 kD. It appears that the cleavage of A generates fragments B and C adding up to the molecular weight of 29 kD.

To obtain information about the location of the three proteolytic fragments in the linear structure of the Ii chain, the same digestion was performed on [^{35}S]cysteine-labeled Ii chain. A single cysteine residue is known to occur in Ii chains and this residue is located in the NH_2-terminal portion (11, 31, 42). Fig. 2, lanes 5–7 shows the results obtained with [^{35}S]cysteine-labeled Ii chains before (lane *5*) or after the digestion with 5 μg/ml (lane *6*) or 50 μg/ml (lane *7*) of V8 protease. Ii chain and the fragments A and C are radioactively labeled with cysteine, but not fragment B. Thus, *Staphylococcus aureus* V8 protease cuts the Ii chain essentially twice giving rise to three polypeptides labeled A, B, and C in Fig. 2.

To demonstrate the location of the fragment C within the polypeptide chain directly, we introduced a gradient of label into Ii chain. mRNA from spleen cells was translated in the presence of microsomal membranes. 1 min after the start of translation, 7-methyl-guanosine was added to synchronize mRNA translation. After different time intervals (Fig. 3), unlabeled methionine was added to a final concentration of

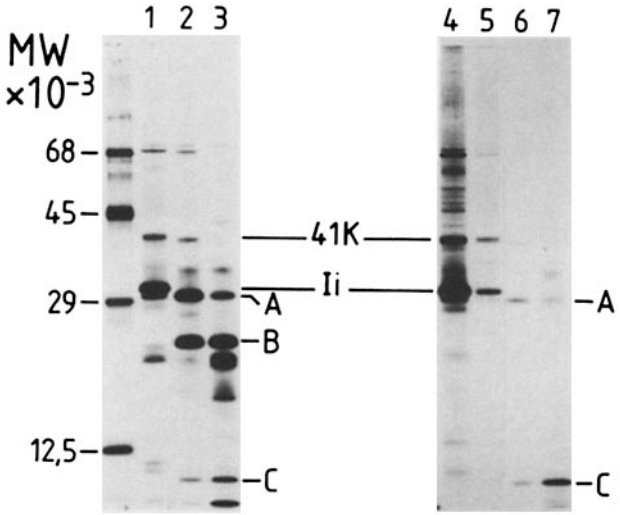

Figure 2. Digestion of Ii chain with *Staphylococcus aureus* V8 protease. Mouse CH1.1. cells were labeled for 1 h with either [^{35}S]-methionine (lanes *1–4*) or [^{35}S]cysteine (lanes *5–7*). Ii antigens were immunoprecipitated and incubated with no protease (lanes *1*, *4*, and *5*) or with 5 μg/ml (lanes *2* and *6*) or 50 μg/ml V8 protease (lanes *3* and *7*) for 15 min at 30°C. The positions of glycosylated Ii chain, the 41-kD polypeptide, and fragments *A*, *B*, and *C* which are generated upon cleavage with *Staphylococcus aureus* V8 protease are indicated.

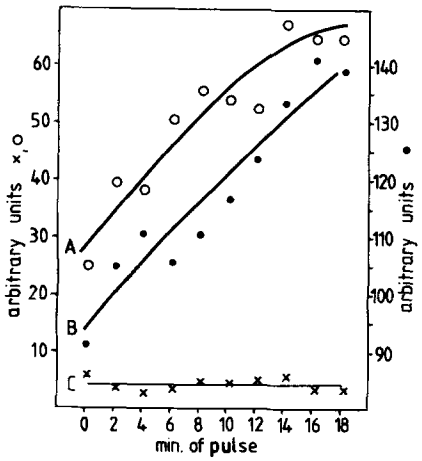

Figure 3. Pulse labeling of Ii chain and its fragments generated by *Staphylococcus aureus* V8 protease digestion. mRNA from mouse spleen cells was translated in a wheat germ cell free system in the presence of dog pancreas microsomes with [^{35}S]methionine as label. To synchronize translation 7-methyl-guanosine-5′-monophosphate was added after 1 min. Different lengths of pulse were achieved by adding unlabeled methionine at the times indicated in the figure. Antigens were immunoprecipitated as described, digested with 10 μg/ml V8 protease for 15 min at 30°C, and characterized by SDS PAGE and fluorography. Radioactivity in bands *A* (○), *B* (●), and *C* (×) (see Fig. 2) was quantitated by densitometry. The constant amount of label in peptide *C* indicates that it is localized close to the NH_2-terminal end. Two scales were used because of different amounts of label in the fragments *A*, *B* and *C*.

