The EMBO Journal vol.14 no.2 pp.217–223, 1995

Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane

Ulrike Kutay, Gudrun Ahnert-Hilger[1], Enno Hartmann, Bertram Wiedenmann[1] and Tom A.Rapoport[2]

Max-Delbrück-Centre for Molecular Medicine, Robert-Rössle-Strasse 10, 13122 Berlin-Buch and [1]Benjamin Franklin Medical Centre, Department of Internal Medicine, Division of Gastroenterology, Free University of Berlin, Medical School, Germany

[2]Corresponding author

Communicated by T.Rapoport

Synaptobrevin/vesicle-associated membrane protein is one of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. It is proposed to provide specificity for the targeting and fusion of vesicles with the plasma membrane. It belongs to a class of membrane proteins which lack a signal sequence and contain a single hydrophobic segment close to their C-terminus, leaving most of the polypeptide chain in the cytoplasm (tail-anchored). We show that in neuroendocrine PC12 cells, synaptobrevin is not directly incorporated into the target organelle, synaptic-like vesicles. Rather, it is first inserted into the endoplasmic reticulum (ER) membrane and is then transported via the Golgi apparatus. Its insertion into the ER membrane *in vitro* occurs post-translationally, is dependent on ATP and results in a trans-membrane orientation of the hydrophobic tail. Membrane integration requires ER protein(s) different from the translocation components needed for proteins with signal sequences, thus suggesting a novel mechanism of insertion.

Key words: protein transport/SNARE proteins/synaptobrevin/tail-anchored proteins/translocation

Introduction

Synaptobrevin/vesicle-associated membrane protein (VAMP) belongs to the family of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins which are believed to provide specificity in intracellular vesicle targeting and fusion (Bennett and Scheller, 1993; Söllner *et al.*, 1993a,b; Südhof *et al.*, 1993). According to the SNARE concept, vesicle-bound v-SNARE proteins pair with t-SNAREs located in the target membrane. The partners of synaptobrevin, a v-SNARE located in synaptic vesicles in neuronal cells and in synaptic-like vesicles in neuroendocrine cells, are syntaxin and SNAP-25, both constituents of the plasma membrane (Söllner *et al.*, 1993a,b; Calakos *et al.*, 1994). Homologs of these proteins direct vesicles to the plasma membrane in other cells, and different pairs of v- and t-SNAREs are known to be involved in the transport of vesicles from the endoplasmic reticulum (ER) membrane to the Golgi apparatus and in other transport routes (Bennett and Scheller, 1993). How the different SNARE proteins are targeted to the various organelles and retained there is unknown, but the answer is crucial to allow an understanding of their function.

Most membrane proteins located in compartments of the mammalian secretory pathway or in organelles derived from it contain signal sequences which direct them to a translocation apparatus of the ER membrane that is also used by secretory proteins (Rapoport, 1992). In general, the signal sequence of a ribosome-bound polypeptide chain is recognized by the signal recognition particle (SRP) and is then targeted to the ER membrane by an interaction of the SRP with its membrane receptor (also called docking protein). The ensuing process of co-translational translocation and membrane insertion requires the Sec61p complex, consisting of three membrane protein subunits and, in most cases, the translocating chain-associating membrane (TRAM) protein (Görlich and Rapoport, 1993). The insertion of proteins into the ER membrane is triggered by hydrophobic polypeptide segments that may follow the signal sequence or may coincide with it (the latter are called signal–anchor sequences).

SNARE proteins do not possess signal sequences. Instead, most of them have a hydrophobic segment close to the C-terminus that anchors them in the membrane, leaving most of the polypeptide chain in the cytoplasm (Kutay *et al.*, 1993). They may therefore be called 'tail-anchored'. They are predicted to be inserted into the membranes post-translationally since at least part of the membrane anchor must be buried in the translating ribosome when it reaches the termination codon. A post-translational mode of membrane insertion implies that the process is independent of the function of the SRP and of its receptor since SRP only interacts with a signal sequence of a nascent chain that is still bound to the ribosome (Wiedmann *et al.*, 1987). Consequently, it is possible that the proteins are not inserted initially into the ER membrane; instead, they could conceivably be incorporated directly into the target organelle or perhaps into a precursor of it, or they could initially be inserted randomly into all membranes.

So far, little is known about the mechanism of membrane integration and intracellular targeting of tail-anchored proteins. The examples studied seem to represent different modes of membrane integration. The ER form of cytochrome b_5 can be inserted *in vitro* into all kinds of membrane, including protein-free liposomes (Daily and Strittmatter, 1978; Takagaki *et al.*, 1983), but it is uncertain whether this reflects the physiological process since *in vivo* the protein is found exclusively in the ER membrane (for a discussion see Borgese *et al.*, 1993). Bcl-2, a tail-

anchored protein whose intracellular location is debated, is reported to be inserted *in vitro* with equal efficiency into all membranes that have a cytosolic surface (Janiak *et al.*, 1994) or preferentially into the outer membrane of mitochondria (Nguyen *et al.*, 1993). The middle-t antigen of the polyoma virus seems to be located in various subcellular membranes by an association with the submembraneous part of the cytoskeleton (Andrews *et al.*, 1993).

In this paper we demonstrate that in neuroendocrine cells, synaptobrevin is first inserted into the ER membrane and then transported via the Golgi apparatus to synaptic-like vesicles. Using *in vitro* systems, we show that the insertion of synaptobrevin into ER membranes occurs post-translationally, is independent of SRP but requires ATP. It results in a trans-membrane orientation of its hydrophobic tail. The integration requires ER protein(s) different from those known to be needed for membrane proteins with signal sequences, thus suggesting a novel mechanism of insertion.

