Structure of Synaptogyrin (p29) Defines Novel Synaptic Vesicle Protein

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Abstract. Synaptogyrin (p29) is a synaptic vesicle protein that is uniformly distributed in the nervous system (Baumert et al., 1990). We have cloned and sequenced the cDNA encoding synaptogyrin, and the sequence predicts a protein with a molecular mass of 25,900 D with four membrane-spanning domains. The topology of the protein was confirmed by limited proteolysis using domain-specific antibodies. Database searches revealed several cDNA sequences coding polypeptides with sequence identities ranging from 32 to 46%, suggesting that synaptogyrin is a member of a multigene family. When the synaptogyrin cDNA is expressed in COS cells, the generated protein is indistinguishable from native synaptogyrin. To study intracellular sorting, synaptogyrin was expressed in CHO cells that revealed a punctate staining that was very similar to that of synaptophysin and endogenously expressed cellubrevin. Significant overlap with transferrin staining was also observed, suggesting that synaptogyrin is targeted to a recycling compartment involved in membrane traffic to and from the plasma membrane.

SYNAPTIC vesicles are storage organelles for neurotransmitters in nerve terminals. Upon arrival of an action potential, voltage-gated Ca^{2+}-channels open, and with a delay that may be as short as 200 μs, synaptic vesicles fuse with the presynaptic plasma membrane, discharging their transmitter content into the synaptic cleft. The membrane is then retrieved and used to regenerate exocytosis-competent synaptic vesicles, possibly involving passage through clathrin-coated vesicles (Maycox et al., 1992) and early endosomes as intermediate steps (for review see Jahn and Südhof, 1994; Bennett and Scheller, 1994).

Although neuronal exocytosis is highly specialized, many properties of the synaptic vesicle pathway resemble that of constitutive membrane traffic to and from the plasma membrane, which is an integral component of every eukaryotic cell (De Camilli and Jahn, 1990; Kelly, 1993). In fact, recent developments have shown that these similarities extend to the molecular steps underlying intracellular membrane fusion (Bennett and Scheller, 1994). It is presently understood that membrane fusion is mediated by specific protein families that are conserved from relatively simple eukaryotic cells such as yeast to highly differentiated cells such as neurons (Ferro-Novick and Jahn, 1994).

To achieve a comprehensive understanding of the molecular elements involved in membrane traffic, it is essential to characterize the protein constituents of intracellular trafficking organelles. These proteins not only define the identity of a trafficking organelle but also serve as the basis for essentially all functions an organelle must perform. Ideally, it should be possible to achieve a complete molecular description of a prototype trafficking organelle that can then be used as a model for vesicles functioning in different pathways of other cells. In the past few years, synaptic vesicles have served as such a prototype organelle. Due to their small size, the number of protein molecules an individual vesicle can carry is limited (see Jahn and Südhof, 1993 for further discussion). The protein composition of purified vesicles appears to be rather simple, containing only a few major polypeptides. Furthermore, synaptic vesicles can easily be purified in large quantities. For these reasons, their protein composition is better understood than that of any other trafficking organelle (for review see Jahn and Südhof, 1994; Bennett and Scheller, 1994; Südhof, 1995). In many cases, proteins identified first in synaptic vesicles were later found to be representatives of larger protein families that function in all cells (Zhong et al., 1992; McMahon et al., 1993; Leube, 1994; Li et al., 1995).

While the proteins responsible for neurotransmitter uptake can be studied easily using functional approaches, structural characterization of vesicular trafficking proteins has been an essential prerequisite to understand their function. Almost all of the vesicular trafficking proteins were identified before their roles were understood. Presently, well-founded working models have been established for most of their functions. Thus, the synaptobrevins are essential components of a presynaptic fusion complex that mediates exocytosis in concert with soluble protein factors

(Söllner et al., 1993), synaptotagmin is the leading candidate for the Ca^{2+}-receptor that is responsible for the fast exocytotic response upon Ca^{2+}-influx (Brose et al., 1992; Geppert et al., 1994a), and rab3A is involved in vesicle docking (Lledo et al., 1993; Geppert et al., 1994b). The synapsins are important regulators that facilitate synaptic vesicle membrane traffic but are not required for basic trafficking functions (Pieribone et al., 1995; Rosahl et al., 1995). Synaptophysin, the first of the vesicle proteins characterized in molecular terms, has been implicated recently in regulating the availability of synaptobrevin to enter the presynaptic fusion complex (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995).

For these reasons, it is very important to complete the molecular characterization of synaptic vesicle constituents. Previously, we have identified a membrane protein of synaptic vesicles with an apparent molecular mass of 29,000 D, referred to as p29 (Baumert et al., 1990). P29 is highly expressed in neurons and neuroendocrine cells where it is colocalized with synaptophysin on small synaptic vesicles and synaptic-like microvesicles, respectively. Like synaptophysin, p29 is phosphorylated on tyrosine residues. Furthermore, some antibodies (including two monoclonal IgM antibodies) raised against p29 showed weak cross-reactivity with synaptophysin, indicating some structural similarity. In contrast to synaptophysin, no evidence for N-linked glycosylation was found (Baumert et al., 1990).

Here we report the molecular characterization of p29, now renamed synaptogyrin (from Greek γυρος = circle, as a reference to the circular structure of synaptic vesicles). Furthermore, we have characterized its topology and sorting upon expression in nonneuronal cells. The protein has an overall structure similar to that of synaptophysin although the sequences are only distantly related. A search of databases containing human cDNA sequences revealed the existence of polypeptides homologous to synaptogyrin, suggesting that synaptogyrin is the first characterized member of a novel protein family.

