The topogenic fate of the polytopic transmembrane proteins, synaptophysin and connexin, is determined by their membrane-spanning domains

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SUMMARY

The synaptophysins and connexins are polytopic transmembrane proteins of similar secondary structure that accumulate as multiple homo-oligomers in specialized membrane regions, the presynaptic transmitter vesicles or gap junctions. Transfection and expression of the respective genes in cultured epithelial cells results in the de novo formation of either small cytoplasmic, synaptophysin-rich vesicles, or functional gap junctions consisting of clustered connexin molecules. To examine the molecular requirements for the specific enrichment and topogenesis of both types of molecule, chimeric cDNAs were constructed composed of different parts of the rat synaptophysin and rat liver connexin32 genes. Expression of the encoded chimeric polypeptides in hepatocellular carcinoma-derived cells showed that only chimeras with all four transmembrane domains from either parent molecule were delivered to their specific destination. In contrast, chimeras with transmembrane domains from both connexin32 and synaptophysin were always retained in the endoplasmic reticulum. The topogenic nature of the transmembrane domains was further demonstrated by deletion mutagenesis, indicating that removal of cytoplasmic end domains or intravesicular loops does not abolish targeting. On the other hand, excision of individual transmembrane domains or introduction of point mutations in transmembrane segments resulted in retention in the endoplasmic reticulum.

Key words: targeting, transmembrane protein, synaptic vesicle, cell junction, synaptophysin, connexin

INTRODUCTION

The ordered structure of cells relies on the sorting and assembly of their polypeptide components into specialized membrane-bound compartments. Selective recruitment and retention of transmembrane (TM) proteins are required for the functional and structural maintenance of these cellular subdomains. Two different mechanisms may determine the specific localization: (i) extrinsic receptor molecules contained in the surrounding matrix or cytoskeleton interact with specific signal sequences to either transport or retain membrane proteins; or (ii) membrane proteins associate with each other independently of extrinsic cues to form specialized membrane domains.

Using molecular biology methods it has been possible, by deletion and point mutagenesis and/or chimeric protein construction, to identify segment(s) of membrane proteins that are responsible for their site-directed transport and specific membrane retention. The majority of sequence elements characterized so far, such as the signal sequences for endocytosis, transcytosis, lysosomal targeting, basolateral sorting, localization to the endoplasmic reticulum (ER) or the trans-Golgi network (for recent reviews, see e.g. Nilsson and Warren, 1994; Sandoeval and Bakke, 1994), support the first model as they are localized in regions of the molecules that are accessible to extrinsic receptors. On the other hand, recent evidence has linked single TM segments to sorting processes by their ability to assemble into topogenic complexes (for review, see e.g. Machamer, 1991; Nilsson and Warren, 1994).

We have been interested in the polytopic channel-forming TM proteins of the synaptophysin and connexin type that are highly enriched, in specialized membrane regions, as multiple homooligomers (Thomas et al., 1988; Johnston and Südhof, 1990; cf. Beyer et al., 1990; Dermietzel et al., 1990; Bennett et al., 1991). While connexins are targeted to gap junctions in the plasmamembrane (cf. Beyer et al., 1990; Dermietzel et al., 1990; Bennett et al., 1991), synaptophysins are specifically sorted to small, electron-translucent, transmitter-containing vesicles in the presynaptic region (Jahn et al., 1985; Wiedenmann and Franke, 1985; Fykse et al., 1993). Furthermore, it has been shown that ectopic expression leads to formation of functional gap junctions (e.g. see Eghbali et al., 1990; Fishman et al., 1990) or cytoplasmic microvesicles in epithelial cells (Leube et al., 1989, 1994; for contrasting results in fibroblasts, see Johnston et al., 1989a; Cameron et al., 1991; Linstedt and Kelly, 1991b; Fykse et al., 1993), respectively, suggesting the existence of inherent membrane targeting information in these proteins.

Although rat synaptophysin and rat liver connexin32 show no obvious homologies in their primary amino acid sequences (Paul, 1986; Kumar and Gilula, 1986; Leube et al., 1987; Südhof et al., 1987), they share several structural features (see also Unwin, 1989; Betz, 1990): both are integral TM proteins of similar size with four membrane-spanning domains, cyto-
plasmically oriented amino and carboxy termini and two intravesicular/exoplasmic loop domains including several conserved cysteine residues that are linked by intramolecular disulfide bonds (Milks et al., 1988; Johnston et al., 1989b; Johnston and Südhof, 1990; Rahman and Evans, 1991). Furthermore, the carboxy termini are extensively phosphorylated (Pang et al., 1988; Barnekow et al., 1990; Sáez et al., 1990; Rubenstein et al., 1993).

On the basis of these striking similarities it was therefore decided to construct and express chimeric molecules consisting of different segments taken from rat liver connexin32 and rat synaptophysin for the identification of localization signals. It was hypothesized that by exchange of corresponding protein domains specific sequence-related targeting signals may be found. Using this approach in combination with deletion and point mutagenesis it is shown that the TM domains are critical for targeting of synaptophysin to small cytoplasmic vesicles and of connexin32 to gap junctions.

