

# P29: A Novel Tyrosine-phosphorylated Membrane Protein Present In Small Clear Vesicles of Neurons and Endocrine Cells

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**Abstract.** A novel membrane protein from rat brain synaptic vesicles with an apparent 29,000  $M_r$  (p29) was characterized. Using monospecific polyclonal antibodies, the distribution of p29 was studied in a variety of tissues by light and electron microscopy and immunoblot analysis. Within the nervous system, p29 was present in virtually all nerve terminals. It was selectively associated with small synaptic vesicles and a perinuclear region corresponding to the area of the Golgi complex. P29 was not detected in any other subcellular organelles including large dense-core vesicles. The distribution of p29 in various subcellular fractions from rat brain was very similar to that of synaptophysin and synaptobrevin. The highest enrichment occurred in purified small synaptic vesicles. Outside the nervous system, p29 was found only in endocrine cell types specialized for peptide hormone secretion. In these cells, p29 had a distribution very similar to that of synaptophysin. It was associated with

microvesicles of heterogeneous size and shape that are primarily concentrated in the centrosomal-Golgi complex area. Secretory granules were mostly unlabeled, but their membrane occasionally contained small labeled evaginations. Immunoprecipitation of subcellular organelles from undifferentiated PC12 cells with anti-synaptophysin antibodies led to a concomitant enrichment of p29, synaptobrevin, and synaptophysin, further supporting a colocalization of all three proteins.

P29 has an isoelectric point of  $\sim 5.0$  and is not *N*-glycosylated. It is an integral membrane protein and all antibody binding sites are exposed on the cytoplasmic side of the vesicles. Two monoclonal antibodies raised against p29 cross reacted with synaptophysin, indicating the presence of related epitopes. P29, like synaptophysin, was phosphorylated on tyrosine residues by endogenous tyrosine kinase activity in intact vesicles.

CHEMICAL signaling between neurons is mediated by regulated exocytosis of secretory vesicles that contain quanta of neurotransmitters. At least two types of secretory vesicles coexist in neurons: small synaptic vesicles (SSVs),<sup>1</sup> which contain only nonpeptide neurotransmitters, and large dense-core vesicles (LDCVs), which contain neuroactive peptides, other polypeptides, and some nonpeptide transmitters. The molecular mechanisms involved in the biogenesis of these organelles and in their life cycles, including exocytosis, are still largely unknown (for review see Reichardt and Kelly, 1983; Kelly, 1988; De Camilli and Jahn, 1990). To understand these processes in more detail, it is essential to obtain detailed information about the properties of the protein components present in their membranes.

Recently, several proteins have been identified in mammalian brain that are associated with virtually all SSVs independent of their neurotransmitter content (De Camilli and

Jahn, 1990). These include the synapsins—a family of homologous proteins associated with the cytoplasmic surface of the vesicles (De Camilli and Greengard, 1986)—and several integral membrane proteins—synaptophysin (Jahn et al., 1985; Wiedenmann and Franke, 1985), synaptobrevin (Baumert et al., 1989; Südhof et al., 1989a; [the two isoforms are also referred to as VAMP 1 and VAMP 2] Trimble et al., 1988; Elferink et al., 1989), p65 (Matthew et al., 1981), and SV2 (Buckley and Kelly, 1985). Some of these proteins have been thoroughly characterized, and the primary structure of the synapsins (Südhof et al., 1989b), synaptophysin (Leube et al., 1987; Südhof et al., 1987; Buckley et al., 1987), and synaptobrevin (Südhof et al., 1989a; Trimble et al., 1988; Elferink et al., 1989) has been elucidated.

In neurons, the synapsins, synaptophysin, and synaptobrevin are selectively concentrated on SSVs (Navone et al., 1984, 1986; Baumert et al., 1989). Outside the nervous system, the synapsins are largely absent, whereas synaptophysin, synaptobrevin, p65, and SV2 are present in certain endocrine tissues. In these tissues, the subcellular localization of

1. *Abbreviations used in this paper:* LDCV, large dense-core vesicle; SSV, small synaptic vesicle.

synaptophysin was studied in detail (Wiedenmann and Franke, 1985; Navone et al., 1986). It is associated with a population of pleomorphic microvesicles (synaptic-like microvesicles) and absent from the membrane of secretory granules (Navone et al., 1986; Wiedenmann et al., 1988; Johnston et al., 1989b). These microvesicles are concentrated at microtubule-organizing centers at the *trans*-side of the Golgi complex, can fuse with the plasmalemma, and are labeled by extracellular tracers. They most probably represent recycling organelles (Navone et al., 1986; Johnston et al., 1989b). Based on these findings, we have suggested that synaptic-like microvesicles in endocrine cells are related to SSVs in neurons (Navone et al., 1986; De Camilli and Jahn, 1990).

In the present study, we report the characterization of a new membrane protein of 29,000 *M<sub>r</sub>*, present in SSVs. The protein is immunologically related to synaptophysin, undergoes endogenous tyrosine phosphorylation in SSV fractions, and is expressed by both neurons and endocrine cells.

## Materials and Methods

### Materials

Nitrocellulose membrane filters (pore size 0.2  $\mu\text{m}$ ) were obtained from Schleicher & Schuell, Inc. (Dassel, FRG).  $^{125}\text{I}$ -labeled donkey anti-rabbit Ig (3.7 MBq/ml) and [ $\gamma$ - $^{32}\text{P}$ ]ATP (37 MBq/ml) was purchased from Amersham Buchler GmbH (Braunschweig, FRG). Affinity-purified goat anti-mouse IgG, coupled to horseradish peroxidase, was from Bio-Rad Laboratories (Munich, FRG). Rhodamine-conjugated goat anti-rabbit IgGs were from Organon Teknika (Malvern, PA); colloidal gold-conjugated goat anti-rabbit IgGs (GAR 5) was from Janssen Pharmaceutica (Olen, Belgium); and monoclonal anti-phosphotyrosine antibody was from Boehringer Mannheim GmbH (Mannheim, FRG). Protein A-colloidal gold complexes were prepared as described in Slot and Geuze (1983). Ampholines (pH 3.5–10 and pH 5–7) were from Pharmacia Fine Chemicals (Freiburg, FRG); recrystallized sodium cholate was from Calbiochem-Behring Corp. (Frankfurt, FRG); and peptide *N*-glycosidase F (*N*-Glycanase) was from Genzyme (Boston, MA). Eupergit C1Z beads (1  $\mu\text{m}$  diameter) were purchased from Röhm Pharma (Darmstadt, FRG). PC12 cells (C 251) were kindly provided by Drs. G. D. Borasio and H. Thoenen (Max-Planck-Institute for Psychiatry, Martinsried, FRG). All other reagents were of analytical grade and obtained from standard commercial sources.

### Immunochemical Techniques

Monoclonal and polyclonal antibodies were generated essentially as described earlier, using p29 as an antigen which was purified by preparative electrophoresis (Baumert et al., 1989). The polyclonal rabbit serum was affinity-purified using p29 excised from preparative immunoblots as immunoadsorbent (Olmstedt, 1987). Monoclonal and polyclonal antibodies against synaptophysin and synaptobrevin were prepared as described (Jahn et al., 1985; Baumert et al., 1989).