Materials and Methods

Purification and Microsequencing of Synaptogyrin

Synaptic vesicles were purified as previously described (Nagy et al., 1976; Huttner et al., 1983; Hell and Jahn, 1994), using controlled pore glass bead chromatography as the last purification step. Peripheral membrane proteins were removed by incubation in 0.1 M Na_2HCO_3 at pH 11 for 15 min on ice. The proteins were separated by two-dimensional gel electrophoresis according to Mcfarlane (1989). The protein spot corresponding to p29 was identified by immunoblotting using a polyclonal antibody (Baumert et al., 1990) and excised from electrophoresis gels. For peptide microsequencing, protein spots from several gels were pooled and submitted to the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT). The protein in the Coomassie stained gel pieces were digested with trypsin in situ, and the resulting tryptic fragments eluted from the gel matrix. The tryptic fragments were purified using reverse phase HPLC and subjected to laser desorption mass spectrometry before peptide microsequencing (Williams and Stone, 1995). The peptide microsequences obtained were checked against various nucleotide and protein databases for potential matches, and none were found. Two peptide sequences (AGGAFDPYTLVR and DNPLNEGTDAA) were used to construct degenerate oligonucleotides for PCR.

cDNA Cloning and Sequencing of Synaptogyrin

The oligonucleotides were used to amplify a 400-bp cDNA sequence by

PCR from rat brain cDNA. This sequence served as a probe for screening a rat brain λZAP cDNA library purchased from Stratagene (La Jolla, CA). Four different clones were isolated, mapped and sequenced by subcloning the fragments into a M13 vector and sequencing the vector according to standard procedures using an automated DNA sequencer from Applied Biosystems, Inc. (Foster City, CA). All the clones overlapped in sequence, and the two longest clones contained an open reading frame that encodes a 234-amino acid protein. The coding region and most of the 5' and 3' untranslated regions were sequenced on both strands. A 940-bp EcoRI/XbaI insert containing the coding sequence and 20 bp of 5' untranslated sequence and 200 bp of 3' untranslated sequence was subcloned into a pCMV5 vector (Andersson et al., 1989) for transfection of fibroblasts.

Cell Culture and Expression of Synaptogyrin in CHO and COS Cells

Both the CHO and COS-7 cell lines were grown in DMET containing 10% heat-inactivated fetal calf serum, 10 mM Hepes, pH 7.4, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37°C in 10% CO_2. A cell line stably expressing synaptophysin (p38CHO; Johnston et al., 1989b) was grown in DMET supplemented with 10% heat-inactivated fetal calf serum, 20 mM Hepes, pH 7.4, 34 g/ml proline, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and maintained under constant selection with 700 μg/ml active G418 (GIBCO BRL, Gaithersburg, MD) at 37°C in 10% CO_2. Only during transfection experiments was media devoid of fetal calf serum, penicillin, streptomycin and G418 used.

COS-7 cells were transfected with the pCMV5-construct using the calcium phosphate precipitation method (Chen and Okayama, 1987). The cells were harvested using a rubber policeman and solubilized for 30 min on ice in 2% Triton X-100 in 20 mM Hepes, pH 7.4. The Triton X-100 insoluble material was pelleted at 900 g for 20 min. Aliquots of the pellet and the supernatant obtained from synaptogyrin-transfected COS cells and from untransfected control COS cells were analyzed by immunoblotting for synaptogyrin.

CHO cells were transfected using transfectamine liposomes (GIBCO BRL, Gaithersburg, MD). 8 μg DNA (see above) and 32 μl of transfectamine were added to 200 μl media lacking serum, penicillin, streptomycin, and G418. After incubation for at least 20 min the transfection mixture was added to the cells. The cells were incubated for 3–4 h before the mixture was replaced with normal media.

Immunocytochemistry

Cells were passed onto polyornithine-coated coverslips in 6-well plates the day after transfection, and allowed to grow for another 24–48 h. For transferrin labeling, the cells were allowed to internalize exogenously added iron-saturated human transferrin (100 μM final concentration) through receptor-mediated endocytosis in serum-free media added 25 min before fixing. For fixation, all cells were rinsed with PBS supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS*) and fixed for 40 min in 2.5% paraformaldehyde in PBS*. All subsequent blocking, incubation and washing steps were performed using a blocking solution of 1% bovine serum albumin and 0.03% saponin in PBS*. The cells were examined through a Zeiss-Axiophot epifluorescence microscope (Carl Zeiss Inc., Thornwood, NY) and photographed using Kodak Gold 100 ASA color print film. Confocal images were collected using a MRC-600 system attached to a Zeiss Axiovert compound microscope. The program ENHANCE was used on images as they were collected. For photo reproduction, image files were transferred to a Macintosh IIfx computer and arranged and annotated using Adobe Photoshop version 3.0 (Adobe Systems, Inc., Mountainview, CA) as software.

Antibodies

Rabbit IgG specific for human transferrin was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). The secondary antibodies used in the immunofluorescence experiments with CHO cells were anti-mouse FITC and Texas Red-conjugated goat antibodies, anti-rabbit dichlorotriazinylamino fluorescein (DTAF)[1] and indocarbocyanine (CY3) conjugated goat antibodies purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

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1. *Abbreviations used in this paper:* DTAF, dichlorotriazinylamino fluorescein; ECL, enhanced chemiluminescence.