**MATERIALS AND METHODS**

**Construction of cDNA clones**

cDNA clones coding either for rat liver connexin32 (pCR1; Troyanovsky et al., 1993) or rat synaptophysin (pSRS; Leube et al., 1989) were used as templates in polymerase chain reactions (PCRs) to construct chimeric cDNAs using the following primers (for schematic representation of the encoded polypeptides see Fig. 1; for nucleic acid sequences see Kumar and Gilula, 1986; Paul, 1986; Leube et al., 1987): CX-O-516 (5′-GTA AGC TTA GAC AGG ATG AAG TTG ACA GGT-3′), CX-O-605 (5′-ACT CTA GAT GTA GAA TCG GCA TCA AGC CC-3′), SY-O-604 (5′-AAT CTA GAC CCC TTC TGG CTC TGC TGA AGG-3′) and SY-O-603 (5′-GTT GAT CCC CCG GGC TGC AGG-3′) for construct CSR5; CX-O-516, CX-O-602 (5′-AAT CTA GAT GCA GCC ACC ACC AGC ACC AT-3′), SY-O-606 (5′-AAT CTA GAG TTC GGC TGA GGG AG-3′) and SY-O-603 for construct CSR4; CX-O-516, CX-O-594 (5′-AAT CTA GAT TGG TGA GGC GGC TGC TGC GTC-3′), SY-O-601 (5′-CCT CTA GAC AGA ACA AGT ACC GAG AGA AC-3′) and SY-O-603 for construct CSR3; CX-O-516, CX-O-592 (5′-AAT CTA GAC ACC ATG GCA TAG CCC GGG TA-3′), SY-O-593 (5′-GAT CTA GAA AAG GGC TCT CCG ATG AG A-3′) and SY-O-603 for construct CSR2; and SY-O-1713 (5′-TAT CGA TAA GCT TGA TAT CAC GC-3′), SY-O-1714 (5′-TGT TGC TCT CGG TAC CTG TGC TGC AG-3′), CX-O-1715 (5′-AAG GGA TCA ACA ACA GAG GAA AAT G-3′), CX-O-592; SY-O-1716 (5′-AAT CTA GAA AAG GCC TGT CCG ATG AG A-3′) and SY-O-603 for construct SCSRI. Amplified DNA fragments were digested with restriction endonucleases recognizing sites contained in the amplimers, purified and used in ligation reactions with the Bluescript vector (Stratagene, La Jolla, CA) for expression in E. coli K12. Amplified DNA fragments were cleaved with restriction endonucleases recognizing sites contained in the amplimers, purified and used in ligation reactions with the Bluescript vector (plasmid constructs were designated with p followed by the respective construct denomination) into M13 vectors (designation: m followed by construct denomination) for nucleic acid sequence analysis. In addition, cRNAs were prepared from all clones after linearization with BamHI with the help of T3 RNA polymerase and were used for in vitro translation assays with or without canine pancreatic microsomes (Promega, Madison, WI).

The construction of a cDNA clone encoding chimera Sy-Dsg1 consisting of the amino-terminal four transmembrane regions of rat synaptophysin and the cytoplasmic part of bovine desmoglein 1 has been described recently (Troyanovsky et al., 1993).

**DNA transfection**

For transfection, plasmid inserts were subcloned either into the eukaryotic expression vector pJ3 or pBEHpac18 (Morgenstern and Land, 1990; Horst et al., 1991; clones were named with S and their respective construct name), or into pBHA-P1-neo (Gunning et al., 1987; clones were named with A and the respective construct name). Plasmid DNA was purified by cesium chloride centrifugation.

Human hepatocellular carcinoma cells of lines PLC (ATCC CRL8024), SK-Hep-1 (ATCC HTB52) and human vulvar carcinoma-derived A431 cells (clone E3; cf. Leube et al., 1988) were grown in DME (Gibco, Karlsruhe, FRG) supplemented with 10% fetal calf serum. Neuroendocrine rat pheochromocytoma cells of line PC-12 (ATCC CRL1721) and synaptophysin-expressing cells of lines PLC-6S4 and A431-5S4 were passaged as described (Leube et al., 1994). Transfection was done using the calcium phosphate precipitation method and cells were selected as reported previously (Leube et al., 1989, 1994; Troyanovsky et al., 1993). For control of transfection and expression efficiency, co-transfections were performed using the chloramphenicol acetyltransferase (CAT) encoding vector pSV2-cat (Gorman et al., 1982) as reference, and expression was examined by immunofluorescence microscopy with specific CAT antibodies (see below).