10 mM to chase the radioactive methionine. The Ii chain was immunoprecipitated, digested with *Staphylococcus aureus* V8 protease, and characterized by SDS PAGE and autoradiography. The amount of label in fragments A, B, and C was then determined by densitometry of the autoradiograph (Fig. 3). It can be predicted that label in a polypeptide fragment located close to the NH_2-terminal end will remain constant, whereas label in fragments located close to the COOH-terminal end will increase with time of the pulse. As can be seen in Fig. 3, the label in fragment C remains constant while label in fragments A and B increases with the length of the pulse. This clearly demonstrates that fragment C is located close to the NH_2-terminal end of Ii chain.

To demonstrate that the C fragment from in vivo synthesized Ii chain is also recognized by In-1 antibody, we characterized Ii chain and its fragments by immunoblotting with In-1 antibody. Total protein from CH1.1. cells either before or after digestion with *Staphylococcus aureus* V8 protease was separated by SDS PAGE, blotted onto nitrocellulose filter, and developed with In-1 antibody. Fig. 4 shows that Ii chain and the 41-kD protein are recognized by In-1 antibody (lane *1*) as well as after V8 protease digestion the 10-kD fragment C (lane *2*). Thus, we conclude that also in vivo the NH_2-terminal fragment of Ii chain carries the determinant required for the binding to In-1 antibody.

To locate the first cleavage site in Ii chain, which removes a segment of ~1 kD (see Fig. 2, lane *2*), membrane-inserted Ii chain was digested with *Staphylococcus aureus* V8 protease on intact microsomes and then immunoprecipitated. As no molecular weight shift could be detected under these conditions, it can be concluded that all cleavage sites for V8 protease must be located on the lumenal side (data not shown).

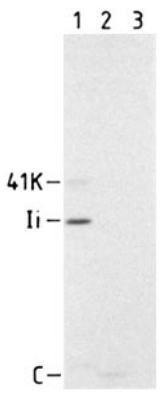

Figure 4. In-1 antibody recognizes proteolytic fragment *C* of Ii chain synthesized by CH1.1. cells. 1 × 10^7 mouse CH1.1. cells were detergent solubilized and nuclei removed by centrifugation at 5,000 *g* for 10 min. Solubilized antigens were treated either with no protease (lane *1*) or with 5 μg/ml (lane *2*) or 50 μg/ml (lane *3*) V8 protease for 15 min at 30°C and then precipitated with 10% TCA, 50% acetone. After resolubilization in SDS-containing sample buffer, proteins were separated by SDS PAGE. After transfer onto nitrocellulose, proteins were reacted with In-1 antibody and visualized by peroxidase-coupled anti-rat antibody and diaminobenzidine staining.

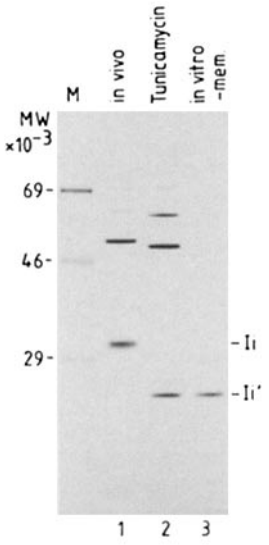

Figure 5. Ii chain is synthesized without a cleavable signal sequence. Mouse CH1.1. cells were labeled for 1 h with [^{35}S]methionine in the absence (lane *1*) or presence (lane *2*) of 3 μg/ml tunicamycin (added 150 min before labeling). mRNA from mouse spleen cells was translated in a wheat germ cell free system (lane *3*). Antigens were precipitated with In-1 antibody. *Ii*, glycosylated Ii chain, *Ii'*, unglycosylated Ii chain integrated (lane *2*) or not integrated (lane *3*) into membranes of the ER. *M*, molecular weight markers.