The following antibodies have been described previously: rabbit serum directed against synaptogyrin (p29) (Baumert et al., 1990), rabbit serum directed against synaptophysin (Jahn et al., 1985), monoclonal antibody directed against the NH$_2$ terminus of synaptotagmin (clone Cl 604.4, Chapman and Jahn, 1994), rabbit serum directed against the first intravesicular loop of synaptophysin (p38-2; Johnston et al., 1989a), and rabbit serum directed against cellubrevin (McMahon et al., 1993). For synaptogyrin, we generated new polyclonal and monoclonal antibodies. Rabbit sera were raised against two synthetic peptides immobilized to keyhole limpet hemocyanine using standard procedures (Schneider et al., 1983). One of the peptides corresponded to the first predicted intravesicular loop (CIYNRNPNACSYG, residues no. 59–71), and the second corresponded to the cytoplasmic tail region (CQDYMDPSQDSSMPY, residues no. 182–196). These antibodies were affinity purified using peptide immobilized to thiopropyl Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's protocol. A third rabbit serum was raised using cytoplasmic tail region of synaptogyrin as antigen. For this purpose, residues no. 169–234 were expressed in bacteria as a GST-fusion protein and purified on GST-Sepharose according to the protocol provided by Pharmacia Biotech AB. Finally, a new IgG$_2$-secreting mouse hybridoma line was established (clone Cl 80.1) according to standard procedures (Köhler and Milstein, 1975; Jahn et al., 1985) using the same fusion protein as antigen.

Miscellaneous Procedures

Nerve terminals were isolated according to McMahon and Nicholls (1991). Pronase digestion of synaptic vesicles was performed as described by Johnston et al. (1989a). SDS-PAGE was performed according to Laemmli (1970) using a Bio-Rad Protean II minigel apparatus. Immunoblotting was carried out as described in Jahn et al. (1985) with the enhanced chemiluminescence (ECL) detection kit (Amersham Life Science, Buckinghamshire, England) or ^{125}I-conjugated protein A (Amersham Chemical Co., Arlington Heights, IL) as detection methods. The images in Figs. 3 and 5 were generated by scanning the autoradiographs with an Apple Color OneScanner (Apple Computer, Inc., Cupertino, CA) using the following software: Ofoto (Light Source Computer Images, Inc., Larkspur, CA), Adobe Photoshop (Adobe Systems, Inc., Mountainview, CA), and Canvas (Deneba Software, Miami, FL). The protein bands in the figure have not been altered, enhanced or otherwise manipulated compared with the original autoradiograph.

Results

Synaptogyrin Structure

To obtain peptide sequence from synaptogyrin, we pooled synaptic vesicles from several preparations, stripped them of membrane-associated proteins with carbonate buffer at pH 11, and separated the proteins by two-dimensional electrophoresis. The position of synaptogyrin was identified by immunoblotting. The corresponding spots were excised and subjected to proteolytic cleavage, followed by peptide purification and microsequencing.
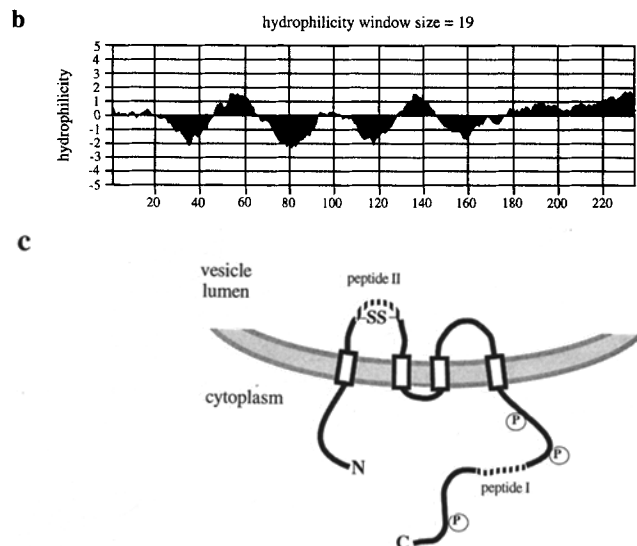
Based on the peptide sequences, a cDNA was cloned and sequenced. It contains an open reading frame predicting a protein of 234 amino acids with a molecular mass of 25,683 D (Fig. 1 a). The hydrophilicity plot (Fig. 1 b) revealed four hydrophobic stretches that fulfill the criteria for transmembrane domains. Thus, the sequence predicts that the protein spans the membrane four times, with the NH$_2$ and COOH termini on the cytoplasmic face of the vesicle (Fig. 1 c). This structure is reminiscent of synaptophysin. Sequence comparison revealed that the proteins are different (Fig. 2 b), with identities being confined to a few small clusters. However, the cytoplasmic tails of synaptophysin and synaptogyrin share an abundance of the amino acids tyrosine, glutamine, glycine, proline, and aspartate/glutamate, comprising 74 and 57%, respectively, of

Figure 1. (a) Nucleotide and amino acid sequence of synaptogyrin. The putative transmembrane domains are boxed, and the peptide sequences identified by microsequencing are underlined. (b) Hydrophobicity plot of synaptogyrin, plotted according to Kyte and Doolittle (1982). Note the four hydrophobic stretches that represent putative transmembrane domains. (c) Model of the topology of synaptogyrin in the vesicle membrane. P, phosphotyrosine. Note that the number and location of the phosphotyrosine residues are arbitrary and have not been determined. Both the NH$_2$ and the COOH termini are predicted to face the cytoplasm. The positions of the two peptides used to raise domain-specific antisera are included.

all residues. It is possible that small clusters of these amino acids are responsible for the cross-reactivity of some of the monoclonal antibodies (clones Cl 7.1 and 13.1) described previously (Baumert et al., 1990). Unlike in synaptophysin, however, these amino acids are not organized in repetitive sequences.