**Immunofluorescence microscopy and immunoelectron microscopy**

For immunohistological experiments the following antibodies were used: (i) monoclonal antibody SY38 against synaptophysin (Wiedemann and Franke, 1985); (ii) an affinity-purified rabbit serum against synaptophysin (Leube et al., 1994); (iii) monoclonal antibody HP24 against protein disulfide isomerase (kindly provided by C. S. Kaetzl; Kaetzl et al., 1987); (iv) monoclonal antibody MYC 1-9E10.2 (ATCC CRL 1729) against a defined myc epitope (Evan et al., 1985); (v) rabbit antibodies against CAT (ABCR GmbH, Karlsruhe, FRG); (vi) rabbit antibodies against β-galactosidase (ABCR GmbH); (vii)
murine monoclonal antibodies B3/25 (Boehringer, Mannheim, FRG) and OKT9 (ATCC CRL8021) against the human transferrin receptor; (vii) monoclonal antibodies against the lysosomal membrane proteins LAMP-1 (antibody H4A3) and LAMP-2 (antibody H4B4) as culture supernatant or ascites (Developmental Studies Hybridoma Bank, Iowa City, IA); (ix) monoclonal antibody Dg3.10 against bovine desmoglein (Schmelz et al., 1986).

For generation of antibodies against the cytoplasmic carboxy terminus of rat liver connexin32, fusion proteins were produced in Escherichia coli. Plasmid pCR1 (see above) was cleaved with HpaI, treated with T4 DNA polymerase in the presence of deoxynucleotides, and further digested with PstI. A resultant 300 bp fragment was subcloned into vector pQE-11 (Diagen GmbH, Düsseldorf, FRG) which had been treated consecutively with BamHI, T4-DNA-polymerase and PstI. From this plasmid the insert was also subcloned into pAX5+ (Medac, Hamburg, FRG). Proteins were purified from cultures as described recently (Leube et al., 1994) or as suggested by the manufacturers, and were used as immunogens in immunizations of rabbits. The ammonium sulfate precipitate obtained from sera of immunized animals could be used directly for immunofluorescence microscopy after appropriate dilution or was affinity purified in some instances, as described by Leube et al. (1994).

Cultured cells were either fixed in methanol/acetone or with 3% formaldehyde for 20 minutes followed by 0.1% saponin treatment for 10 minutes and were processed for light and electron microscopy as described (cf. Leube et al., 1994). In some cases, cells were grown on coverslips precoated with a 0.1% (w/v) poly-L-lysine solution (Sigma), and for co-localization experiments secondary antibodies were pre-incubated with 5% low fat milk powder for 5 minutes.

Cell fractionation, gel electrophoresis and immunoblotting
Cultured cells were sometimes suspended directly in two times concentrated solubilization buffer (2% (w/v) SDS, 100 mM DTT, 0.001% (w/v) BPB, 10% (w/v) glycerol, 60 mM Tris-HCl, pH 6.8). Alternatively, cells were lysed in hypotonic buffer containing 10 mM triethanolamine (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT and 0.2 mM PMSF, and homogenized in a tight-fitting Dounce homogenizer by 30 up and down strokes. Nuclei were spun down at 1,000 g for 5 minutes resulting in pellet P1 and ‘postnuclear’ supernatant S1. The S1 fraction was again centrifuged for 5 minutes at 10,000 g resulting in pellet P2 and ‘postmicrosomal’ supernatant S2.

SDS-PAGE, blot transfer, and immunoblotting were essentially as described (cf. Leube et al., 1994). To increase sensitivity, an enhanced chemiluminescence system (Amersham Int.) was sometimes used following the procedures suggested by the manufacturer.

RNA analyses
RNA was purified, separated by electrophoresis through formaldehyde-containing gels, blotted onto nylon membranes, and hybridized with random prime labelled probes under stringent conditions essentially as described (Leube et al., 1988; Leube, 1994). In some instances, a digital recording system was used for documentation (PhosphorImagine™, Molecular Dynamics GmbH, Sunnyvale, CA).

RESULTS

Construction and transient expression of synaptophysin/connexin chimeras
For the identification of molecular domains involved in the targeting of rat synaptophysin and rat liver connexin32 in cDNA-transfected cells, genes were constructed that encoded chimeric polypeptides each with four complete TM domains, thereby maintaining the original membrane topology. Hybrid molecules composed of various connexin and synaptophysin segments should then aid in the identification of signals that are needed for protein targeting either to gap junctions or to cytoplasmic synaptophysin-rich microvesicles.

Fig. 1i depicts, schematically, the chimeric polypeptides whose distribution was examined in human hepatocellular carcinoma-derived PLC cells two days after cDNA transfection by immunofluorescence microscopy. As expected, wild-type rat synaptophysin (SR) was detected in multiple cytoplasmic dots (Fig. 1a) due to enrichment in small electron-translucent vesicles (Leube et al., 1989, 1994). Substitution of the synaptophysin amino terminus by the corresponding domain of rat liver connexin32 (chimera CSR5) did not alter the staining pattern notably (Fig. 1b). On the other hand, wild-type connexin32 (CR) was primarily targeted to regions of cell contact where it was localized in gap junctions (Fig. 1c; see also Eghbali et al., 1990). Substitution of the carboxy-terminal connexin32 domain by the synaptophysin carboxy terminus (chimera CSR1) led also to integration of transgenic polypeptides in plasma membrane clusters (Fig. 1f), although a significant amount of immunoreactivity was seen in cytoplasmic structures, most notable in strongly positive cells. The establishment of functional gap junctions was evident in Lucifer Yellow injection experiments of selected cell lines stably expressing CSR1, showing an increased coupling comparable to that observed for cells expressing the connexin-desmocollin 1a chimera Co-Dsc1a (data not shown; and Troyanovsky et al., 1994).