Results

Transport route of synaptobrevin in PC12 cells

The site of membrane insertion of synaptobrevin 2 (Syb2) and its intracellular transport were studied in neuroendocrine PC12 cells that contain synaptic-like vesicles (SLVs) (Baumert *et al.*, 1989). The cells were pulse-labeled with [^{35}S]methionine and either analyzed directly or incubated further for different chase periods with unlabeled methionine. The cells were homogenized and post-mitochondrial supernatants were submitted to equilibrium sucrose density gradient centrifugation. This procedure separates the light SLVs, containing endogenous Syb2, from the dense rough ER, containing the α-subunit of the 'translocon-associated protein' (TRAPα; Hartmann *et al.*, 1993). Both marker proteins could be identified by immunoblotting with specific antibodies (Figure 1). Metabolically labeled Syb2 was identified in the fractions of the sucrose gradients by immunoprecipitation with specific antibodies followed by SDS–PAGE. It represented the major precipitated band and co-migrated with the product obtained by *in vitro* translation (Figure 1). The band was not seen if the antibodies were pre-saturated with the peptide against which they were raised (data not shown).

The results of the pulse–chase experiment demonstrated that after short labeling periods, the majority of newly synthesized Syb2 was found in dense fractions corresponding to the ER (Figure 1, upper panel). With a half-life of ~20 min, labeled Syb2 was shifted to the position of endogenous Syb2. The process was almost complete after a chase period of 40 min (Figure 1, second and third panels).

To demonstrate that vesicular transport is required for the transport process, brefeldin A was added to the cells prior to labeling (Klausner *et al.*, 1992). Under these conditions, even after prolonged incubation (60 min), essentially none of the newly synthesized Syb2 was found in light vesicles (Figure 1, fourth panel), although the positions of the ER marker and of endogenous Syb2 in sucrose gradients remained unchanged (results not shown). In the parallel incubation in the absence of brefeldin A,

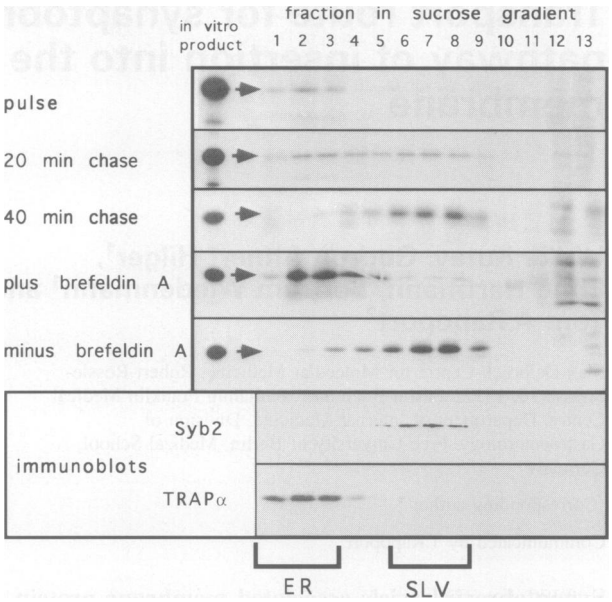

Fig. 1. Transport route of Syb2 in PC12 cells. PC12 cells were pulse-labeled with [^{35}S]methionine and subsequently incubated with unlabeled methionine for different periods of time (chase). A cell homogenate was submitted to equilibrium sucrose density gradient centrifugation to separate the SLVs (identified by immunoblotting for endogenous Syb2) from the ER (identified by immunoblotting for TRAPα). Labeled Syb2 in the fractions was analyzed by immunoprecipitation with specific antibodies, followed by SDS–PAGE and fluorography. In a separate experiment, PC12 cells were incubated in the presence or absence of brefeldin A and labeled for 30 min, followed by a chase for 30 min. The arrow indicates the position of Syb2. For size comparison, Syb2 was also synthesized *in vitro* (*in vitro* product). The bands seen in lanes 12 and 13 represent unspecifically precipitated cytosolic proteins present in large excess in these fractions.

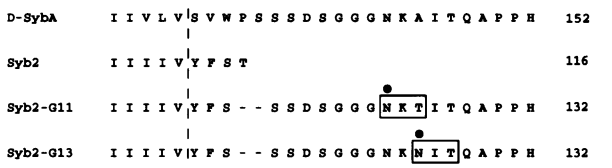

Fig. 2. C-terminal sequences of human (Syb2) and *Drosophila* synaptobrevin (D-Syb) and of their derivatives. Syb2-G11 and Syb2-G13 were constructed by PCR mutagenesis to extend the coding region of Syb2 by a sequence resembling that of D-Syb (Südhof *et al.*, 1989). Glycosylation sites (boxed) were introduced at distances of 11 and 13 residues from the membrane anchor, respectively, by changing the indicated Ala to either Thr or Asn. The potentially glycosylated Asn residues are marked by dots. Numbers to the right give the lengths of the polypeptide chains. The C-terminal border of the membrane anchor is indicated by a dashed line.

almost all labeled Syb2 was found in fractions corresponding to SLVs (Figure 1, fifth panel). These results indicate that Syb2 cannot be incorporated directly into the target organelle; rather, it is first inserted into the ER and subsequently sorted by vesicular transport.

We next tested whether Syb2 is transported through the Golgi apparatus by analyzing the glycosylation of a mutant of synaptobrevin (Syb2-G13). Syb2-G13 contains at its C-terminus a short extension corresponding to the sequence of *Drosophila* synaptobrevin (Südhof *et al.*, 1989), except that an *N*-glycosylation site is present at a distance of 13 amino acids from the membrane anchor (Figure 2). As shown below, this modification does not

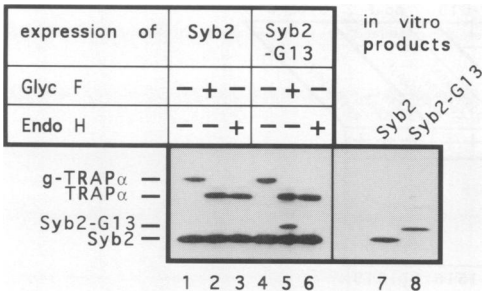

Fig. 3. Syb2-G13 passes the Golgi in PC12 cells. PC12 cells were transfected with constructs coding for Syb2 or Syb2-G13, that carries a glycosylation site C-terminal of its membrane anchor (see Figure 2). Proteins in a 100 000 *g* membrane fraction were treated with *N*-glycosidase F (Glyc F) or endoglycosidase H (Endo H) and analyzed by SDS–PAGE followed by immunoblotting with antibodies directed against Syb2 and against TRAPα. Control samples were mock-treated. For size comparison, Syb2 and Syb2-G13 were also synthesized *in vitro* (*in vitro* products). g-TRAPα, glycosylated TRAPα.