No consensus site for N-glycosylation was found in the synaptogyrin sequence, agreeing with our earlier observations that the protein is not glycosylated. The first intrave-

a

b
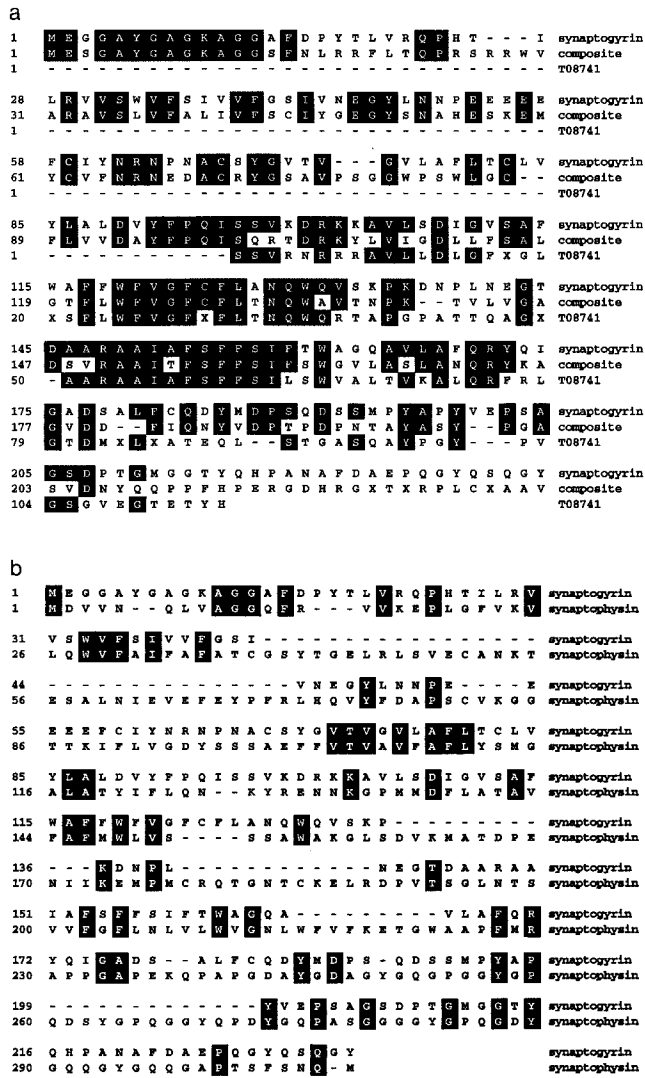
Figure 2. Amino acid sequence alignment of synaptogyrin with homologous proteins. (*a*) Alignment with translated cDNA sequences from the database of expressed sequence tags (dbest) derived from human infant brain and spleen/liver libraries (Adams et al., 1993). For alignment, cDNA sequences were translated to the closest fit with synaptogyrin that included internal frameshifts in the nucleotide sequences. The composite sequence represents a synthesis of five sequences (these sequence data are available from EMBL/Genbank/DDBJ accession numbers H20630, R61783, T26382, T77418, T96193, and T96194) that contain overlapping regions of high similarity suggesting that they are representing the same protein. Alignment was performed using the program Lasergene (DNAStar, Inc., Madison, WI) using the clustal method with a PAM250 residue weight table. (*b*) Alignment of synaptogyrin with rat synaptophysin. Alignment was performed using the same software and parameters as above.

sicular loop contains two cysteine residues that may form an intramolecular disulfide bond. Interestingly, a disulfide bond is present within both of the intravesicular loops of synaptophysin (Johnston and Südhof, 1990). It remains to be established which of the tyrosines are phosphorylated since both cytoplasmic termini of the protein contain tyrosine residues.

With exception of the limited similarity to synaptophysin,
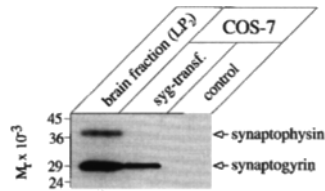
Figure 3. Comparison of native and recombinant synaptogyrin (expressed in COS-7 cells), demonstrating identical migration behavior of both proteins in SDS-PAGE. The figure shows an immunoblot of a brain fraction enriched in synaptic vesicles (*left lane,* see Huttner et al., 1983 for details) and of extracts of COS-7 cells transfected with synaptogyrin cDNA (*middle lane*) and of nontransfected COS-7 cells (*right lane*). COS cell homogenates were extracted with Triton X-100 and cleared by centrifugation before analysis (10 μg of protein/lane, detection by the enhanced chemiluminescence method). For immunodetection, a rabbit serum directed against p29 was used that was described earlier and that also reacts with synaptophysin due to synaptophysin contaminated antigen (Baumert et al., 1990).

no homologies to known proteins were found when various databases were searched for matches. Recently, however, a database was made public that contains human cDNA sequences generated by expressed sequence tags (Adams et al., 1993). Five cDNA sequences of this database showed significant homologies to synaptogyrin when translated into peptide sequences (Fig. 2 *a*). Sequence comparison revealed that they might encode two different human homologues of synaptogyrin. Thus, synaptogyrin could represent the first characterized member of a new protein family. Since two of these sequences were obtained from a liver-spleen library, it is possible that synaptogyrin isoforms are expressed in nonneuronal/nonendocrine cells.