For the preparation of immunobeads, antibodies were immobilized on nonporous methacrylate microbeads using a recently developed procedure (Burger et al., 1989). For coupling of IgM-antibodies (C1 13.1), the protocol was slightly modified. IgM was enriched from ascites by fractionated ammonium sulfate precipitation (30–50%) and extensively dialyzed against 150 mM NaCl. 500 mg of Eupergit C1Z methacrylate microbeads were washed once with water and incubated with 10 mg IgM (final concentration 1 mg/ml) for 45 min at 20°C. Inactivation of remaining binding sites and washing was performed as described (Burger et al., 1989).

For immunoprecipitation of p29, synaptic vesicle protein (final concentration 0.4 mg/ml) was solubilized in 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, containing 1% (wt/vol) sodium cholate. The extract was incubated for 30 min on ice and spun for 20 min at 50,000 rpm in a Beckman Instruments, Inc. (Fullerton, CA) TLA 100.2 rotor. The supernatant was removed, and 20  $\mu\text{l}$  C1 13.1 immunobeads were added followed by end-over-end rotation for 90 min at 4°C. Thereafter, the beads were sedimented by centrifugation at 12,000 *g* for 30 s, washed three times with solubilization buffer, and finally resuspended in electrophoresis sample buffer.

### Subcellular Fractionation

Synaptic vesicles were prepared essentially as described earlier (Nagy et al., 1976; Huttner et al., 1983; Jahn et al., 1985). Briefly, cerebral cortices of 20 rats were homogenized in 150 ml of sucrose buffer (0.32 M sucrose, 0.1 mM PMSF, 2  $\mu\text{g}/\text{ml}$  pepstatin A). The homogenate (H) was spun for 10 min at 800 *g*, yielding a pellet (P<sub>1</sub>). The supernatant was centrifuged for 15 min at 9,200 *g*. The resulting pellet was washed once with sucrose buffer, yielding a crude synaptosomal pellet (P<sub>2</sub>). The supernatant was centrifuged at 165,000 *g* for 1 h, yielding pellet P<sub>3</sub> and supernatant S<sub>3</sub>. P<sub>2</sub> was resuspended in 12 ml of sucrose buffer and lysed by addition of 9 vol of distilled water, followed by homogenization. Hepes/NaOH buffer was added to a final concentration of 10 mM at pH 7.4. After 30 min of incubation, the fraction was spun at 25,000 *g* for 20 min (pellet LP<sub>1</sub>), and the supernatant was further centrifuged for 2 h at 165,000 *g*, yielding a crude synaptic vesicle pellet (LP<sub>2</sub>) and a supernatant (LS<sub>2</sub>). Vesicles were further purified by centrifugation of LP<sub>2</sub> on a continuous sucrose density gradient, yielding fraction SGV. This was chromatographed on controlled pore glass beads, resulting in purified synaptic vesicles (fraction CPG). For preparative purposes, the preparation method described by Hell et al. (1988) was used.

Synaptophysin-containing microvesicles were isolated from PC12 cells using a modification of the recently described immunoisolation procedure for synaptic vesicles (Burger et al., 1989). Cells were grown under standard conditions (Greene and Tischler, 1976). After harvesting, cells were collected by centrifugation ( $\sim 3 \times 10^7$  cells) and homogenized in 0.5 ml of 0.32 M sucrose, 3 mM imidazole, pH 7.4, 0.1 mM PMSF, 2  $\mu\text{g}/\text{ml}$  pepstatin A by repetitively passing through a 27-gauge needle. The homogenate was adjusted to 1 ml and centrifuged for 10 min at 10,000 *g*. To 1 ml of supernatant, 50 mg of C 7.2 immunobeads were added as a 100- $\mu\text{l}$  solution in 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, and incubation was allowed to proceed for 1 h at 4°C under slow rotation. Beads were collected by centrifugation for 10 min at 320 *g* and washed three times in 0.32 M sucrose, 10 mM Tris-Cl, pH 7.4, 0.1 mM PMSF, 2  $\mu\text{g}/\text{ml}$  pepstatin A. The final pellet was incubated for 15 min in 100  $\mu\text{l}$  of the washing solution containing 2% SDS. The beads were removed by centrifugation, and an aliquot of the supernatant was used for SDS-PAGE and immunoblotting.

### Immunocytochemistry

For light microscopy immunocytochemistry, tissue fixation, preparation of 10- $\mu\text{m}$ -thick frozen tissue sections, and immunofluorescence staining were performed as described (De Camilli et al., 1983a; Navone et al., 1986). Immunogold labeling of agarose-embedded homogenates of bovine tissues (hypothalamus, anterior pituitary, and adrenal medulla) and of rat brains, was performed as described (De Camilli et al., 1983b; Navone et al., 1986). A lytic fixation method was used for the brain tissues (to open revealed nerve endings) and an isoosmotic fixation method for the endocrine tissues (for a discussion of lytic and nonlytic fixation see De Camilli et al., 1983b). Unless indicated otherwise, affinity-purified rabbit antibody directed against p29 was used.

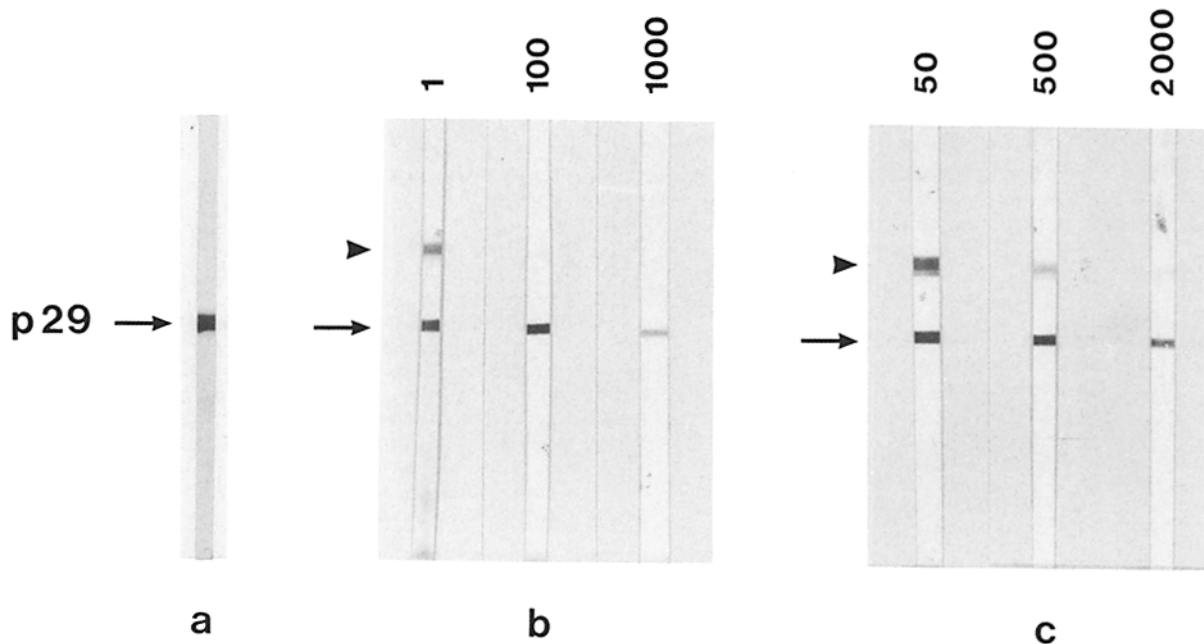
### Other Analytical Techniques

SDS-PAGE was performed according to Laemmli (1970). Immunoblotting was carried out according to Towbin et al. (1979), using either radioiodinated (dilution 1:200) or horseradish peroxidase-coupled secondary antibodies as detection systems (Jahn et al., 1985). Phase partition in Triton X-114 was carried out according to Bordier (1981). Phosphorylation of vesicle proteins was performed essentially as described by Hirano et al. (1988). Protein was determined either by the method of Bradford (1976) or by a modified Lowry procedure (Peterson, 1977).