change its mode of membrane insertion *in vitro*. When Syb2-G13 was expressed in PC12 cells, immunoblot analysis only showed the presence of endogenous Syb2 (Figure 3, lane 4). However, if the membrane fraction was first treated with peptidyl *N*-glycosidase F, an enzyme known to remove all *N*-linked carbohydrate chains, a band appeared (lane 5) that exactly comigrated with unglycosylated Syb2-G13 synthesized *in vitro* (lane 8). This band was not seen if the cells were transfected with wild-type Syb2 (lane 2). Thus, Syb2-G13 synthesized *in vivo* must have been glycosylated, but is presumably too heterogeneous in size to be visible in SDS gels. Treatment with endoglycosidase H did not result in the appearance of unglycosylated Syb2-G13 (Figure 3, lane 6), although the enzyme was active as demonstrated by the size shift of the glycosylated ER protein TRAPα which was present in the same sample (see lanes 4 and 6, upper bands). Similar results were obtained when the glycosylation of metabolically labeled Syb2-G13 was studied (results not shown). Thus, the carbohydrate chains on Syb2-G13 must be insensitive to endoglycosidase H, a characteristic of Golgi glycosylations.

Post-translational insertion into ER membranes in vitro

We next investigated the insertion of synaptobrevin into microsomes *in vitro*. Syb2 was synthesized in the reticulocyte system in the presence of [^{35}S]methionine, and microsomes from PC12 cells (results not shown) or from dog pancreas were added either during or after translation (Figure 4A). In both cases a high proportion of Syb2 (50–70%) was found in the membrane pellet (lanes 3 and 4). In the co-translational reaction, a read-out of membrane-bound ribosomes present in the microsomes was observed, resulting in a number of additional bands (lane 3).

Syb2 is stably integrated into the ER membrane because it was found to float with the microsomes in an alkaline sucrose gradient (Figure 4B). Again, after post-translational incubation, a high percentage of the total labeled Syb2 was inserted into the membranes (see lanes 1 and 5). Very little material was found in the floated fraction in the absence of microsomes (lane 4).

To make sure that in the post-translational reaction the translocated molecules were released from ribosomes, the translation mixture was treated with puromycin or, in addition, the ribosomes were removed by sedimentation before incubation with the microsomes. Even with these stringent criteria for post-translational membrane insertion, a high proportion of Syb2 was found inserted in the microsomes (lanes 7 and 9; lanes 2 and 3 show the corresponding total material before floatation).

Topology of membrane-inserted synaptobrevin

Membrane-inserted Syb2 was sensitive to treatment with proteases (results not shown), indicating that most of the polypeptide chain faces the cytoplasm. The C-terminus can reach the lumenal side of the ER membrane, as indicated by the *in vivo* experiments with Syb2-G13 (see above). This conclusion could be confirmed by *in vitro* experiments in which Syb2-G13 was found to be glycosylated in both co- and post-translational incubations with microsomes (Figure 4A, lanes 11 and 12; see open arrow). Proof of the identity of the glycosylated species of Syb2-G13 could be obtained by its binding to concanavalin A (see lanes 17 and 18) and to antibodies against Syb2 (lane 19). In contrast to Syb2-G13, the secretory protein prepro-α-factor that contains an N-terminal signal sequence was only glycosylated in a co-translational reaction (lane 15 versus lane 16). A mutant of Syb2, in which the glycosylation site had only a distance of 11 amino acids from the membrane anchor (Syb2-G11; see Figure 2), was not glycosylated (Figure 4A, lanes 5–8). These results are in agreement with other data that demonstrate for a co-translationally transported protein, that a minimum distance of 12 residues for an *N*-glycosylation site is required (Nilsson and von Heijne, 1993). This distance seems to be dictated by the location of the active site of the oligosaccharyltransferase relative to the lumenal membrane surface, and is apparently the same whether the substrates are presented co- or post-translationally. Syb2-G13 behaved in an identical manner to Syb2 in post-translational ribosome-independent membrane insertion (Figure 4B, lanes 10–18). One may thus conclude that Syb2-G13 can reach a trans-membrane orientation and be glycosylated in an efficient post-translational reaction. Also, the C-terminal extension of Syb2 has not converted its membrane anchor into a conventional type II signal–anchor sequence which would have functioned only in co-translational reactions.

ATP requirement

The post-translational membrane insertion of Syb2 was greatly reduced if nucleotides were removed by gel filtration, as determined by floatation in an alkaline sucrose gradient (Figure 5, upper panel, lane 5). A high efficiency of insertion could be restored by re-addition of ATP (lane 7 versus lane 4). The poorly hydrolyzable analog ATPγS could not substitute for ATP (lane 8), and at a concentration of 10 mM it inhibited the reaction induced by the endogenous ATP in the reticulocyte lysate (results not shown); thus, ATP hydrolysis seems to be necessary. GTP was much less effective than ATP in supporting the post-translational membrane insertion of Syb2 (lane 9).

Similar results were obtained for Syb2-G13 (Figure 5, lower panel). In this case, the membrane insertion was analyzed by its co-sedimentation with microsomes.