To confirm that the cloned synaptogyrin cDNA encodes the entire p29 protein, we expressed the cDNA in COS-7 cells. The product was analyzed by SDS-PAGE and immunoblotting using a previously characterized anti-p29 rabbit serum (Baumert et al., 1990). As shown in Fig. 3, the serum reacted with a protein band that comigrates with native synaptogyrin.

Membrane Orientation of Synaptogyrin

To map the membrane topology of synaptogyrin, we generated a new set of antibodies that are specific for defined domains of the protein. First, two rabbit sera were generated using immobilized synthetic peptides as antigens that correspond to domains in the first intravesicular loop (residues no. 59–71, syg-loop peptide) and the cytoplasmic tail (residues no. 182-196, syg-tail peptide), respectively. Second, the COOH-terminal 65 amino acids, corresponding to the cytoplasmic tail of synaptogyrin (syg-tail), were expressed in *Escherichia coli* as a glutathione-*S*-transferase fusion protein. The purified fusion protein was used to generate a rabbit serum and a mouse monoclonal antibody (hybridoma line Cl 80.1, IgG₂). In a synaptosomal preparation, all antibodies react with a major band of an apparent molecular mass of 29,000 D comigrating with that recognized by our previous anti-p29 antibody (Fig. 4). With the exception of some minor immunoreactive bands recognized by the syg-loop peptide antibody, no cross-reactivity with other proteins was observed demonstrating that the antibodies are specific for synaptogyrin.
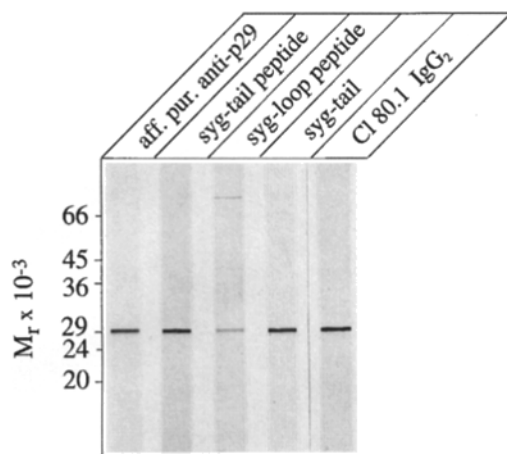
Figure 4. Characterization of antibodies raised against synaptogyrin fragments and peptides using immunoblot analysis of isolated nerve terminals (12.5 µg of protein/lane). (*Left to right*) Anti-p29 serum, affinity-purified (Baumert et al., 1990); syg-tail peptide and syg-loop peptide: rabbit sera raised against synthetic peptides corresponding to sequences in the COOH-terminal tail and in the first intravesicular loop, respectively, of synaptogyrin (all diluted 1:500); syg-tail: rabbit serum raised against the bacterially expressed COOH-terminal domain (dilution 1:20,000); Cl 80.1 IgG$_2$ monoclonal antibody raised against the same antigen as syg-tail.

A fraction enriched in synaptic vesicles was treated with the enzyme pronase for increasing amounts of time to progressively digest exposed cytoplasmic domains of synaptogyrin. The digests were then separated by SDS-PAGE and subsequently analyzed with domain-specific antibodies. As shown in Fig. 5 (*left*), protease treatment generated a fragment of synaptogyrin with an apparent mass of 18,000 D that was recognized by the loop peptide antibody but not by any of the tail-specific antibodies. Thus, the epitope recognized by the loop peptide antibody is protected from protease attack whereas the COOH terminus is not. This demonstrates that the COOH terminus is exposed on the cytoplasmic side of the vesicle whereas the epitope recognized by the loop peptide is facing the lumenal side, confirming the topology suggested in Fig. 1 c. Prolonged incubation in pronase did not result in further degradation of the 18,000-D fragment. This is in contrast to the digestion pattern of synaptophysin (Fig. 5, *middle* and *right*). Here, a corresponding loop peptide antibody (Johnston et al., 1989a) reveals the appearance of a larger fragment that is progressively degraded into a smaller fragment. The larger of them corresponds to a fragment devoid of both the NH$_2$ and COOH termini but containing all transmembrane domains and both intravesicular loops whereas the smaller fragment represents the first two transmembrane domains and the first intravesicular loop (Johnston et al., 1989a). Apparently, the small cytoplasmic loop between the second and the third transmembrane domains is more resistant to protease cleavage in synaptogyrin than in synaptophysin. This may be explained by the fact that in synaptogyrin the loop is predicted to be very short, comprising only 6–7 residues, compared to 9–10 residues in synaptophysin. That only cytoplasmic domains were digested was confirmed by an analysis of synaptotagmin, a

transmembrane protein with a different topology (Perin et al., 1991). Protease treatment generated a fragment that was recognized by a monoclonal antibody specific for the NH$_2$ terminus and that contains the transmembrane and lumenal domains of the protein (Fig 5, *far right*).

Expression of Synaptogyrin in Fibroblasts

In the following experiments, we transiently expressed synaptogyrin in fibroblasts (CHO cells) in order to study its sorting in nonneuronal cells. Previous work has established that in non-neuronal cell lines synaptophysin is sorted to an intracellular compartment that contains transferrin receptors and is involved in the recycling of transferrin between the plasmalemma and early endosomes (Johnston et al., 1989b; Leube et al., 1989; Clift-O'Grady et al., 1990). In addition, the protein cellubrevin seems to be present in the same compartment (McMahon et al., 1993). Recent work has demonstrated that the expression pattern of other synaptic vesicle proteins is different from that of synaptophysin. This is most conspicuous for synaptotagmin that is found predominantly on the plasma membrane of transfected cells (Feany and Buckley, 1993; Feany et al., 1993). For these reasons, we have compared the expression pattern of synaptogyrin with that of synaptophysin, cellubrevin, and transferrin.