Ii Chain Is Synthesized without a Cleavable Signal Sequence

Membrane proteins which expose the NH_2 terminus on the lumenal side of the ER are usually synthesized with a cleavable signal sequence (35, 51). In contrast, proteins that expose the NH_2-terminal end on the cytoplasmic side, like the influenza neuraminidase and the rat and human asialoglycoprotein receptor, are synthesized without a cleavable signal sequence (15, 20, 41). We therefore asked whether Ii chain is also synthesized without a cleavable signal sequence. In glycoproteins, the presence or absence or a cleavable signal sequence can be determined by comparing the molecular weight of the translation products synthesized in a cell free system in the absence of microsomal membranes with those synthesized in vivo in the presence of tunicamycin. Tunicamycin is known to prevent glycosylation but does not interfere with membrane insertion and cleavage of a signal sequence. As can be seen in Fig. 5, Ii chain is synthesized in CH1.1. cells in the presence of tunicamycin as a 25-kD polypeptide chain. The identical molecular weight is found for the Ii chain when it is synthesized in a wheat germ cell free system in the absence of microsomal membranes.

Membrane Insertion of Ii Chain Is SRP Dependent

For proteins that are translocated across the membrane of the ER, it is known that SRP and DP are required for their

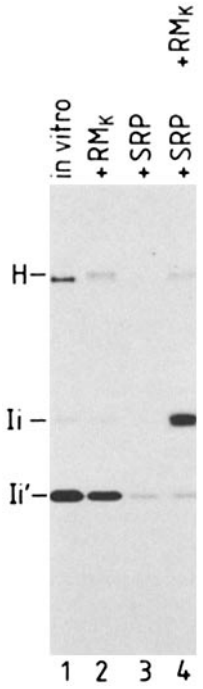

Figure 6. SRP-dependent insertion of Ii chain into microsomal membranes. Spleen cell mRNA was translated in the wheat germ cell free system and synchronized after 5 min by 7-methyl-guanosine (lane *1*). Translation was supplemented with salt-washed microsomal membranes (RM_K) (lane *2*) or SRP (lanes *3* and *4*). In lane *4*, RM_K were added after 15 min of synthesis in the presence of SRP.

insertion into the membrane (33, 42). For one membrane protein, which is synthesized without cleavable signal sequence, it has been found that SRP and DP are required for membrane insertion but no arrest in elongation was observed (2). As demonstrated above, Ii chain is synthesized without a cleavable signal sequence, spans the membrane, and exposes the NH_2 terminus on the cytoplasmic side. We tested therefore whether this type of protein requires SRP and DP for membrane insertion and whether elongation could be arrested by SRP. When Ii chain was translated in a wheat germ cell free translation system, the 25-kD unglycosylated form of Ii chain was synthesized (Fig. 6, lane *1*). When salt-washed rough microsomes, depleted of SRP, were added, no shift of molecular weight could be detected (Fig. 6, lane *2*). When SRP alone was added to the cell free translation system, synthesis of Ii chain was arrested as well as that of heavy chain of IgG (Fig. 6, lane *3*). The arrest in translation could be released when salt-washed rough microsomes (RM_k) were added 15 min after initiation of translation (Fig. 6, lane *4*). Glycosylated Ii chains now accumulated.

Discussion

Orientation of Ii across the ER Membrane

Several lines of evidence suggest that Ii chain spans the membrane and exposes the NH_2-terminal end on the cytoplasmic and the COOH-terminal end on the lumenal side of the membrane of the ER. (*a*) Sequence data derived from a cDNA for Ii chain locate a single stretch of 26 hydrophobic or uncharged amino acid residues close to the NH_2-terminal end (11, 31, 42). Hydrophobic sequences are known to occur in regions of proteins that span the membrane. (*b*) When protease is used to digest the cytoplasmically exposed portion of Ii chain, a segment comprising ~30 amino acid residues can be removed. This segment corresponds to the length found between the NH_2 terminus and the stretch of uncharged amino acid residues in the Ii chain. The remaining 170 amino acids are protected by the membrane barrier and must thus be located on the lumenal side of the membrane. (*c*) The monoclonal antibody In-1 recognizes a determinant located on a part of the Ii chain that is exposed on the cytoplasmic side of the membrane. Protease digestion of intact microsomal vesicles destroys this binding site. It is the NH_2 terminally located fragment C which is recognized by In-1 antibody. Two independent methods were used to locate fragment C within Ii chain: (*i*) After digestion of Ii chain with *Staphylococcus aureus* V8 protease, fragment C was found to be the only one that labeled with [^{35}S]cysteine. The only cysteine in Ii chain occurs 28 amino acid residues away from the NH_2-terminal end (see Fig. 7). (*ii*) When a gradient of [^{35}S]cysteine label was introduced into Ii chain, the amount of label in fragment C remained constant throughout the chase period. This is only consistent with a location of fragment C close to NH_2-terminal end. (*d*) There is no cleavable signal sequence found in Ii chain. The unglycosylated precursor of Ii chain synthesized in the wheat germ cell free system in the absence of microsomal membranes has the identical molecular weight as unglycosylated and membrane-inserted Ii chain synthesized by CH1.1 cells. Cleavable signal sequences have a length between 13 and 45 amino acid residues (45). A difference of five amino acids would have been detected by the SDS PAGE system used.