Surprisingly, all other mutants (CSR4, CSR3, CSR2, and SCSR1) containing TM segments from both synaptophysin and connexin32 showed a new type of distribution. The cytoplasmic immunofluorescence with pronounced perinuclear reactivity was localized along ‘tubular’ structures that formed a continuous network (Fig. 1c-e,h). Furthermore, a common observation in these experiments was the scarcity of positively stained cells, although the levels of RNA transcribed from the transfected gene constructs were as expected (Fig. 2, upper panel). In co-transfection experiments with a CAT-encoding vector, wild-type synaptophysin and chimeras CSR5 or CSR1 were seen in most CAT-positive cells while less than 4% of the CAT-positive cells were also positive for mutants CSR4, CSR3, CSR2 or SCSR1 (Fig. 2, lower panel). Yet, the detectability of mutant polypeptides by immunofluorescence microscopy indicated that the carboxy-terminally located antigenic epitope (for epitope characterization, see Knaus and Betz, 1990) was accessible and that consequently the complete mutants were synthesized in transfected cells.

Identification of the endoplasmic reticulum as a specific retention compartment for synaptophysin/connexin transmembrane chimeras
In order to learn more about the compartment(s) to which synaptophysin/connexin transmembrane chimeras were localized we stably transfected SK-Hep-1 cells, which are also derived from a human hepatocellular carcinoma and are known to contain only trace amounts or no connexin32 and synaptophysin (Eghbali et al., 1990; Leube, 1994), thereby minimizing possible contributions of endogenous connexin32 or synaptophysin to the distribution of chimeric proteins.

Representatively, one of the isolated cell clones (subline Hep-CS2-3) is described in detail here as it contained a large amount of RNA coding for chimera CSR2, exceeding that
produced by any other transgene or by the endogenous synaptophysin gene in neuroendocrine PC-12 cells (Fig. 3a). This high level of RNA expression was in sharp contrast to the low amount of protein that was barely detectable in the 10,000 g pellets from postnuclear supernatants (Fig. 3b). However, when cells were kept at 23°C overnight prior to analysis, a significant increase in the level of CSR2 was noticed in total cell lysates (Fig. 3c). The intracellular distribution was not affected by the temperature shift as judged from immunofluorescence microscopy (not shown) and the observation that most immunoreactivity was still pelleted at 10,000 g (compare Fig. 3d and e).

Chimera CSR2 was detected by immunofluorescence microscopy in a cytoplasmic network extending from the nucleus to the cell periphery of Hep-CSR2-3 cells (Fig. 4a and b), similar to that seen in transiently transfected PLC cells (Fig. 1e). By immunoelectron microscopy this network was resolved as a system of tubules that was in continuity with the outer nuclear membrane and contained the transgene product mostly in its expected membrane integration, i.e. with the epitope-carrying carboxy terminus facing the cytoplasm (Fig. 4c). Golgi apparatus, plasma membrane, and vesicles of various size and shape, were not labelled by antibodies (Fig. 4d). In co-localization experiments using antibodies against synaptophysin and the ER resident protein disulphide isomerase, it was possible to identify the compartment containing most, if not all, of the chimeric polypeptides as the ER (Fig. 5).

It was therefore concluded that the connexin32/synaptophysin chimera, CSR2, was specifically retained in the ER of Hep-CSR2-3 cells. Similarly, chimeras CSR4, CSR3 and SCSR1 co-localized with protein disulphide isomerase (not shown). Together, these observations indicate that combination of TM domains from connexin32 and synaptophysin results in ER retention and interference with targeting.

**Cytoplasmic end domains and topogenesis**

To examine in which way amino- and carboxy-terminal domains influence topogenesis, co-localization and co-transfection studies were performed (Fig. 6). Comparison of the distribution of wild-type synaptophysin to carboxy-terminal mutants showed a significant co-localization (Fig. 6a,a′ shows the results for mutant Sy-Dsg1 but the same localization was observed for SRDTmyc (Fig. 7) or Sy-Dsc1a; Troyanovsky et al., 1993). In the absence of endogenous synaptophysin such mutants were also detected in small cytoplasmic dots (e.g. see Fig. 7c; see also Troyanovsky et al., 1993). In addition, co-distribution was evident for the amino-terminal synaptophysin