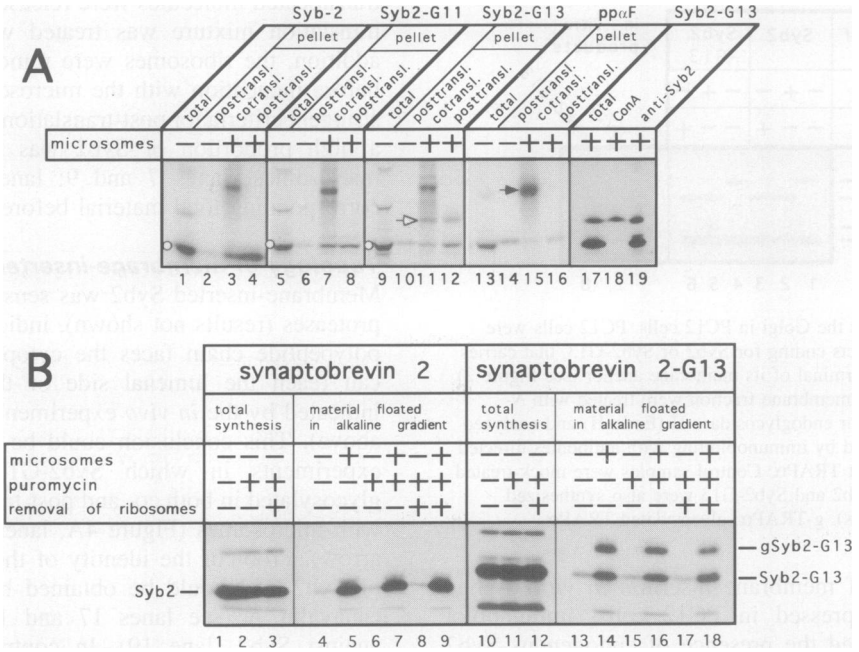

Fig. 4. Membrane insertion of synaptobrevin *in vitro*. (**A**) Radiolabeled Syb2 and two mutants of it, Syb2-G11 and Syb2-G13 (see Figure 2), were synthesized *in vitro* with rough microsomes present either during (cotransl.) or after (posttransl.) translation. The membranes were sedimented and the bound products were analyzed by SDS–PAGE and fluorography. The total products and the material sedimenting in the absence of microsomes are also shown. The band indicated by an open arrow is glycosylated Syb2-G13, as it is bound to concanavalin A–Sepharose (ConA) and is immunoprecipitated by antibodies against Syb2 (anti-Syb2; lanes 17–19). Lanes 13–16 show controls with prepro-α-factor (ppαF) which is only glycosylated in co-translational reactions (solid arrow). The open circles indicate the positions of Syb2 and of its derivatives. (**B**) After synthesis *in vitro* of Syb2 or Syb2-G13, the samples were either treated with puromycin to release nascent chains from ribosomes or, in addition, the ribosomes were sedimented. To these samples (total synthesis) microsomes were added and, after incubation, submitted to floatation in an alkaline sucrose gradient. The floated material was analyzed by SDS–PAGE and fluorography. gSyb2-G13, glycosylated Syb2-G13.

Glycosylation of the protein was only observed with ATP (see lanes 7 and 4) or, much less efficiently, with GTP (lane 9). In these experiments, the background levels were somewhat higher than with the floatation assay (see, for example, lanes 3 and 5). For unknown reasons, in the absence of nucleotide triphosphates and in the presence of microsomes the background bands appeared as a smear (e.g. lane 6 versus lane 5).

Insertion of synaptobrevin into proteoliposomes

Next, we investigated the requirement of ER membrane proteins for the insertion of synaptobrevin using proteoliposomes of different composition. Preprolactin, the translocation of which is dependent on the functions of the SRP receptor and the Sec61p complex (Migliaccio *et al.*, 1992; Görlich and Rapoport, 1993), was used as a control.

Proteoliposomes, produced from an unfractionated detergent extract of canine pancreas microsomes, showed the same activity as native microsomes (PK-RM) in post-translational insertion of Syb2 (insertion determined by the alkaline floatation assay; Figure 6, lowest panel, lane 4 versus lane 3). They were also active in translocation and processing of preprolactin, as indicated by the appearance of signal sequence-cleaved prolactin and its resistance to externally added protease (upper panels, lane 4 versus lane 3).

Proteoliposomes, deficient in either the SRP receptor or the Sec61p complex, were produced from immunodepleted detergent extracts of microsomes. To exclude unspecific inactivation of the depleted proteoliposomes, control vesicles were produced by complementing the depleted

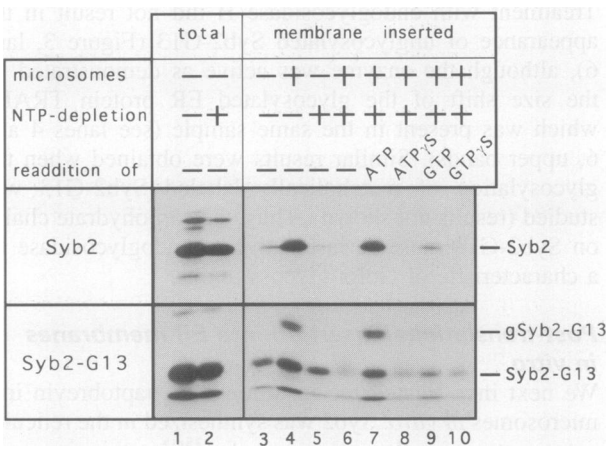

Fig. 5. Post-translational insertion of synaptobrevin requires ATP. Syb2 or Syb2-G13 were synthesized *in vitro*. Where indicated, the samples were subsequently submitted to gel filtration (NTP depletion) and 10 mM nucleotide triphosphates or their analogs were re-added during the incubation with microsomes. In the case of Syb2, the membrane-inserted material was analyzed by floatation in an alkaline sucrose gradient, in the case of Syb2-G13 by co-sedimentation with the membranes. gSyb2-G13, glycosylated Syb2-G13.

extracts with the corresponding purified protein complexes (Görlich and Rapoport, 1993). Whereas the translocation of preprolactin was drastically reduced in the absence of SRP receptor (lane 5 versus lane 6) or Sec61p complex (lane 7 versus lane 8), the membrane insertion of Syb2 was unaffected (lower panel, lanes 5 and 7). Similar results were obtained with Syb2-G13 (data not shown).