Synaptogyrin cDNA was transfected into CHO cells and its expression pattern was compared to that of cellubrevin using double labeling immunocytochemistry. As shown in Fig. 6 (*top*), the staining pattern of both proteins was virtually identical as evidenced by double exposure. To further differentiate the localization of both proteins, double labeled cells were also analyzed by confocal microscopy (Fig. 6, *second panel*). As evident from the color overlay, the majority of both proteins are colocalized. However, some differential localization was observed in the periphery of the cells (note green and red puncta in the color overlay). To compare the localization of synaptogyrin with that of synaptophysin, we transfected synaptogyrin into a CHO cell line that stably expresses synaptophysin and that has been extensively characterized (Johnston et al., 1989b; Cameron et al., 1991). Both conventional immunofluorescence (not shown) and confocal microscopy (Fig. 6, *third panel*) indicated that synaptophysin and synaptogyrin distributed in an almost identical pattern. Labeling resulted in the staining of small puncta that are scattered throughout the cytoplasm and concentrated in a paranuclear region, probably representing the location of an endosomal compartment involved in recycling of receptors. No significant labeling of the plasma membrane was observed. Furthermore, we compared the labeling pattern of synaptogyrin with that of internalized transferrin (Fig. 6, *bottom*). Again, the confocal staining pattern was similar but the color overlay showed more pronounced differences between the compartments containing the two proteins. Both proteins are colocalized in the paranuclear region but in the cell periphery many fine puncta positive for synaptogyrin were devoid of transferrin, possibly due to the presence of synaptogyrin in late endosomes and in membranes of the Golgi apparatus which do not participate in transferrin recycling. Conversely, a minority of the larger puncta labeled for transferrin are negative for synaptogyrin, indi-
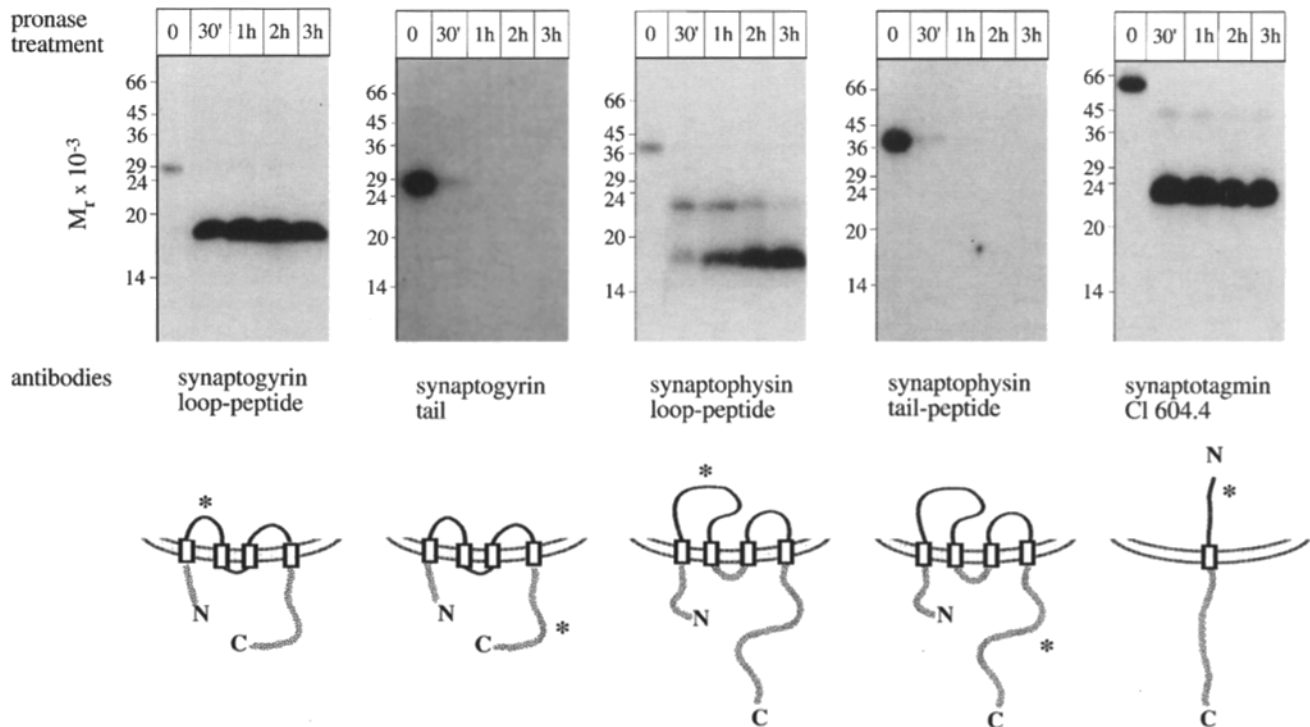
Figure 5. Mapping of the transmembrane orientation of synaptogyrin using limited proteolysis of synaptic vesicles followed by immuno-blot analysis of the fragments with side-specific antibodies. As reference, the partial digestion of synaptophysin and synaptotagmin, two integral membrane proteins of synaptic vesicles with an established orientation, was also monitored. The asterisks in the models indicate the position of the epitopes of the antibodies used for immunoblotting. Dotted lines in the models indicate regions expected to be de-graded by pronase. Note that only one fragment of synaptogyrin is generated the size of which indicates that the cytoplasmic loop be-tween transmembrane regions 2 and 3 is not cleaved. The size of all other fragments conform with the predictions of the models.

cating some microheterogeneity between the two proteins. We conclude that synaptogyrin and synaptophysin are cosorted to the same recycling compartment. This compartment overlaps to a large extent with cellubrevin and transferrin, indicating that it is involved in constitutive membrane traffic between the plasma membrane and early endosomes.