## Results

### Preparation and Characterization of Antibodies Directed against p29

Electrophoretic analysis of membrane proteins extracted from a highly purified SSV fraction revealed the presence of a protein band with an apparent 29,000 *M<sub>r</sub>* (not shown; see Baumert et al., 1989). This protein band, referred to as p29, was purified by preparative electrophoresis and used for immunization of a rabbit and mice.



**Figure 1.** Characterization of polyclonal and monoclonal antibodies directed against p29 by immunoblotting. 2  $\mu$ g of synaptic vesicle protein per lane was separated by SDS-PAGE on a 12% gel and blotted onto nitrocellulose filters. (a) Labeling with rabbit antibody (affinity purified; see Materials and Methods) diluted 1:200. (b) Dilution series of monoclonal antibody C1 7.1 (unprocessed ascites fluid). (c) Dilution series of monoclonal antibody C1 13.1 (unprocessed ascites fluid). In b and c, the dilution factor of the ascites ( $\times 10^{-2}$ ) is indicated on top of each lane. For visualization, the strips were subsequently incubated with a species-specific anti-Ig antibody (linked to horseradish peroxidase) followed by development with 4-chloro-1-naphthol. Arrowheads in b and c point to the position of an additional immunoreactive band of 38,000  $M_r$  (synaptophysin; see text).

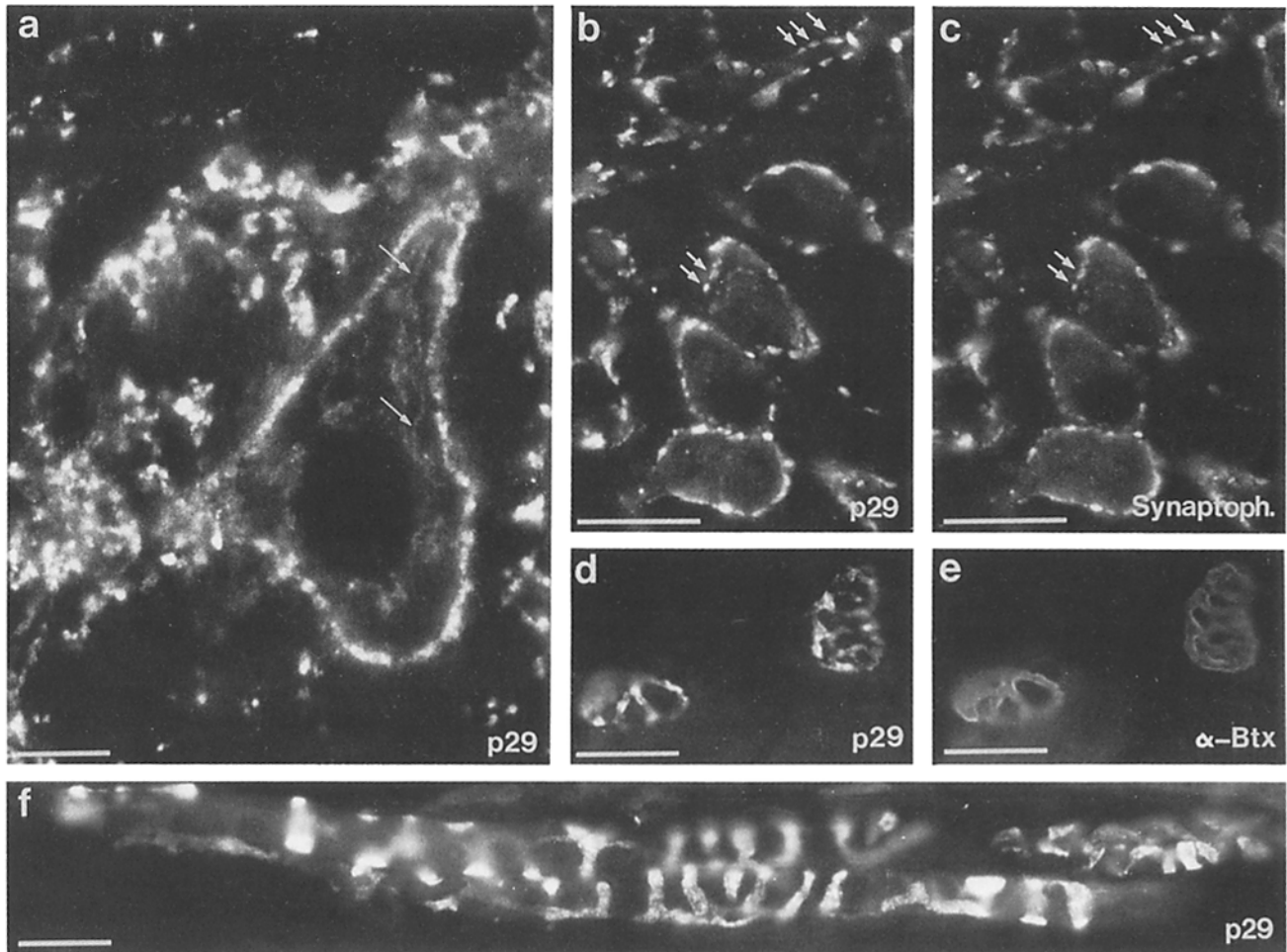
Rabbit polyclonal antibodies were affinity purified from serum by blot adsorption. The antibodies reacted with a single band of an apparent 29,000  $M_r$  in an immunoblot of vesicle proteins (Fig. 1 a). In addition, two monoclonal antibodies (clones C1 7.1 and C1 13.1, both IgM) were generated from two independent fusions. Both antibodies reacted specifically with two protein bands of 29,000 and 38,000  $M_r$  (Fig. 1, b and c). The identity of the 29,000- $M_r$  protein, recognized by the polyclonal and monoclonal antibodies, respectively, was confirmed by immunoprecipitation from a detergent extract of rat brain homogenate. In further immunoprecipitation experiments, it was shown that the 38,000- $M_r$  protein band is identical to synaptophysin (data not shown). To examine the degree of cross-reactivity with synaptophysin, various dilutions of the monoclonal antibodies C1 7.1 and C1 13.1 were tested by immunoblotting (Fig. 1 b). At higher dilutions (e.g., 10,000-fold), both antibodies displayed a preference for p29, showing a stronger reaction with p29 than with synaptophysin. These results indicate that both proteins share similar, although not identical, epitopes.