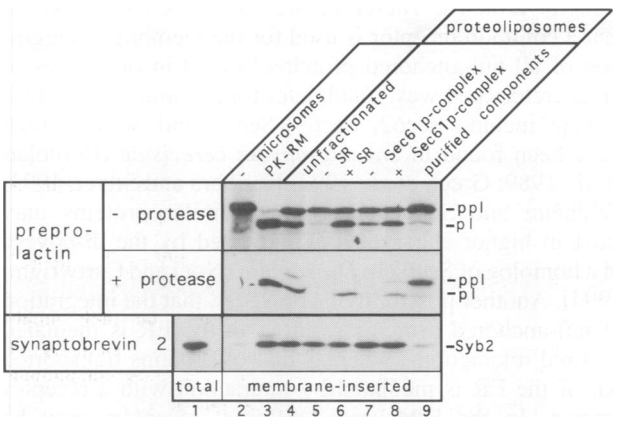

Fig. 6. Insertion of synaptobrevin into proteoliposomes. Syb2 was synthesized *in vitro* and its insertion into microsomes stripped of ribosomes (PK-RM) and into proteoliposomes was analyzed after floatation in an alkaline sucrose gradient. Proteoliposomes were produced from either an unfractionated detergent extract of PK-RM, from extracts which were immunodepleted of the SRP receptor (−SR) or the Sec61p complex (−Sec61p), or from extracts which were first depleted and then replenished with purified SRP receptor (+SR) or Sec61p complex (+Sec61p). Lane 9 shows an experiment with proteoliposomes containing only the purified SRP receptor, Sec61p complex and the TRAM protein. As a control, the co-translational translocation of preprolactin into the vesicles was tested. Translocated material was analyzed after incubation with proteinase K (+protease). ppl, preprolactin; pl, prolactin.

Immunoblot analysis demonstrated that the depleted proteoliposomes contained <5% of the amounts of both translocation components in native microsomes (data not shown).

Further evidence for essential differences between the translocation of preprolactin and Syb2 was obtained using proteoliposomes containing only the purified SRP receptor, the Sec61p complex and the TRAM protein. Whereas these vesicles were active in the translocation of preprolactin (Figure 6, upper panels, lane 9) and in the insertion of all tested single-spanning membrane proteins containing a signal sequence (Görlich and Rapoport, 1993; S.High and B.Jungnickel, personal communication), they did not support the insertion of Syb2 (lower panel, lane 9) above levels observed with protein-free liposomes (results not shown). Replacement of the artificial lipid mixture by a lipid preparation from ER membranes did not increase the activity of the proteoliposomes (results not shown), suggesting that the missing component is one or more proteins.

Requirement for a protease-sensitive membrane component

The requirement for a protein component was supported by the observation that the insertion of Syb2 into microsomes stripped of ribosomes (PK-RM) was drastically reduced (up to 85%) if the membranes were pretreated with trypsin (Figure 7, lanes 5–9 versus lane 4). The residual level corresponded to that seen with protein-free liposomes. Similar results were obtained with ribosome-containing rough microsomes (results not shown). Controls showed that preprolactin was not transported into microsomes pretreated with trypsin above 10 μg/ml (data not shown; see also Meyer *et al.*, 1982).

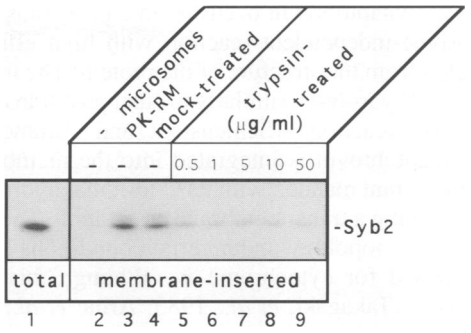

Fig. 7. Requirement for a protease-sensitive membrane component. Microsomes stripped of ribosomes (PK-RM) were pretreated with different concentrations of trypsin and then tested for post-translational insertion of Syb2. Controls demonstrated that the protease was completely inhibited before Syb2 was added (data not shown).

Discussion

Our results indicate that in neuroendocrine cells, synaptobrevin is first inserted into the rough ER membrane and is then transported in vesicles through the Golgi apparatus to its final destination, small SLVs. Our evidence is based on pulse−chase labeling experiments and subsequent cell fractionations in sucrose gradients, as well as on the modification of carbohydrate chains of a glycosylation mutant of synaptobrevin, presumably in the Golgi apparatus. The experiments with brefeldin A, which blocks the exit of proteins from the ER, demonstrate further that synaptobrevin cannot be inserted directly into SLVs. We therefore conclude that despite its post-translational mode of insertion, synaptobrevin takes an obligatory route through the ER. Its intracellular route thus corresponds to the general pathway used by proteins containing signal sequences.

Presumably, the route of synaptobrevin is shared by other tail-anchored SNARE proteins. Indeed, plasma membrane syntaxin shows, to some extent, localization in the Golgi and can be chased away in the presence of cycloheximide (Banfield *et al.*, 1994). Also, expression of its yeast homolog Sso2 in mammalian cells indicates an initial ER localization followed by transport through the Golgi to the plasma membrane (J.Jäntti, S.Keränen, J.Toikkanen, E.Kuismanen, C.Ehnholm, H.Söderlund and V.M.Olkkonen, personal communication).

An obvious consequence of the suggested route of SNARE proteins through the ER is that there must exist signals and mechanisms for their retention at various points of the secretory pathway. It seems that the membrane-spanning region contains a targeting signal but, at least in Sed5 (a t-SNARE of the Golgi), there seem to be further signals in the cytoplasmic domain (Banfield *et al.*, 1994). One must also postulate that both v- and t-SNAREs are kept in an inactive state *en route* to their destination to avoid premature targeting and fusion of vesicles. v-SNAREs may also be turned off during their recycling after vesicle fusion. Retention and activation of the SNARE proteins may be achieved by their interaction with other membrane proteins in the target organelle. Alternatively, inhibitory partners may accompany the SNARE proteins until they have reached their site of action.