Discussion

In the present study, we have characterized synaptogyrin, a previously identified protein (p29) of synaptic vesicles. Our structural analysis revealed that despite some similar-ities to synaptophysin, synaptogyrin is a novel protein with no significant/sequence homology to other known pro-teins. Homologies to cDNA sequences in the expressed se-quence tag database indicate that it represents the first member of a new protein family.

Synaptogyrin represents a new addition to the growing list of proteins that contain four transmembrane domains, with both termini facing the cytoplasm, and that reside on intracellular post-Golgi compartments. The function of these proteins is still unclear. The best known member of this group is synaptophysin (Jahn et al., 1985; Südhof et al., 1987). It is the most abundant representative of a small protein family that also includes synaptoporin (Knaus et al., 1992), a second neuron-specific isoform, and HL-5 (also referred to as panthophysin; Zhong et al., 1992; Leube, 1994), an isoform that appears to be expressed in all tissues. Recently, it was shown that synaptophysin binds

Figure 6. Localization of synaptogyrin in CHO cells by immunofluorescence that transiently express the protein. For synaptogyrin (*syg*), monoclonal antibody Cl 80.1 was used. Note that not all cells express synaptogyrin. (*Top*) Comparison of the distribution of syn-aptogyrin (*syg, green channel*) and cellubrevin (*ceb, red channel*) using conventional immunofluorescence microscopy. Double labeling was performed using a polyclonal serum for cellubrevin. Double exposure shows that in the cells expressing synaptogyrin an almost identical staining pattern is obtained. (*Second panel*) Same as above (synaptogyrin, *red channel*, cellubrevin, *green channel*) except that the analysis was performed by confocal laser scanning microscopy. Note that in the color overlay (*right*) some differentiation between the two proteins is observed in the cell periphery. (*Third panel*) Comparison of the distribution of synaptogyrin (*syg, red channel*) and synaptophysin (*syp, green channel*, color overlay on the right), analyzed by confocal microscopy. For these experiments, a CHO cell line was used that stably expresses synaptophysin (Johnston et al., 1989b). Note that the staining patterns are very similar in the cells ex-pressing both proteins (not all of the synaptophysin-containing cells express synaptogyrin). (*Bottom*) Double labeling of cells that were preincubated with iron-saturated transferrin for synaptogyrin (*syg*) and transferrin (*tfr*), analyzed by confocal microscopy. Note that the staining pattern for transferrin overlaps only partially with that of synaptogyrin, with the overlap being concentrated in a paranuclear spot, as expected for an endosomal location. Bars: (*top*) 9 μm; (*second panel*) 10 μm; (*third panel*) 14 μm; (*bottom*) 9 μm.
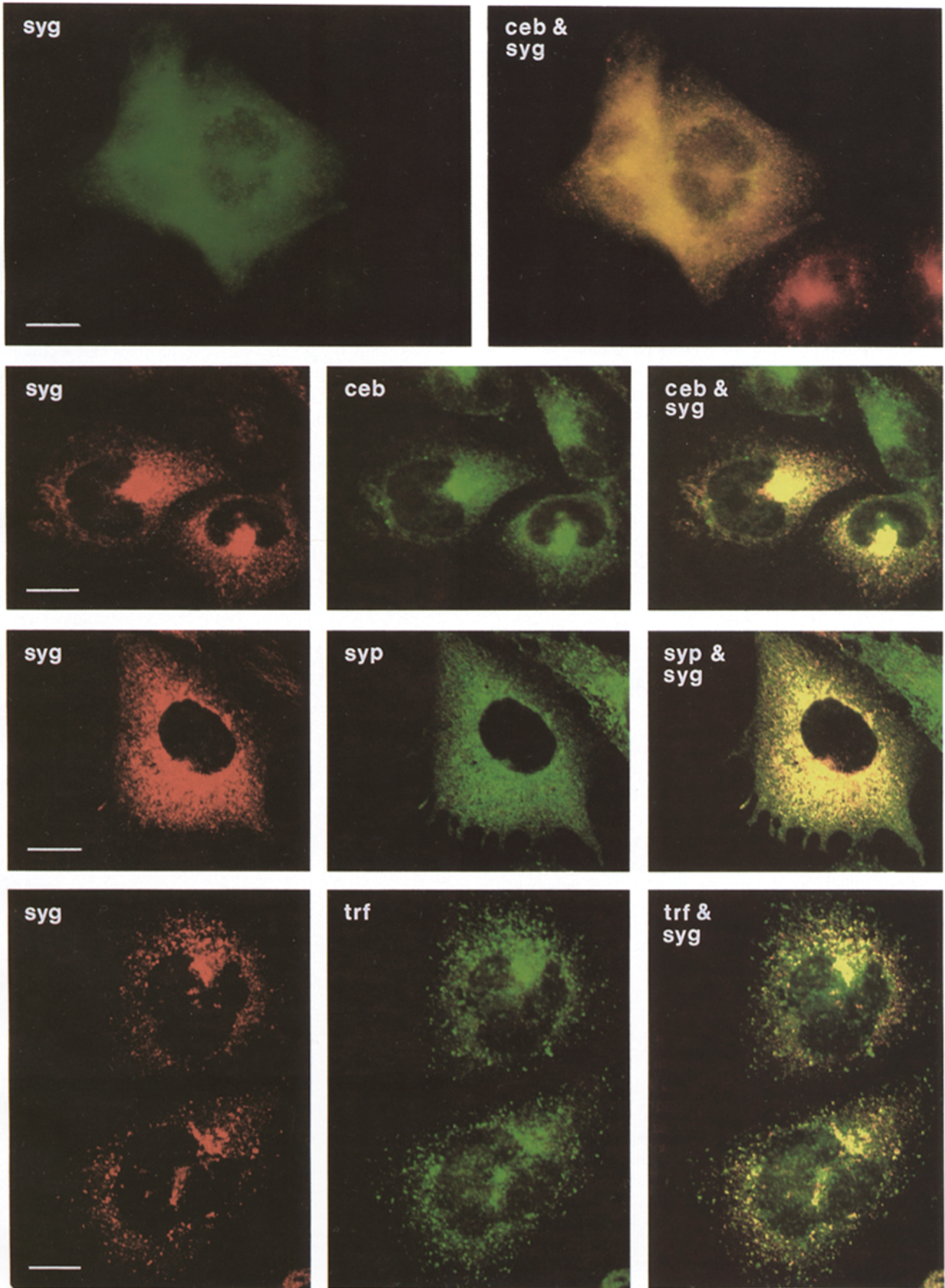
to synaptobrevin suggesting a role in the control of the exocytotic fusion machine (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995). Another vesicle protein of this category is SCAMP (secretory carrier membrane protein), a membrane protein expressed in all tissues that probably has a similar topology but virtually no homology to synaptophysin. It contains putative metal binding domains and regions with high potential for forming amphiphilic helices (Brand et al., 1991; Brand and Castle, 1993). Peripherin/rds, a protein confined to the highly curved rim of the discs in the outer segments of photoreceptors (Molday et al., 1987; Connell and Molday, 1990; Arikawa et al., 1992) has a similar topology and may be involved in stabilizing areas of high membrane curvature. Since there is no significant sequence homology between these protein families it remains to be established whether these proteins, at least in part, have analogous functional roles on their respective trafficking organelle, or whether they are functionally divergent. In addition to their membrane topology, they share a number of similarities that are intriguing: (a) all of these proteins have similar molecular masses, ranging from a molecular mass of 25,000 to 39,000 daltons; (b) with the exception of SCAMP, all proteins contain even numbers of cysteine residues in the lumenal domains suggesting that they form intramolecular disulfide bonds (demonstrated for synaptophysin; Johnston and Südhof, 1990); (c) all proteins reside on highly curved organelles (most conspicuously for peripherin/rds) which led to the suggestion that they may confer stability to highly curved membrane regions; and (d) with the exception of peripherin/rds, all proteins have nonneuronal counterparts suggesting that they are basic elements of constitutive and regulated intracellular membrane traffic.