#### **Cellular and Subcellular Localization of p29 within the Nervous System**

To analyze the precise localization of p29 within the nervous system, the affinity-purified rabbit antibodies were used in immunocytochemical experiments. Immunofluorescence performed on frozen sections from various regions of the rat central nervous system revealed a punctate pattern of immunoreactivity (Fig. 2, a and b). This pattern of immunoreactivity was very similar to that produced by antibodies

directed against other SSV proteins (De Camilli et al., 1983a; Jahn et al., 1985; Navone et al., 1986; Baumert et al., 1989). This is shown in Fig. 2, b and c, by double labeling of the same brain stem section for p29 and synaptophysin. It was previously shown that the puncta correspond to individual nerve terminals. P29 immunofluorescence was also detectable on a network of thread-like structures that is present in neuronal perikarya and extends into the dendrites (Fig. 2 a). This labeling pattern is characteristic of the Golgi complex of neurons (De Camilli et al., 1986). A widespread distribution of p29 was also found in the peripheral nervous system. Immunoreactive nerve terminals were found in the skeletal muscle (Fig. 2, d and f) and, in the muscle layers of the stomach wall, the vas deferens, and the Auerbach plexus (not shown). P29 was also concentrated in sensory nerve endings (Fig. 2 f) and in terminals of the posterior pituitary (not shown), which corresponds with the staining pattern obtained for other small synaptic vesicle proteins (De Camilli et al., 1988; Navone et al., 1989).

The subcellular localization of p29 in the nervous tissue was analyzed by various complementary methods. Fig. 3 shows agarose-embedded cell fragments of rat and bovine brain homogenates that were immunogold labeled for p29. Gold label was only found in nerve terminals and was almost exclusively localized on SSVs. A few larger vesicular structures were labeled, which probably corresponds to intermediate stages in the life cycle of SSVs (Fig. 3 c). Similar structures were also labeled using antibodies directed against synaptophysin (Navone et al., 1986) and synaptobrevin (Baumert et al., 1989). Other membranous elements in nerve ter-



**Figure 2.** Localization of p29 in nerve terminals of the central and peripheral nervous system. Immunofluorescence of frozen rat tissue sections. (a) Brain stem neuron. The profile of the neuronal perikaryon is outlined by bright puncta of immunoreactivity that correspond to nerve endings forming axosomatic synapses. Other large puncta visible in the field represent nerve terminals of axodendritic synapses. Immunoreactivity is also visible on thread-like structures in the neuronal cytoplasm (arrows), which correspond to elements of the Golgi complex. (b and c) Double staining of the same brain stem section for p29 (b; affinity-purified rabbit antibody) and for synaptophysin (c; monoclonal antibody C 7.2). The patterns of immunoreactivity are identical, suggesting a very close colocalization of the two proteins. Immunoreactive nerve terminals outline the profile of neuronal perikarya. Arrows point to the same nerve terminals in b and c. (d and e) Double staining of the same skeletal muscle motor end plates for p29 (d; immunorhodamine) and for alpha-bungarotoxin (e; fluorescein-conjugated toxin), which binds to the acetylcholine receptor and therefore outlines the morphology of the synapse. (f) Immunostaining of skeletal muscle showing immunoreactive sensory nerve endings in a muscle spindle. Bars: (a) 10  $\mu\text{m}$ ; (b-f) 20  $\mu\text{m}$ .

minals, which probably represent tubules and cisternae of the endoplasmic reticulum, were not labeled. Also, no significant labeling of LDCVs or of the presynaptic plasmalemma was observed (Fig. 3, b, d, e, arrowheads). Virtually all nerve terminals with a disrupted plasma membrane (i.e., with SSVs accessible to antibodies; see De Camilli et al., 1983b) were labeled.

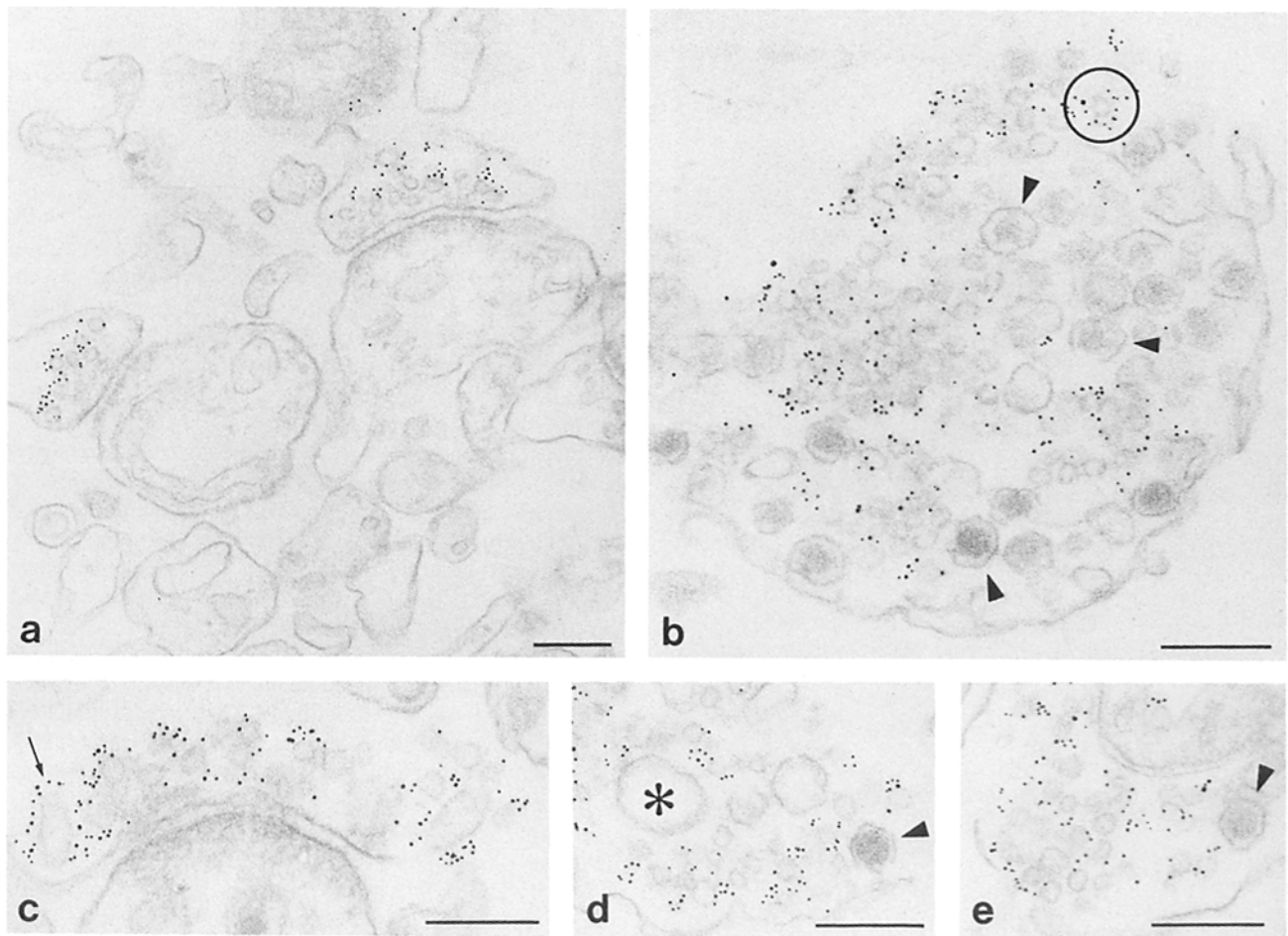
These results were supported by subcellular fractionation experiments. Immunoblot analysis of various fractions obtained during the isolation of SSVs from rat cerebral cortex showed that p29 has a distribution very similar to that of the SSV proteins synaptophysin and synaptobrevin, with the highest enrichment in the purified vesicle fraction (Fig. 4).