These facts demonstrate that Ii chain exposes the NH_2-terminal end on the cytoplasmic and the COOH-terminal end on the lumenal side of the membrane of the ER.

In-1 antibody has previously been used to locate the Ii chain on the cell surface (26). As this antibody, however, does not recognize a determinant exposed on the external surface of the cell, it must be concluded that broken cells were responsible for the observed binding of In-1 antibody. Using fluorescence activated cell sorter analysis, no surface labeling could be detected using this antibody (Arnold, B., and J. Lipp, unpublished results).

Membrane Insertion of Ii Chain, a Type II Membrane-spanning Protein

Proteins that span the membrane once can expose either their COOH-terminal end (type 1 membrane proteins) or their NH_2-terminal end (type 2 membrane proteins) on the cytoplasmic side. Like secretory proteins, type 1 membrane proteins are usually synthesized with cleavable signal sequences. Their membrane insertion proceeds co-translationally and requires SRP and DP. The same requirements were found for

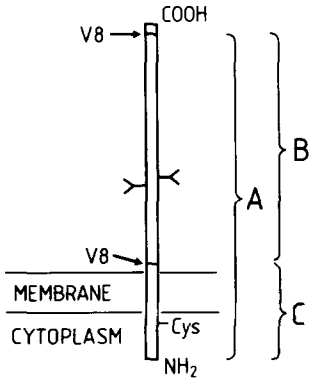

Figure 7. Schematic representation of Ii chain. Ii chain spans the ER membrane and exposes the NH_2-terminal end on the cytoplasmic side and the COOH-terminal end on the lumenal side. Proposed locations of cleavage sites for *Staphylococcus aureus* V8 protease and the fragments *A*, *B*, and *C* are indicated. In-1 antibody recognizes that part of fragment *C* that is exposed on the cytoplasmic side.

membrane insertion of Ii chain for a typical type 2 membrane protein.

What mechanism can be envisaged for a common step in membrane insertion of secretory and type 1 and 2 membrane proteins? A very attractive possibility, first proposed by Inouye and his colleagues for the lipoprotein of *Escherichia coli* and further extended to membrane-spanning proteins, is that the insertion of the NH_2-terminal portion of nascent secretory or membrane-spanning proteins into the membrane of the ER occurs in a loop-like fashion (14, 22, 45). This model is based on the assumption that the NH_2-terminal end of the signal sequence, cleavable or noncleavable, remains exposed on the cytoplasmic side of the ER membrane. Cleavage of the signal sequence then releases the new NH_2-terminal end of the mature protein to the lumen of the ER vesicle. The cleaved signal sequence might remain in some or all cases buried in the membrane. Type 1 membrane-spanning proteins, like the H-2 antigens or VSV G protein, have in addition to a cleavable signal sequence a second stretch of uncharged amino acid residues located close to the COOH-terminal end. This functions as a "stop transfer" sequence and anchors the protein in the membrane. In type 2 membrane proteins with uncleaved signal sequence the single hydrophobic segment might perform two functions: (*a*) as a single sequence mediating SRP-dependent membrane insertion, and (*b*) as a stop transfer sequence anchoring the protein in the membrane. Certainly further direct evidence is required for support of this model for membrane insertion of type 2 membrane proteins.

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