Previously, we reported that synaptogyrin is selectively expressed in neurons and neuroendocrine cells (Baumert et al., 1990). We have reexamined the tissue-distribution of synaptogyrin using protocols that enrich for membrane proteins and thus allow the detection of very low protein levels (see e.g., McMahon et al., 1993). This was prompted by the observation that cellubrevin, a nonneuronal isoform of synaptobrevin, is expressed at much lower levels in nonneuronal cells and was overlooked in our initial screen using a cross-reacting antibody. No detectable amounts of synaptogyrin protein were found in nonneuronal tissues, including liver and a variety of cell lines (unpublished observations). Although our assay would have revealed synaptogyrin levels at least two orders of magnitude less than in the brain, we cannot exclude the presence of very low levels of synaptogyrin as the case is with synaptobrevin I and II (Ralston et al., 1994). In fact, synaptogyrin was also cloned from commercially available liver and thymus cDNA libraries (unpublished observations). We therefore performed Northern blots to determine the RNA expression levels in nonneuronal tissues. A strongly expressed message of ~4 kb was found in brain. Long exposures of the blot showed less abundant messages in other nonneuronal tissues (heart, spleen, lung, liver, skeletal muscle, kidney, and testes, unpublished observations). Therefore, synaptogyrin expression on the RNA level is not restricted to neuronal tissues although the protein levels, if at all present, are very low. However, the existence of related cDNA sequences in a human database derived from nonneuronal

tissues suggests that synaptogyrin, like synaptophysin, has nonneuronal isoforms. Thus it is possible that synaptogyrin represents a group of proteins that is present on trafficking organelles in all tissues.

When synaptogyrin was coexpressed with synaptophysin in fibroblasts, both proteins were distributed in a very similar fashion. Furthermore, synaptogyrin largely overlapped with endogenous cellubrevin and, albeit to a lesser extent, with endocytosed transferrin. Thus, the sorting of synaptogyrin is very similar to that of synaptophysin in nonneuronal as well as in neuronal cells. This agrees with our previous observation that synaptogyrin, like synaptophysin, has not been detected on large dense-core vesicles in the CNS or chromaffin granules, in contrast to other vesicle proteins such as synaptobrevin and synaptotagmin (Baumert et al., 1990; De Camilli and Jahn, 1990). Thus it appears that both proteins share common sorting signals that direct them to an identical subset of cellular organelles.

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