#### **Distribution of p29 outside the Nervous System**

Outside the nervous system, significant levels of p29 immunoreactivity were observed only in various endocrine tis-

suess specialized for regulated exocytotic secretion of proteins. Immunofluorescence of frozen tissue sections revealed p29 immunoreactivity in the anterior pituitary (Fig. 5 a), in the adrenal medulla (Fig. 5 b), in the intermediate pituitary, in the endocrine pancreas, in the parathyroids, and in sparse endocrine cells of the gut (not shown). P29 immunoreactivity was also found in cell lines derived from endocrine tissues (e.g., PC12 cells and insulinoma cells [Rin cells]). In all these cells, immunoreactivity was present throughout the cytoplasm but was concentrated primarily in a paranuclear region corresponding to the location of the Golgi complex (Fig. 5, arrows). Similar fluorescence patterns were obtained after double labeling the same cells for p29 and for synaptophysin (not shown), although the Golgi-like staining pattern was more intense for p29 than for synaptophysin.

Tissues which were both nonneuronal and nonendocrine did not contain any detectable amounts of p29. This was confirmed by immunoblotting (Fig. 6).



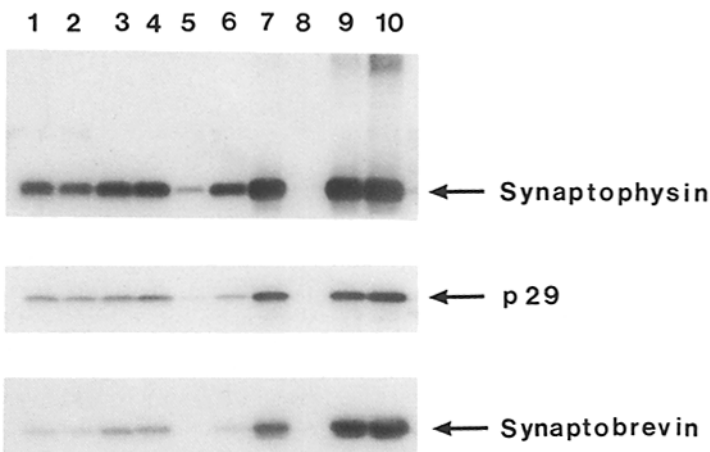
**Figure 3.** Localization of p29 in nerve endings of rat brain (a) and bovine hypothalami (b-e). Immunogold labeling of agarose-embedded cell fragments obtained by mild homogenization. a shows selective labeling of SSVs in nerve terminals. LDCVs, visible in b, d, and e (arrowheads), are unlabeled. An SSV completely decorated by gold particles is outlined by a black circle in b. An arrow in c points to a labeled membranous profile larger than SSVs. An asterisk in d marks an unlabeled vacuole, possibly an element of the endoplasmic reticulum. Note the lack of labeling on the plasma membrane. Bars, 200 nm.

### Subcellular Localization of p29 in Endocrine Cells

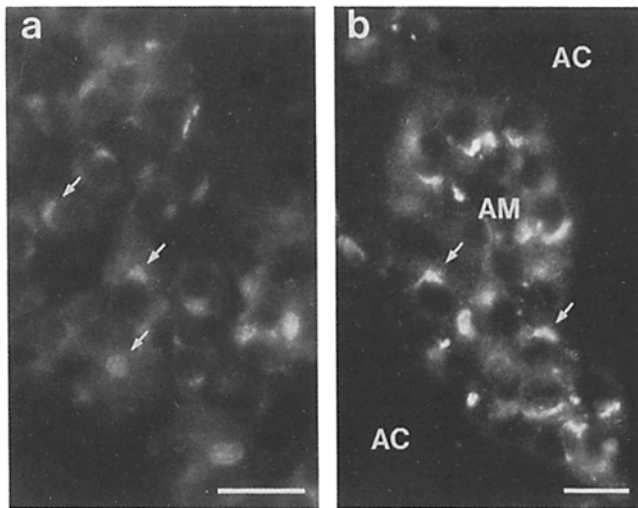
We have previously shown that synaptophysin in endocrine cells is localized on a microvesicle population (synaptic-like microvesicles) distinct from secretory granules (Navone et al., 1986). Since the distribution of p29 in nonneuronal cells

described in the previous section is similar to that of synaptophysin (Navone et al., 1986), it was of interest to determine whether p29 has a similar subcellular localization.

Fig. 7 shows a series of electron micrographs illustrating the localization of p29 by immunogold in subcellular parti-



**Figure 4.** Autoradiography of an immunoblot of various subcellular fractions obtained during the isolation of rat brain synaptic vesicles labeled with affinity-purified serum antibodies for synaptophysin (dilution 1:2,000; top) p29 (dilution 1:300; middle), and synaptobrevin (dilution 1:300; bottom). 5  $\mu$ g of each fraction was separated by SDS-PAGE on a 13% gel and blotted onto nitrocellulose membrane filters. The blot was cut between the position of synaptophysin, p29, and synaptobrevin, respectively, and each part was stained with the respective antibody followed by incubation with  $^{125}$ I-labeled secondary antibody and autoradiography. To increase sensitivity, the exposure time for synaptobrevin was twice as long as that for p29 and synaptophysin. (Lanes 1-10) H, P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>, S<sub>3</sub>, LP<sub>1</sub>, LP<sub>2</sub>, LS<sub>2</sub>, SGV, and CPG, respectively. See Materials and Methods for details.



**Figure 5.** Localization of p29 by immunofluorescence in endocrine tissues. Frozen sections of the rat anterior pituitary (*a*) and adrenal gland (*b*). The adrenal gland cells of the adrenal medulla (*AM*), but not cells of the adrenal cortex (*AC*), are positive. Note the presence of moderate levels of immunoreactivity throughout the cytoplasm and an intense accumulation of immunoreactivity in the proximity of the nucleus (*arrows*) corresponding to the Golgi-centrosomal area. Bars, 20  $\mu$ m.

cles of bovine anterior pituitary (Fig. 7, *a* and *b*) and adrenal medulla (Fig. 7, *c*–*e*). P29 immunoreactivity was found primarily on microvesicles (Fig. 7, *a*–*c*), while no significant labeling was observed on secretory granules. Occasionally, immunogold labeling was observed on relatively small dense-core structures and on small membrane evaginations of larger granules (Fig. 7 *d*). Other labeled elements included large irregularly shaped vesicles with a clear core and tubular extensions of vacuolar structures (possibly endosomes; Fig. 7 *e*). The strong labeling of the entire granule membrane by antibodies directed against the chromaffin granule membrane protein cytochrome *b*<sub>561</sub> (Fig. 7 *f*) showed that the absence of labeling for p29 on the granule membrane is not due to inhibition of antibody access to binding sites.

