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 Ca2+-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 \(\gamma\)\(\rho\)\(\omega\)\(\nu\) = 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 protein spots were removed by incubation in 0.1 M Na\(_2\)HCO\(_3\) at pH 11 for 15 min on ice. The proteins were separated by two-dimensional gel electrophoresis according to Mefarlane (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, peptide 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 was 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 according to Mcfarlane (1989). The protein spot corresponding to p29 was digested with trypsin in situ, and the resulting tryptic fragments were transferred to a Macintosh IIfx computer and arranged and annotated using the program PSD-600 and SeaView (Carl Zeiss Inc., Thornwood, NY) and photographed using Kodak Gold 100 ASA color print film. Confoocal images were collected using a MRC-600 system attached to a Zeiss Axiosvert 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 antirabbit FITC and Texas Red-conjugated goat antibodies, anti-rabbit dcholorotrizinylamino fluorescein (DTAF) and indocarbocyanine (CY3) conjugated goat antibodies purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

1. Abbreviations used in this paper: DTAF, dichlorotrizinylamino 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 NH2 terminus of synaptogyrin (clone CI 604 A, Chapman and Jahn, 1994), rabbit serum directed against the first intravesicular loop of synaptophysin (p38-2; Johnston et al., 1989a), and rabbit body directed against the NH2 terminus of synaptotagmin (clone C1 604.4, serum directed against synaptophysin (Jahn et al., 1985), monoclonal antibody directed against synaptogyrin (p29) (Baumert et al., 1990), rabbit limpet hemocyanine using standard procedures (Schneider et al., 1983). 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 (CYNRRNPNACSYG, residues no. 59-71), and the second corresponded to the cytoplasmic tail region (CODYMPSQDSSMPY, 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 IgG2-secreting mouse hybridoma line was established (clone CI 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 Protein 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 [125I]-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 Genetix (Denysa Software, Miami, FL). The protein bands in the figures 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 hydrophilic stretches that fulfill the criteria for transmembrane domains. Thus, the sequence predicts that the protein spans the membrane four times, with the NH2 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 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 CI 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 intravesicular loop of synaptophysin (p38-2; Johnston et al., 1989a) and rabbit body directed against the NH2 terminus of synaptotagmin (clone CI 604 A, Chapman and Jahn, 1994), rabbit serum directed against the first intravesicular loop of synaptophysin (p38-2; Johnston et al., 1989a), and rabbit body directed against the NH2 terminus of synaptotagmin (clone C1 604.4, serum directed against synaptophysin (Jahn et al., 1985), monoclonal antibody directed against synaptogyrin (p29) (Baumert et al., 1990), rabbit limpet hemocyanine using standard procedures (Schneider et al., 1983).
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 CI 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.
and in the first intravesicular loop, respectively, of synaptogyrin peptides corresponding to sequences in the COOH-terminal tail of syg-loop 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 and syg-loop peptide: rabbit sera raised against synthetic peptides corresponding to sequences in the COOH-terminal tail of synaptogyrin. The digests were then separated by SDS-PAGE and subsequently analyzed with domain-specific antibodies.

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 plasma membrane 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 immunoblot 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 degraded by pronase. Note that only one fragment of synaptogyrin is generated the size of which indicates that the cytoplasmic loop between transmembrane regions 2 and 3 is not cleaved. The size of all other fragments conform with the predictions of the models.

Discussion

In the present study, we have characterized synaptogyrin, a previously identified protein (p29) of synaptic vesicles. Our structural analysis revealed that despite some similarities to synaptophysin, synaptogyrin is a novel protein with no significant sequence homology to other known proteins. Homologies to cDNA sequences in the expressed sequence 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...
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 luminal 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 overlapped 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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