Our studies *in vitro* have shown that the membrane

insertion of synaptobrevin occurred in a post-translational and ribosome-independent reaction with high efficiency, as predicted from the structure of the protein. The insertion required ATP hydrolysis, similarly to other post-translational translocation reactions (Schlenstedt and Zimmermann, 1987). Synaptobrevin is integrated into the membrane in an alkali-resistant manner, with its C-terminal hydrophobic domain adopting a trans-membrane orientation. The attainment of such a topology under certain conditions has also been proposed for cytochrome b_5, although this is still controversial (Takagaki, *et al.*, 1983; Arinc *et al.*, 1987). Recently, Yamasaki *et al.* (1994) also showed that synaptobrevin from *Aplysia* can reach a trans-membrane orientation *in vitro* in a post-translational reaction. However, this synaptobrevin is unusual since it has a domain of ~80 amino acids following its membrane anchor. Its co-translational insertion was predominant and it has not been determined whether the post-translational translocation was independent of the presence of ribosomes.

Using reconstituted proteoliposomes of different composition, we have obtained evidence that synaptobrevin follows a novel pathway of membrane insertion, independently of known translocation components. If the process is at all dependent on the Sec61p complex, much lower concentrations are sufficient than for all proteins with signal sequences studied so far. The situation is reminiscent of a class of 'Sec-independent' proteins in *Escherichia coli* (Wickner *et al.*, 1991) which are transported after depletion from the cells of SecA and SecY (the latter is the bacterial homolog of Sec61p; Görlich *et al.*, 1992). In any case, since synaptobrevin is efficiently inserted into proteoliposomes containing unfractionated ER membrane proteins, but not into proteoliposomes containing known purified translocation components or into protein-free liposomes, it seems that at least one unknown membrane protein is involved. A role for an ER membrane protein in the insertion of synaptobrevin is further indicated by the finding that the protein is exclusively integrated into this organelle *in vivo* and that the pretreatment of microsomes with trypsin prevents insertion *in vitro*.

There appear to be a variety of mechanisms of post-translational membrane insertion. For preprocecropin A and other small proteins it is possible that only the SRP-targeting pathway is bypassed (Klappa *et al.*, 1994), the membrane insertion of the α-subunit of the SRP receptor may be mediated by its interaction with the β-subunit (Hortsch and Meyer, 1988; Andrews *et al.*, 1989), for middle-t antigen of the polyoma virus a special pathway involving the membrane skeleton may exist (Andrews *et al.*, 1993), and cytochrome b_5 (Daily and Strittmatter, 1978; Takagaki *et al.*, 1983), M13 procoat (Ohno-Iwashita and Wickner, 1983), Ubc6 (Sommer and Jentsch, 1993; our unpublished results) and perhaps Bcl-2 (Janiak *et al.*, 1994), may be inserted *in vitro* even into protein-free liposomes. However, in the latter cases, a high spontaneous *in vitro* interaction of the proteins with lipids may have masked the facilitating role of a membrane protein. The fact that the stable and specific insertion of cytochrome b_5 and Bcl-2 requires specific amino acid sequences at their C-terminus, in addition to the hydrophobic polypeptide segments (Mitoma and Ito, 1992; Janiak *et al.*, 1994), would indeed be more consistent with recognition by receptor proteins. Therefore, we still consider it possible that a common receptor is used for the membrane integration of all tail-anchored proteins located in organelles of the secretory pathway. Candidates for a ubiquitous receptor protein include Sec62, Sec63, Sec71 and Sec72 which have been found in *Saccharomyces cerevisiae* (Rothblatt *et al.*, 1989; Green *et al.*, 1992; Kurihara and Silver, 1993; Feldheim and Schekman, 1994); similar proteins may exist in higher eukaryotes as indicated by the discovery of a homolog of Sec62 in *Drosophila* (Noël and Cartwright, 1994). Another possibility is, however, that the integration of tail-anchored proteins residing in the ER is mediated by lipid interactions, whereas that of proteins transported out of the ER is mediated by interaction with a receptor protein. In the latter case, different receptors may be required for different proteins and the receptor may be identical with the postulated retention and sorting proteins needed at a later point of the intracellular transport route.

Materials and methods

Synaptobrevin constructs and antibodies

The cDNA for Syb2 was isolated from a human cerebellum cDNA library (Stratagene), using an oligonucleotide for screening that corresponds to the antisense strand of the gene between positions 142 and 165. The derived sequence coincided with the published one (Archer *et al.*, 1990), except that nucleotide 346 was found to be A instead of T, thus leading to the change of amino acid Ser116 to Thr.

Syb2-G11 and Syb2-G13 were constructed by extending the coding region of Syb2 in a two-step PCR, resulting in the sequences shown in Figure 2.

For *in vitro* transcription, all genes were cloned into the plasmid pGEM3βGL downstream of the 5′ untranslated region of the β-globin gene. For expression of Syb2 and Syb2-G13 in PC12 cells, the genes were cloned into the plasmid pRc/CMV (Invitrogen) behind the CMV promoter. The 5′ and 3′ untranslated regions of the Syb2 gene remained unchanged in both constructs, except for two bases directly following the stop codon. Transfection into the cells was carried out with lipofectamin.

Antibodies against Syb2 were raised in rabbits against the peptide VDKVLERDQKLSDLC. They were affinity-purified on a peptide column and covalently coupled to a mixture of protein A− and protein G−Sepharose, as described (Görlich *et al.*, 1992).