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**Fig. 1.** Immunofluorescence microscopy detecting rat synaptophysin (a), rat liver connexin32 (g), and connexin32/synaptophysin chimeric proteins (b-h), after transfection of recombinant cDNA constructs into hepatocellular carcinoma-derived PLC cells (a-h) and schematic representation of expressed polypeptides (i). (a-h) Cells were fixed with methanol/acetone two days after transfection and reacted either with monoclonal synaptophysin antibody SY38 to detect transgenic polypeptides SR (a), CSR5 (b), CSR4 (c), CSR3 (d), CSR2 (e), CSR1 (f) and SCSR1 (h), or with a rabbit serum produced against the carboxy terminus of rat liver connexin32 to visualize CR (g). Texas Red-conjugated secondary antibodies were used in all instances. Note that the finely multipunctate reaction patterns in (a,b) and the patchy plasmamembrane labelling in (g, arrow in f) differ from the reactions seen in c-e and h. Bars, 20 μm. (i) Shows polypeptides (denotation on left-hand side) encoded by recombinant cDNAs. Synaptophysin parts are depicted as either white or black (TM regions) boxes whereas connexin32 parts are denoted by stippled light or dark (TM domains) areas. The relative position of either the last or first amino acid in each molecular segment is given according to the published sequences (Kumar and Gilula, 1986; Paul, 1986; Leube et al., 1987). Amino acids introduced by cloning procedures at junctions are written in bold italic capitals.
chimera CSR5 and SRDTmyc (Fig. 6b,b'). These observations suggest that neither the amino nor the carboxy terminus of synaptophysin is needed for localization to the abundant small, electron-translucent vesicles that were identified in the cytoplasm of synaptophysin-expressing PLC cells (Leube et al., 1989, 1994).

On the other hand, the carboxy-terminal connexin32 mutant CSR1 co-localized perfectly with wild-type connexin32 in co-transfection experiments (Fig. 6c,c'). However, in many cells expressing high levels of CSR1 a pronounced cytoplasmic staining was noted (Fig. 1f). By immunoelectron microscopy some of these cytoplasmic structures were identified as vesicles that were surrounded by two closely apposed unit membranes separated by an approx. 2 nm gap (not shown; see, for occurrence of similar structures, Larsen and Risinger, 1985). In addition, co-localization with antibodies directed against

**Fig. 2.** Determination of transfection efficiencies by northern blot hybridization (upper panel) and characterization of polypeptide expression by quantification of immunofluorescent cells (lower panel) two days after cDNA transfection of PLC cells. Upper panel: print-outs of digital recordings obtained from northern blots after hybridization with a mixed probe consisting of equal amounts of radioactively labelled fragments recognizing either synaptophysin or connexin32. In each lane approximately 10 µg total RNA was loaded after purification from cells transfected with constructs encoding SR (lane 1), CSR5 (lane 2), CSR4 (lane 3), CSR3 (lane 4), CSR2 (lane 5), CSR1 (lane 6), CR (lane 7) and SCSRI (lane 8). The relative positions of the 28 S and 18 S RNA are indicated on the left margin. Lower panel: histogram showing percentage of cells stained with CAT antibodies that were also reactive with monoclonal synaptophysin antibody SY38 two days after co-transfection of the CAT expression vector pSV2-cat together with eukaryotic expression vectors encoding SR (1), CSR5 (2), CSR4 (3), CSR3 (4), CSR2 (5), CSR1 (6) or SCSRI (8). Cells that were transfected with equal amounts of chimeric constructs and the pSV2-cat marker gene were analyzed by indirect immunofluorescence microscopy. CR (7) expression could not be determined in this way due to the lack of appropriate antibodies (n.d., not determined). In each case 400 CAT-positive cells were scored.

**Fig. 3.** Detection of RNA and polypeptides produced from cDNAs introduced into hepatocellular carcinoma-derived cells. (a) An autoradiograph of a northern blot hybridization. RNA (10 µg total RNA in all lanes) was extracted from SK-Hep-1 subclones stably expressing CSR2 (lane 1), CSR1 (lane 2; despite the low RNA level all cells stained strongly positive with antibody SY38) and SR (lane 3). For comparison, total RNA from neuroendocrine PC-12 cells was loaded in lane 4, resulting in the larger hybridizing mRNA transcribed from the endogenous rat synaptophysin gene in these cells. Arrowheads on the left-hand side denote relative positions of co-electrophoresed marker RNAs (from top to bottom: 4.4 kb, 2.4 kb, 1.4 kb, 0.24 kb). (b) Immunoblot of polypeptides contained either in the postnuclear (1,000 g) supernatant (lane 1) or the 10,000 g pellet (lane 2) of CSR2-expressing clone SK-Hep-CSR2-3 after separation by 12% SDS-PAGE. The blot was incubated with synaptophysin antibody SY38, detecting chimera CSR2, and the immunological reaction was visualized with the alkaline phosphatase detection system. (c) Immunoblot using SY38 and the enhanced chemiluminescence system to detect CSR2 in total cell lysates of SK-Hep-CSR2-3 that were grown either under standard conditions at 37°C (lane 1) or at 23°C for 16 hours prior to harvesting (lane 2). Positions of marker proteins co-electrophoresed with lanes shown in b and c are indicated on the right margin (from top to bottom: BSA, M_r 67,000; ovalbumin, M_r 45,000; glyceraldehyde-2-phosphate dehydrogenase, M_r 36,000; carbonic anhydrase, M_r 29,000; trypsinogen, M_r 24,000). (d,e) Comparative immunoblot analysis (same procedure as in c) of different protein fractions obtained from SK-Hep-CSR2-3 grown either at 37°C (d) or at 23°C for 16 hours (e; only half the amount of protein as in the comparable lanes in d was loaded). Lanes 1, 1,000 g pellets; lanes 2, 10,000 g pellets; lanes 3, 1,000 g supernatants; lanes 4, 10,000 g supernatants. Relative molecular mass markers and SDS-PAGE as in c.
endogenous vesicle populations showed that CSR1-positive cytoplasmic reactivity was not coincident with that for the plasma membrane recycling endosomal marker transferrin receptor (Fig. 6d,d′), but that it often co-localized with lysosomal membrane markers (Fig. 6e,e′). Furthermore, connexin32 mutants carrying the short myc epitope in place of the longer cytoplasmic tail were also targeted to cytoplasmic structures and gap junctions (not shown) in agreement with the reported occurrence of functional connexin32 and connexin43 tail mutants (Fishman et al., 1991; Werner et al., 1991; Dunham et al., 1992; Levine et al., 1993; Troyanovsky et al., 1994).