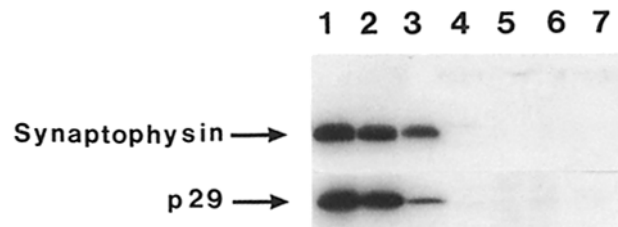
These results show that the vesicle population containing p29 has a morphological appearance similar to the population containing synaptophysin (Navone et al., 1986). This suggests that both proteins are colocalized on the same endomembrane system. To test this hypothesis, synaptophysin-containing vesicles were purified by immunoisolation from undifferentiated PC12 cells and analyzed for the presence of p29. For this purpose, monoclonal antibodies directed against synaptophysin were immobilized on the surface of nonporous methacrylate microbeads that were then used for organelle immunoadsorption. This method allows the one-step purification of synaptophysin-containing organelles from crude cell extracts. As shown in Fig. 8, p29 coenriched with synaptophysin in the immunoadsorbed fraction. Similar enrichment was also found for synaptobrevin. The majority of each protein was removed from the starting fraction by this procedure (not shown), suggesting that all three proteins are colocalized in the same vesicle pool of PC12 cells. However, the relative abundance of the proteins in PC12 cells is different from that in the brain. When compared with synaptophysin, both p29 and synaptobrevin are significantly less abundant in endocrine cells (data not shown).

### Molecular Properties of p29

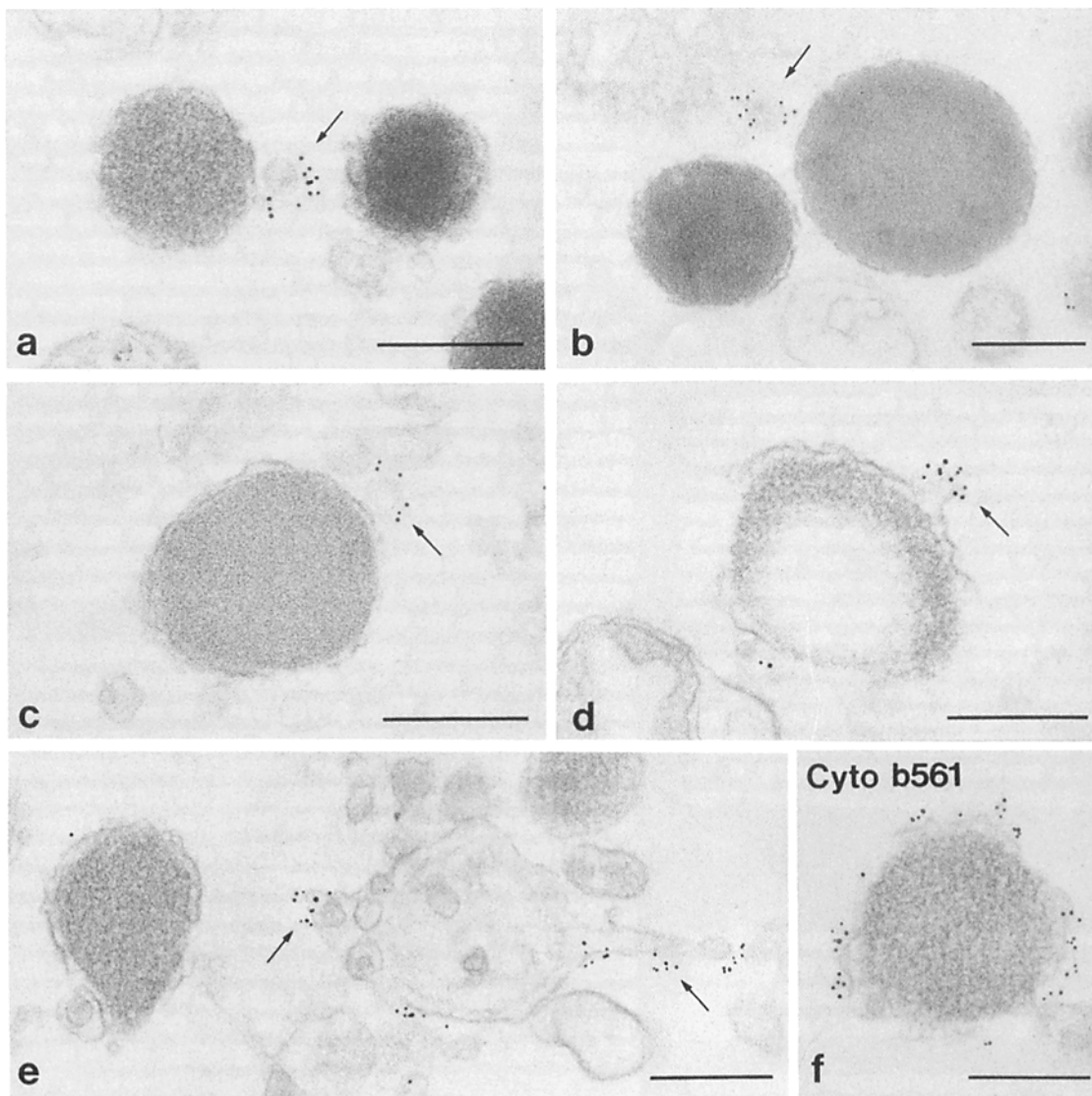
The isoelectric point of p29 was determined by isoelectric focusing followed by SDS-PAGE and immunoblotting of SSV proteins. It was found to be in the range of 5.0 (not shown), which is more acidic than that of synaptobrevin but similar to that of synaptophysin.

To analyze the nature of the association of p29 with the vesicle membrane, phase partition of synaptic vesicle proteins was performed using the detergent Triton X-114 (Bordier, 1981). As shown in Fig. 9 *a*, p29 was selectively enriched in the detergent phase (lane *D*), suggesting that it contains hydrophobic, detergent-binding domains. This is indicative of the presence of transmembrane segments. Since antibodies to p29 bind to the outer surface of SSVs (see electron micrographs above), a portion of the molecule is probably exposed on the cytoplasmic side. Furthermore, we investigated whether p29 is modified by *N*-glycosylation. A detergent extract of SSVs was treated with peptide *N*-glycosidase F to remove *N*-linked sugar moieties. As shown in Fig. 9 *b*, the electrophoretic mobility of p29 remained unchanged whereas synaptophysin (which is known to be *N*-glycosylated; Navone et al., 1986; Rehm et al., 1986) was partially shifted to an apparent 34,000 *M*<sub>r</sub> (compare control sample [lane *C*] with endoglycosidase-treated sample [lane *E*]). Similar results were obtained after treating PC12 cells with tunicamycin to inhibit core glycosylation (not shown). Together, these results indicate that p29 is not *N*-glycosylated in neurons and PC12 cells.