Pulse–chase labeling of PC12 cells and cell fractionation

Semi-confluent PC12 cells (75 cm^2 flasks) were incubated in a methionine-depleted medium with 0.5 mCi/ml [^{35}S]methionine for 10 min. They were then either analyzed directly (pulse) or incubated for different periods of time with DMEM (Gibco) containing unlabeled methionine (chase). When the effect of brefeldin A was tested, the cells were incubated in the absence or presence of 10 μg/ml of the drug and labeled for 30 min with [^{35}S]methionine, followed by a chase for 30 min in DMEM. After incubation, the cells were washed and homogenized with glass beads in a Vortex mixer in 40 μl S buffer [50 mM HEPES/KOH, pH 7.6, 250 mM sucrose, 5 mM magnesium acetate, 2 mM dithiothreitol (DTT), 1 mM cycloheximide, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (10 μg/ml leupeptin, 5 μg/ml chymostatin, 3 μg/ml pepstatin and 1 μg/ml elastatinal)]. The homogenate was centrifuged at 10 000 *g* for 5 min and the supernatant was layered onto a 16–40% linear sucrose gradient over a pad of 1 ml 60% sucrose, both in 50 mM HEPES/KOH (pH 7.6), 125 mM potassium acetate, 5 mM magnesium acetate, 2 mM DTT and protease inhibitors as in S buffer. After centrifugation for 15 h at 4°C and 40 000 r.p.m. in an SW40 rotor (Beckman), the gradient was fractionated and the proteins were precipitated with trichloroacetic acid. The samples were dissolved in SDS sample buffer and either analyzed by SDS–PAGE followed by immunoblotting or submitted to immunoprecipitation. For the latter, the samples were diluted 1:50 in IP buffer (50 mM Tris–HCl, pH 7.8, 0.5 M NaCl, 1% CHAPS, 4% bovine serum albumin and protease inhibitors). They were then incubated at 4°C overnight with 20 μg affinity-purified immobilized antibodies against Syb2. The resin was thoroughly washed with buffers containing 1% Triton X-100, a mixture of 0.1% Triton X-100, 0.5% SDS and 0.5% deoxycholate, and a mixture

of 0.5 M NaCl, 0.1% Triton X-100 and 0.1% deoxycholate. Bound material was released by boiling in SDS sample buffer and analyzed by SDS–PAGE (10–20% linear acrylamide gradient) followed by fluorography.

Immunoblotting and treatment of a 100 000 *g* membrane fraction of PC12 cells with glycosidase F and endoglycosidase H were carried out as described (Tarentino *et al.*, 1989).

In vitro translation and tests for membrane insertion

Transcripts for Syb2, Syb2-G11 and Syb2-G13, obtained by *in vitro* transcription with T7 RNA polymerase, were translated in the reticulocyte lysate system (Promega) at 30°C in the presence of 0.75 mCi/ml [^{35}S]methionine. To a 10 µl reaction, two equivalents (for a definition see Walter *et al.*, 1981) dog pancreatic microsomes were added either during or after translation for 60 or 30 min, respectively. Ribosomes were removed after reaction with puromycin by sedimentation for 20 min at 100 000 r.p.m. (rotor 100) in a Beckman table-top ultracentrifuge. The removal of nucleotide triphosphates was carried out by centrifugation of the translation mixture twice through an equilibrated, prepacked Sephadex G-25 column. The removal of ATP was checked in a parallel sample with [^{32}P]ATP (generally <1/1000 of the original concentration).

An analysis of the membrane-inserted material was carried out by either sedimentation of the membranes for 30 min in a microfuge or floatation in an alkaline sucrose gradient. For the latter, the samples were incubated with 0.1 M Na_2CO_3 (pH 11.5) for 10 min on ice. They were then adjusted to 1.6 M sucrose and overlayered with cushions of 1.25 and 0.25 M sucrose, both in 0.1 M Na_2CO_3 (pH 11.5). After centrifugation for 90 min at 100 000 r.p.m. in a table-top ultracentrifuge, the upper and lower phases were analyzed. The recovery of membranes in the upper phase was checked by immunoblotting for ER membrane proteins and was generally >80%.

Analyses of the products by binding to concanavalin A–Sepharose and by immunoprecipitation have been described previously (Görlich *et al.*, 1992; Görlich and Rapoport, 1993).

Reconstituted proteoliposomes

Proteoliposomes from an unfractionated extract of puromycin/high salt-stripped microsomes (PK-RM) were produced as described (Görlich and Rapoport, 1993), except that 0.7% deoxy-BigCHAP was used for their solubilization. Immunodepletion was carried out with immobilized antibodies against the β-subunit of the Sec61p complex or against the α-subunit of the SRP receptor, as described (Görlich and Rapoport, 1993), except that the detergent extract was incubated three times for 10 h, each time with fresh antibodies. Purified Sec61p complex or SRP receptor was added to the depleted extracts in amounts estimated to be present in the original unfractionated samples. All vesicles were added at a concentration of 0.2 equivalents/µl to the translation mixtures. Incubation was carried out for 30 min at 26°C for preprolactin (tested in the wheatgerm system in a co-translational reaction; Görlich and Rapoport, 1993), and at 30°C for synaptobrevin (tested in the reticulocyte lysate system in a post-translational reaction).

Proteoliposomes containing purified components were produced with proteins in deoxy-BigCHAP using a mixture of phospholipids corresponding approximately to that of ER membranes, as described (Görlich and Rapoport, 1993). The final concentrations of the components in the translocation assay were: SRP receptor, 0.2 equivalents/µl; Sec61p complex, 0.4 equivalents/µl; and TRAM protein, 0.15 equivalents/µl.

Trypsin treatment of microsomes

PK-RM were incubated with TPCK-trypsin for 30 min on ice, the incubation was stopped by the addition of 0.5 mM PMSF and protease inhibitors (see S buffer) and the membranes were centrifuged twice through a 0.5 M sucrose cushion in high salt buffer. The recovery of membranes was checked by immunoblotting with antibodies against TRAPβ and equal amounts were used in the subsequent insertion tests.

Acknowledgements

We thank B.Jungnickel for providing purified translocation components, A.Bergfeld for help in producing some constructs and B.Nentwig, A.Wittstruck and S.Knespel for technical support. We are grateful to D.Görlich, T.Sommer, K.-U.Kalies, B.Jungnickel, T.Kurzchalia, S.Monier and S.M.Rapoport for critical reading of this manuscript. This work was supported by grants from the BMFT, the DFG, the Human Science Frontier Program and the Mildred Scheel Stiftung.