Taking all these observations together, I conclude that the cytoplasmic end domains of synaptophysin and connexin32 play only a minor role in topogenesis.

**Determination of the importance of transmembrane domains for topogenesis by deletion mutagenesis**

Transfection of further deletion mutants showed that, in
Fig. 6. Double immunofluorescence microscopy of methanol/acetone-fixed A431 (a,a') and PLC cells (b-e') two days after transfection with different cDNA constructs. Antibody reactions were visualized as in Fig. 5 and the same focal plane was maintained for each pair of micrographs. (a,a') Detection of wild-type synaptophysin with affinity-purified rabbit antibodies (a) and of synaptophysin/desmoglein chimera Sy-Dsg1 (cf. Troyanovsky et al., 1993) with monoclonal antibody Dg3.10 (a') in a cell of the synaptophysin-expressing clone A431-5S4 (Leube et al., 1994) after transfection with a Sy-Dsg1-encoding cDNA construct. Note that the distribution of both polypeptides is similar (arrows show some identical positions). (b,b') Co-localization of tailless synaptophysin SRDTmyc (see Fig. 7) using monoclonal anti-myc antibody MYC 1-9E10.2 (b) and chimera CSR5 (see Fig. 1) using rabbit synaptophysin antibodies (b') after cDNA co-transfection. Note the extensive similarities in both staining patterns (arrows). (c,c') Staining of cells after co-transfection with cDNA constructs encoding chimera CSR1 and CR (see Fig. 1) by reaction with monoclonal synaptophysin antibody SY38 to detect CSR1 (c) and rabbit connexin32 antibodies to detect CR (c'). Note that the immunoreactivity co-localizes in large membrane patches corresponding to gap junctions (arrows) and in cytoplasmic structures (arrowheads). (d,d') Double labelling of cells expressing high levels of CSR1 with rabbit antibodies against synaptophysin to detect CSR1 (d) and monoclonal transferrin receptor antibody B3/25 (d'). Note that the overall distribution is very different (structures only positive in d are marked by arrows; structures only positive in d' by arrowheads). (e,e') Immunofluorescence microscopy of cells transiently expressing CSR1 after reaction with rabbit antibodies against synaptophysin to detect CSR1 (e) and monoclonal antibody HA3 directed against the lysosomal membrane protein LAMP-1 (e'). Some vesicles positive in (e) and (e') are circled, those positive only in (e') are marked by arrowheads and structures only positive in (e) are labelled by arrows (upper arrows denote cytoplasmic vesicles, lower arrows gap junctions in the plasma membrane). Bars, 20 µm.
addition to the carboxy terminus, almost the entire intravesicular loop regions 1 and 2 could be excised from synaptophysin without interference with its sorting to cytoplasmic vesicles (mutants SRDIV1 and SRDIV2; Fig. 7a and b), although each of the excised segments contains a highly conserved pair of cysteine residues that form intramolecular disulfide bonds in the intact molecule (Johnston and Südhof, 1990). Similarly, cysteine residues in the exoplasmic loop domains of connexin32 do not affect transport to the cell surface (Dahl et al., 1991, 1992; for intramolecular disulfide bond formation, see Rahman and Evans, 1991).

On the other hand, deletion of TM domains resulted in retention of mutants in the ER. Again, hardly any transfected cells could be detected by immunofluorescence microscopy and only temperature reduction resulted in an increased number of positively stained cells in which the mutants could be localized in the ER (as an example, the distribution of mutant SRDTM3 is shown in Fig. 7d).

Identification of amino acid residues within the transmembrane regions critical for targeting
To identify amino acid residues within the TM domains that are critical for targeting of synaptophysin, point mutations were introduced and tested for their capacity to interfere with sorting. The selection of amino acids to be altered by site-directed mutagenesis was based on the observed contribution of charged amino acid and cysteine residues for assembly and transport of multimeric membrane proteins (Bonifacino et al., 1991; Cosson et al., 1991; Aoki et al., 1992; Rutledge et al., 1992; Fra et al., 1993) and of cysteine residues for covalent attachment to phospholipids (for review, see e.g. Olson, 1988;
Synaptophysin contains three such residues that are conserved between the ray fish Torpedo californica, rat, cow and man, and are also present in the related pantophysin (Leube, 1994); lysine 24 and cysteine 38 in TM domain 1, and aspartic acid 137 in TM domain 3.