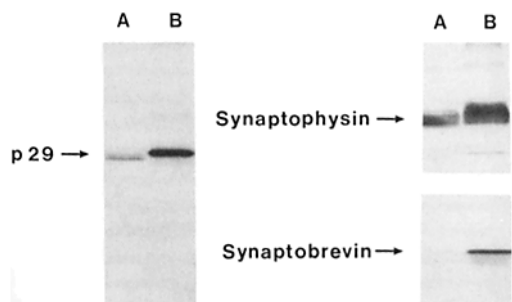
In a final series of experiments, we investigated whether p29, like synaptophysin, undergoes endogenous tyrosine phosphorylation. This possibility was suggested by recent studies reporting the endogenous tyrosine phosphorylation of synaptophysin and of additional proteins in the molecular mass range of 26–30 kD in synaptic vesicle fractions (Pang et al., 1988; Hirano et al., 1988). To examine whether the 30-kD band described in these studies is identical with p29, a vesicle-enriched fraction was incubated with [ $\gamma$ <sup>32</sup>P]ATP under conditions optimal for tyrosine phosphorylation. As shown in Fig. 10 *a*, a number of protein bands were labeled, indicating phosphorylation by endogenous protein kinases. Three of the bands (corresponding to 38,000, 29,000, and 24,000 *M*<sub>r</sub>) contain phosphotyrosine (Pang et al., 1988; Hirano et al., 1988; our unpublished observations). Three



**Figure 6.** Immunoblot of various rat tissue homogenates (10  $\mu$ g/lane), labeled for p29 with affinity-purified serum antibodies, using radiolabeling with an <sup>125</sup>I-linked secondary antibody for detection as described in the legend to Fig. 4. For comparison, the upper part of the blot was cut off and labeled for synaptophysin using the same protocol. To increase sensitivity, the strip labeled for p29 was exposed six times longer than that for synaptophysin. (Lanes 1–7) Cortex, cerebellum, spinal cord, skeletal muscle, liver, kidney, and lung, respectively.



**Figure 7.** Subcellular particles of bovine anterior pituitary (*a* and *b*) and adrenal medulla (*c-f*) immunolabeled for p29 (*a-e*) or for cytochrome *b*<sub>561</sub> (*f*). Immunogold labeling of homogenates embedded in agarose. Arrows point to membranous profiles immunolabeled for p29. p29 immunolabeling is localized on microvesicles (*a-c*) and on the tubular evaginations of a larger membranous organelle that may represent an endosome (*e*). Secretory granule membranes are unlabeled. The vacuole with a partially dense core shown in *d* may represent an immature granule. (*f*) Cytochrome *b*<sub>561</sub> immunoreactivity is abundant on the entire secretory granule membrane. Bars, 200 nm.

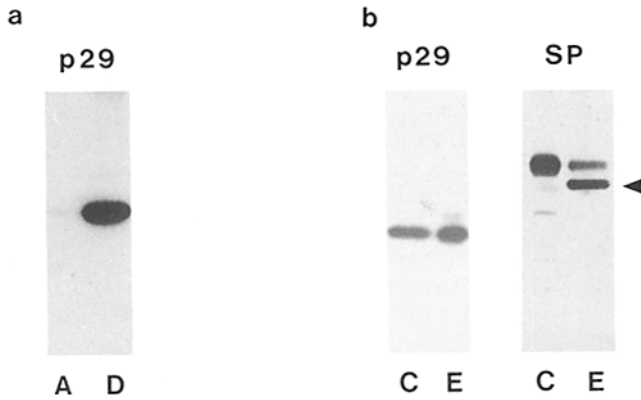


**Figure 8.** Autoradiography of an immunoblot of a PC12 cell homogenate (lanes *A*; 10  $\mu$ g of protein) and of a membrane fraction isolated using anti-synaptophysin monoclonal antibody C 7.2 coupled to methacrylate beads (lanes *B*). SDS-PAGE, immunoblotting, and visualization were performed as given in the legend to Fig. 4. (*Left*) Labeling for p29; (*upper right*) labeling for synaptophysin; (*lower right*) labeling for synaptobrevin.

identical bands were recognized by a phosphotyrosine-specific monoclonal antibody (Fig. 10 *b*, lane *tyr-P*). To demonstrate that the 29,000-*M*<sub>r</sub> band is identical with p29, the vesicle fraction was extracted with cholate at the end of the phosphorylation reaction and subjected to high-speed centrifugation. P29 was immunoprecipitated from the resulting supernatant using monoclonal antibody C1 13.1 conjugated to methacrylate beads, which recognizes p29 as well as synaptophysin. As shown in Fig. 10 *c*, both p29 and synaptophysin were selectively immunoprecipitated (lane *13.1*) and shown to contain phosphotyrosine (lane *tyr-P*). We conclude that p29 is a substrate for endogenous tyrosine kinase.

### Discussion

In the present study, we have characterized p29, a new membrane protein of SSVs. P29 is a nonglycosylated, integral



**Figure 9.** Molecular properties of p29. (a) Detergent binding analyzed by phase partition in Triton X-114. 2  $\mu$ g of synaptic vesicle proteins (fraction CPG) was used for the partition followed by SDS-PAGE on a 12% gel and transferred to nitrocellulose membranes. The blot was incubated with affinity-purified rabbit antibody against p29 followed by incubation with  $^{125}$ I-labeled secondary antibody and autoradiography. (lane A) Aqueous phase; (lane D) detergent phase. (b) Analysis of p29 for the presence of *N*-glycosylation by endoglycosidase treatment. 15  $\mu$ g of vesicle proteins was extracted with Triton X-114 and then treated with peptide *N*-glycosidase F (*N*-glycanase) according to the manufacturer's instructions (lanes E). A control sample (lanes C) was processed in parallel with the enzyme being omitted from the reaction mixture. The samples were separated by SDS-PAGE on 16% gels and labeled in parallel for p29 and synaptophysin (SP) as described in the legend to Fig. 4. The arrowhead points to the position of the deglycosylated form of synaptophysin.

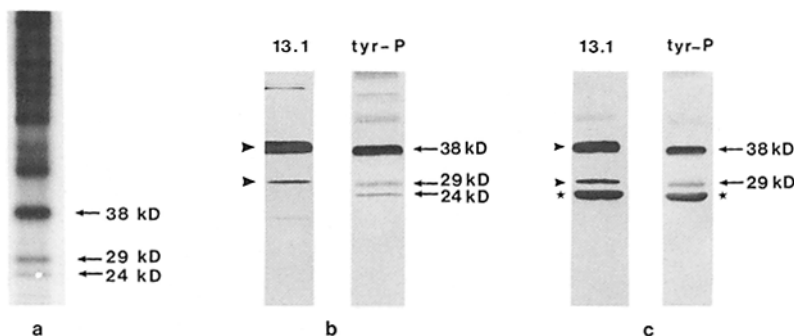
membrane protein with 29,000 *M*, and an acidic isoelectric point. Portions of the molecule are exposed at the cytoplasmic surface of the vesicle as indicated by antibody binding (see Fig. 6) and protease digestion experiments (our unpublished observations).

p29 appears to have some structural similarity with synaptophysin. Both monoclonal antibodies raised against denatured p29 cross react with synaptophysin in immunoblotting and immunoprecipitation assays. The high degree of speci-

ficity in both assay systems suggests that this cross-reactivity is not due to a nonspecific side reaction, which is known to occur with antibodies of the IgM subclass. However, it can be clearly excluded that p29 is a breakdown product of synaptophysin. The polyclonal antibody used in our study is monospecific for p29, and both monoclonal antibodies display a preference for p29 (see Fig. 1). Furthermore, none of the available anti-synaptophysin antibodies, including those specific for internal epitopes (Jahn et al., 1985; Johnston et al., 1989a), react with p29 (our unpublished observations).