References

Andrews,D., Lauffer,L., Walter,P. and Lingappa,V. (1989) *J. Cell Biol.*, **108**, 797–810.

Andrews,D.W., Gupta,J. and Abisdris,G. (1993) *Mol. Cell. Biol.*, **13**, 4703–4713.

Archer,B.T., TMzcelik,T., Jahn,R., Francke,U. and Südhof,T. (1990) *J. Biol. Chem.*, **265**, 17267–17273.

Arinc,E., Rzepecki,L.M. and Strittmatter,P. (1987) *J. Biol. Chem.*, **262**, 15563–15567.

Banfield,D.K., Lewis,M.J., Rabouille,C., Warren,G. and Pelham,H.R.B. (1994) *J. Cell Biol.*, **127**, 357–371.

Baumert,M., Maycox,P.R., Navone,F., de Camilli,P. and Jahn,R. (1989) *EMBO J.*, **8**, 379–384.

Bennett,M.K. and Scheller,R.H. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 2559–2563.

Borgese,N., D'Arrigo,A., De Silvestris,M. and Pietrini,G. (1993) *Subcell. Biochem.*, **21**, 313–341.

Calakos,N., Bennett,M.K. and Scheller,R.H. (1994) *Science*, **263**, 1146–1149.

Daily,H.A. and Strittmatter,P. (1978) *J. Biol. Chem.*, **253**, 8203–8209.

Feldheim,D. and Schekman,R. (1994) *J. Cell Biol.*, **126**, 935–944.

Görlich,D. and Rapoport,T.A. (1993) *Cell*, **75**, 615–630.

Görlich,D., Prehn,S., Hartmann,E., Kalies,K.U. and Rapoport,T.A. (1992) *Cell*, **71**, 489–503.

Green,N., Fang,H. and Walter,P. (1992) *J. Cell Biol.*, **116**, 597–604.

Hartmann,E., Görlich,D., Kostka,S., Otto,A., Kraft,R., Knespel,S., Bürger,E., Rapoport,T.A. and Prehn,S. (1993) *Eur. J. Biochem.*, **214**, 375–381.

Hortsch,M. and Meyer,D.I. (1988) *Biochem. Biophys. Res. Commun.*, **150**, 111–117.

Janiak,F., Leber,B. and Andrews,D.W. (1994) *J. Biol. Chem.*, **269**, 9842–9849.

Klappa,P., Zimmermann,M. and Zimmermann,R. (1994) *FEBS Lett.*, **341**, 281–287.

Klausner,R.D., Donaldson,J.G. and Lipincott-Schwartz,J. (1992) *J. Cell Biol.*, **116**, 1071–1080.

Kurihara,T. and Silver,P. (1993) *Mol. Biol. Cell.*, **4**, 919–930.

Kutay,U., Hartmann,E. and Rapoport,T.A. (1993) *Trends Cell Biol.*, **3**, 72–75.

Meyer,D.I., Krause,E. and Dobberstein,B. (1982) *Nature*, **297**, 503–508.

Migliaccio,G., Nicchitta,C.V. and Blobel,G. (1992) *J. Cell Biol.*, **117**, 15–25.

Mitoma,J.Y. and Ito,A. (1992) *EMBO J.*, **11**, 4197–4203.

Nguyen,M., Millar,D.G., Yong,V.W., Korsmeyer,S.J. and Shore,G.C. (1993) *J. Biol. Chem.*, **268**, 25265–25268.

Nilsson,I. and von Heijne,G. (1993) *J. Biol. Chem.*, **268**, 5798–5801.

Noël,P.J. and Cartwright,I.L. (1994) *EMBO J.*, **13**, 5253–5261.

Ohno-Iwashita,Y. and Wickner,W. (1983) *J. Biol. Chem.*, **258**, 1895–1900.

Rapoport,T.A. (1992) *Science*, **258**, 931–936.

Rothblatt,J.A., Deshaies,R.J., Sanders,S.L., Daum,G. and Schekman,R. (1989) *J. Cell Biol.*, **109**, 2641–2652.

Schlenstedt,G. and Zimmermann,R. (1987) *EMBO J.*, **6**, 699–703.

Söllner,T., Bennett,M.K., Whiteheart,S.W., Scheller,R.H. and Rothman,J.E. (1993a) *Cell*, **75**, 409–418.

Söllner,T., Whiteheart,S.W., Brunner,M., Erdjument-Bromage,H., Geromanos,S., Tempst,P. and Rothman,J.E. (1993b) *Nature*, **362**, 318–323.

Sommer,T. and Jentsch,S. (1993) *Nature*, **365**, 176–179.

Südhof,T., Baumert,M., Perin,M.S. and Jahn,R. (1989) *Neuron*, **2**, 1475–1481.

Südhof,T.C., de Camilli,P., Niemann,H. and Jahn,R. (1993) *Cell*, **75**, 1–4.

Takagaki,Y., Radhakrishnan,R., Wirtz,K.W. and Khorana,H.G. (1983) *J. Biol. Chem.*, **258**, 9136–9142.

Tarentino,A.L., Trimble,R.B. and Plummer,T.H. (1989) In Tartakoff,A.M. (ed.), *Laboratory Methods in Vesicular and Vectorial Transport.* Academic Press, New York, pp. 299–329.

Walter,P., Ibrahimi,I. and Blobel,G. (1981) *J. Cell Biol.*, **91**, 545–550.

Wickner,W., Driessen,A.J.M. and Hartl,F.U. (1991) *Annu. Rev. Biochem.*, **60**, 101–124.

Wiedmann,M., Kurzchalia,T.V., Bielka,H. and Rapoport,T.A. (1987) *J. Cell Biol.*, **104**, 201–208.

Yamasaki,S., Hu,Y., Binz,T., Kalkuhl,A., Kurazono,H., Tamura,T., Jahn,R., Kandel,E. and Niemann,H. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 4688–4692.

Received on September 13, 1994