Immunofluorescence microscopy of PLC cells expressing synaptophysin point mutants demonstrated that a cysteine to alanine mutation in TM domain 1 (mutant SR C/A38) or an aspartic acid to alanine mutation in TM domain 3 (mutant SR D/A137) did not notably affect the intracellular distribution (Fig. 8).

In contrast, several lysine 24 mutants exhibited an altered distribution. The most dramatic effect was observed for mutant SR K/L24 containing a lysine to leucine mutation. In routine transfections no cells were seen that could be stained by synaptophysin antibodies and only temperature reduction resulted in stabilization of the ER-localized mutant (Fig. 9a). Somewhat more variable effects were noted for mutant SR K/A24 containing a lysine to alanine alteration in the same position; again, a significant number of cells showed an ER-like distribution (Fig. 9b) although a cytoplasmic, ‘vesicular’ staining pattern was also seen in a minority of cells (Fig. 9c). A lysine to arginine mutation (SR K/R24) did not visibly change the distribution in comparison to wild-type synaptophysin (Fig. 9d), indicating that a positively charged amino acid is sufficient for correct topogenesis. Most complex were the immunofluorescence patterns obtained for mutant SR K/E24 in which lysine 24 was replaced by glutamic acid. Although this mutant showed a characteristic ER-like distribution in the majority of positively stained cells, it exhibited quite different patterns in other cells, including small dots often accumulated in the cell periphery (Fig. 9e) or large cytoplasmic dots (Fig. 9f). A similar critical contribution of certain amino acids within the TM domains of connexin32 is suggested by the recent identification of three families with the inherited Charcot-Marie-Tooth neuropathy, who carry point mutations in the connexin32 gene resulting in alterations of amino acids located in the third TM domain (Bergoffen et al., 1993; Ionasescu et al., 1994).

**DISCUSSION**

In an attempt to dissect the pathways and molecular principles that define the sorting of the polytopic TM proteins synaptophysin and connexin32 to specialized membrane domains, the ER was identified as the primary compartment in which several chimeras and mutants were retained. Exit from the ER and consequent targeting were shown to be dependent on the presence of all four TM domains of either molecule. It has been shown for other integral membrane proteins that single TM domains can determine targeting to the plasma membrane (Bonifacino et al., 1990), the nuclear pore (Wozniak and Blobel, 1992), the crystalloid ER (Jingami et al., 1987), the inner nuclear membrane (Smith and Blobel, 1993), or the Golgi apparatus (for review see Machamer, 1991; Nilsson and Warren, 1994). These TM segments assemble either with other proteins or with each other to form topogenic multimers that are retained in specific membrane domains (e.g. see Bonifacino et al., 1990; Cosson et al., 1991; Lemmon et al., 1992; Nilsson and Warren, 1994). Similar principles might guide the topogenesis of the multiple membrane-spanning proteins synaptophysin and connexin32, and the following events would be prerequisites for correct localization: (i) folding in the ER which is subject...
to the local quality control mechanisms; (ii) homo-oligomerization that may also occur in post ER compartments as reported for connexin43 (Musil and Goodenough, 1993); and (iii) cluster formation in a membrane patch with specific lipid composition in a certain location. Mutants have been identified that may have deficits in either one of these processes: connexin32/synaptophysin TM chimeras, synaptophysin TM deletion mutants and synaptophysin point mutants were retained in the ER (Figs 1c-e,h, 4, 5, 7d, 9a,b), certain synaptophysin lysine 24 point mutants could exit the ER but were localized in unusual cytoplasmic structures (Fig. 9e,f), and hybrids between connexin32 and the desmosomal cadherin desmoglein reached the cell surface but did not form gap junctions (Troyanovsky et al., 1993, 1994). Thus, topogenesis of the connexins and synaptophysins may rely on and be the result of proper folding, oligomerization and cluster formation. Our experiments indicate that the TM domains are the most important determinants for these events probably by formation of multiple contact points in the fully assembled multimer.

The tight association of the TM helices with each other and the phospholipid bilayer likely imposes specific sequence requirements on the molecules. The highest degree of sequence conservation among members of the synaptophysin and connexin multigene families in various species was indeed found in the TM domains (cf. Bennett et al., 1991; Kumar and Gilula, 1992; Leube, 1994). On the basis of the reported importance of paired charged amino acids for the stabilization and targeting of other intramembrane helices (Bonifacino et al., 1990, 1991; Cosson et al., 1991), it was assumed that such an interaction may occur between lysine 24 and aspartic acid 137 in synaptophysin. It was suggested that mutation of either one of these charged residues would result in a charge imbalance rendering the polypeptide incompetent to exit the ER. However, only the lysine 24 mutation (remarkably, this residue is not present in the related synaptoporin; Knaus et al., 1990) led to ER retention. Maybe mutation of lysine 24 resulted in exposure of retention and degradation signals while mutation of aspartic acid residue 137 did not.