A few reports have been published concerning proteins of brain coated and synaptic vesicles with molecular weights similar to that of p29. Analysis of membrane proteins present in brain coated vesicles revealed the presence of a 29,000-*M*, protein that was probably derived from synaptic vesicles (Pfeffer and Kelly, 1985; Wiedenmann et al., 1985). Furthermore, a membrane protein of 30,000 *M*, has been described in guinea pig brain synaptic vesicles (Obata et al., 1987). Since synaptic vesicles contain several membrane proteins of similar molecular weight (our unpublished observations), further work is required to determine whether any of these proteins are related to p29.

Within the nervous system, p29 is present in virtually all nerve terminals where it is selectively associated with the membranes of SSVs. Thus, p29 appears to be a new member of a group of proteins, including the synapsins, synaptophysin, and synaptobrevin, that are present in all SSVs irrespective of the specific neurotransmitter content (for review see De Camilli and Jahn, 1990). The SSV proteins SV2 (Buckley and Kelly, 1985) and p65 (Matthew et al., 1981) apparently share this widespread distribution, although the idea that synaptophysin and p65 are colocalized has been disputed (Volknaend et al., 1988). The absence of p29 from the membranes of LDCVs confirms our previous observations that the membrane compositions of SSVs and LDCVs are dissimilar (Navone et al., 1984, 1986; Baumert et al., 1989). This indicates that SSVs do not originate from recycling LDCV membranes as suggested by other investigators (Lowe et al., 1988; Kelly, 1988; Boarder, 1989) unless extensive protein sorting within the nerve terminal is involved. Rather, the data add further experimental support to our view that SSVs and



**Figure 10.** Tyrosine phosphorylation of p29. (a) Autoradiograph of a vesicle-enriched fraction after phosphorylation with  $[\gamma^{32}\text{P}]\text{ATP}$  and SDS-PAGE (7-15% gradient gel). (b) Immunoblots of a vesicle fraction after phosphorylation with unlabeled ATP and separation by SDS-PAGE (13% gel). (Lane 13.1) Labeling with monoclonal antibody C1 13.1; (lane *tyr-P*) labeling with anti-phosphotyrosine monoclonal antibody. Both antibodies were visualized with a horseradish peroxidase procedure. (c) Immunoprecipitation of p29 and synaptophysin from a cholate extract of synaptic vesicles after phosphorylation with unlabeled ATP followed by SDS-PAGE and immunoblotting of the immunoprecipitate.

precipitate. For the precipitation, monoclonal antibody C1 13.1, immobilized on methacrylate microbeads, was used. (Lane 13.1) Labeling with monoclonal antibody C1 13.1; (lane *tyr-P*) labeling with anti-phosphotyrosine monoclonal antibody. Both antibodies were visualized with a horseradish peroxidase procedure. The position of synaptophysin and p29 in b and c is indicated by arrowheads. The asterisks in c indicate the positions of the light chain of C1 13.1 that was partially removed from the immunobeads and reacted with the secondary antibody used in this assay. No signals with synaptophysin or p29 were obtained with either antibody when beads coupled to control Ig were used (not shown).



LDCVs have an independent biogenesis and independent life cycles (Navone et al., 1986; De Camilli and Jahn, 1990).

Outside the nervous system, p29 was detected only in cells specialized for endocrine secretion by regulated exocytosis (neuroendocrine cells). Again, its subcellular localization is very similar to that of synaptophysin (Navone et al., 1986) and synaptobrevin (cf. Fig. 10; Baumert et al., 1989), indicating a colocalization of all three proteins. This was confirmed by their parallel enrichment upon immunoisolation of subcellular membranes. Structures labeled for p29 were mostly microvesicles with clear content. The membranes of secretory granules were unlabeled, with the exception of occasional labeling of focal evaginations. This may indicate that segregation of p29 from secretory granule proteins is completed during granule maturation after leaving the Golgi complex. Protein segregation by focal evagination of membrane patches has been shown to be involved in the secretory pathway of liver cells where the asialoglycoprotein receptor is separated from secretory proteins by such a mechanism (Geuze et al., 1987).

Together, these results are consistent with our previous suggestion that the presence of SSV proteins in endocrine cells reflects the presence of a population of microvesicles (synaptic-like microvesicles) distinct from secretory granules (Navone et al., 1986; De Camilli and Jahn, 1990). It should be noted, however, that the absence of SSV membrane proteins from secretory granules has recently been disputed. Based on immunoisolation or subcellular fractionation experiments, it was reported that synaptophysin, p65, and SV2 are common to SSVs and to endocrine secretory granules (Lowe et al., 1988; Obendorf et al., 1988). At present, it is difficult to explain this discrepancy. It is possible that synaptophysin, and also the other SSV proteins, are present in the membranes of mature secretory granules at a concentration below the detection limit of our immunocytochemical techniques. However, the secretory granule fractions may be contaminated with synaptic-like microvesicles that remain attached to secretory granules after isolation (e.g., Fig. 7). The latter interpretation is supported by our recent observation that synaptophysin-containing microvesicles can be separated from chromaffin granules by immunoadsorption, leaving behind a granule fraction containing high amounts of cytochrome *b*<sub>561</sub> but no synaptophysin (our unpublished observations).

The function of synaptic-like microvesicles in endocrine cells remains to be established. Recent studies on synaptophysin-containing microvesicles in PC12 cells and in fibroblasts transfected with the cDNA of synaptophysin have provided important insights into the relationship of these membranes with other vesicle-routing pathways. It appears that synaptic-like microvesicles are organelles that recycle between the region of the Golgi complex and the plasmamembrane (Johnston et al., 1989b). When synaptophysin is expressed in Chinese hamster ovary cells, which naturally do not express the protein, it is targeted to a microvesicle population similar to that involved in the recycling of transferrin receptors. These findings raise the possibility that synaptic-like microvesicles in endocrine cells may be related to vesicles that recycle plasmalemma receptors. SSV exocytosis and recycling in synaptic terminals could then be the neuronal adaptation of a general membrane recycling pathway.

The function of p29 remains unknown. However, an im-

portant clue is provided by our finding that p29 can undergo endogenous tyrosine phosphorylation in SSV fractions, adding another element of similarity to synaptophysin, which is the major substrate for tyrosine kinase activity in synaptic vesicles (Pang et al., 1988). It is likely that the protooncogene pp60<sup>c-src</sup> is responsible for this phosphorylation since pp60<sup>c-src</sup> is present in synaptic vesicles (Hirano et al., 1988). The concentration of pp60<sup>c-src</sup> and of some of the major substrates for tyrosine phosphorylation in SSVs is intriguing. It was suggested that pp60<sup>c-src</sup> expression in the nervous system is correlated not only with cell proliferation but also with the development or maturation of neuronal processes and synaptic contacts (Cotton and Brugge, 1983; Sorge et al., 1984). Tyrosine phosphorylation of p29 and synaptophysin may, therefore, be involved in a major regulatory mechanism during the life cycle of SSVs and/or in controlling the assembly of SSV clusters at presynaptic sites.

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