The importance of the TM domains for topogenesis was further supported by the observations that neither the cytoplasmic ends nor the intravesicular loop domains were needed for exit from the ER. Although the TM regions appear to be sufficient for specific intracellular localization, non-membrane domains may modify and/or support this inherent topogenic information. We observed that some of the cells that were transfected with synaptophysin mutants lacking either intravesicular loop 1 or 2 did not exhibit the characteristic multipunctate immunofluorescence pattern but contained instead larger-appearing fluorescent structures possibly corresponding to cytoplasmic aggregates or, more likely, to lysosomes (data not shown). The interaction of the disulfide-linked intravesicular loop domains with a low molecular mass protein that was identified in synaptic vesicles (Johnston and Südhof, 1990) could be critical or protein stability may be affected by the spatial restrictions imposed by the shortened loop domains on the mature protein complex.

It is also remarkable that the carboxy termini could be deleted without abolishing topogenesis. Their unusual amino acid composition and several observations had suggested their participation in topogenesis: (i) the multiple tyrosine residues in the cytoplasmic carboxy terminus of synaptophysin have been proposed to act as signals for endocytosis (Linstedt and Kelly, 1991a). This could explain why synaptophysin localizes with endocytotic markers in cDNA-transfected non-neuroendocrine cells (Johnston et al., 1989a; Leube et al., 1989, 1994; Cameron et al., 1991; Linstedt and Kelly, 1991b), although it does not explain the segregation of synaptophysin into synaptophysin-rich vesicles in the hepatocellular carcinoma cells used in this study or in neuronal and neuroendocrine cells (Leube et al., 1989, 1994; Cameron et al., 1991; Linstedt and Kelly, 1991b; Régnier-Vigoroux et al., 1991; Maycox et al., 1992). The lack of significant co-localization of the connexin32/synaptophysin tail chimera CSR1 together with the transferrin receptor in cytoplasmic vesicles (Fig. 6d,d') is also in contrast with the function of the tyrosine residues as strong and specific endocytotic signals. (ii) The multiple tetrapeptide repeats in the carboxy terminus of synaptophysin may interact with certain cytoskeletal elements as has been shown in vitro for a 24 kDa membrane protein from Dicytostelium discoideum that contains similar repeats (Stratford and Brown, 1985; Noegel et al., 1990). (iii) Secondary factors which are likely to act on the accessible carboxy termini (e.g. by phosphorylation) are able to control topogenesis as shown for connexins (Musil and Goodenough, 1991; Hendrix et al., 1992; Krutovskikh et al., 1994). (iv) Dominant signals can override the capacity of the four TM domains to form clusters in specific membrane regions. We have constructed and expressed mutants in which such dominant signals were fused to the carboxy terminus of tailless connexin32 or synaptophysin and showed that the chimeras were either incapable of forming clusters (Troyanovsky et al., 1993) or were integrated into ‘foreign’ clusters (S. M. Troyanovsky, R. E. Leube and W. W. Franke, unpublished observations). Either way, it is important to note that the tailless deletion mutants SRD'Tmyc and Sy-Dsg1 were primarily detected in small cytoplasmic structures (Figs 6a-b', 7c) and were not arrested at the cell surface as implicated by Linstedt and Kelly (1991a), and that tailless connexin mutants are apparently integrated into functional gap junctions at the cell surface (Fig. 6c,c'; Fishman et al., 1991; Werner et al., 1991; Dunham et al., 1992; Levine et al., 1993; Troyanovsky et al., 1994). Therefore, the TM domains are sufficient for specific localization and topogenesis appears not to rely on extramembranous components.

The retention of chimeras in the ER may be coupled to degradation as judged from the difference between high mRNA and low polypeptide levels and the increased polypeptide accumulation at reduced temperatures. This pathway guarantees cellular homeostasis by enabling cells to dispose of abnormal or superfluous, predominantly integral membrane proteins, particularly those that are neither synthesized completely, nor properly modified, folded and assembled (for review see Klausner and Sítia, 1990; Bonifacino and Lippincott-Schwartz, 1991). Since all chimeras were completely synthesized and neither N-glycosylation of synaptophysin nor phosphorylation of connexin32 is a prerequisite for intracellular topogenesis (Leube et al., 1989; Werner et al., 1991), ER retention and degradation are likely due to incorrect folding and/or assembly. The retained connexin32/synaptophysin chimeras may expose molecular segments that act as retention and/or degradation signals. Conversely, these cryptic signals must be hidden in the completely folded molecule, which is then able to exit the ER.
Conclusion

In this report the exit from the ER was identified as the first sorting step in the topogenesis of synaptophysin and connexin32 in cDNA-transfected cells and the TM domains were shown to be primarily involved in this process. The fact that most of the mutants capable of leaving the ER were seen either in small cytoplasmic vesicles or in gap junctions suggests strongly that the ensuing sorting steps are driven by molecular signals that are also located in the TM regions through incorporation of homo-oligomeric subunits into extended membrane patches by lateral interaction and association.

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