

Specific Association of the Proto-Oncogene Product pp60^{c-src} with an Intracellular Organelle, the PC12 Synaptic Vesicle

Adam D. Linstedt,* Monica L. Vetter,* J. Michael Bishop,†§|| and Regis B. Kelly‡¶

*The Program in Neuroscience, the ‡Department of Biochemistry and Biophysics, the §G. W. Hooper Research Foundation, the ||Department of Microbiology and Immunology, and ¶the Hormone Research Institute, University of California, San Francisco, CA 94143-0534

Abstract. The protein product of the proto-oncogene *c-src* is a membrane-associated tyrosine kinase of unknown function. Identification of pp60^{c-src} target membranes may elucidate the function of the *c-src* protein. The available evidence indicates that pp60^{c-src} associates with distinct membranes within single cell types and has different distributions in different cell types. Our experiments demonstrate targeting of pp60^{c-src} to an isolatable and biochemically identified membrane fraction in the neuroendocrine cell line PC12. The *c-src* protein was found to be specifically associated with synaptic vesicles since: (a) the pp60^{c-src} im-

munofluorescent pattern overlapped with a synaptic vesicle marker, synaptophysin; (b) a significant proportion (44%) of the pp60^{c-src} from PC12 but not fibroblast postnuclear supernatants was recovered in a small vesicle fraction; (c) an anti-synaptophysin cytoplasmic domain antibody immunodepleted all of the pp60^{c-src} vesicles in this fraction, and (d) pp60^{c-src} copurified during a 100-fold purification of PC12 synaptic vesicles. These results suggest a role for the *c-src* protein in the regulation of synaptic vesicle function.

THE gene responsible for the transforming activity of Rous sarcoma virus was derived from a normal cellular gene, *c-src* (Stehelin et al., 1976). Both genes encode membrane-associated tyrosine kinases. The *c-src* protein, pp60^{c-src}, has been well characterized but its physiological function remains unknown. Although pp60^{c-src} is expressed in all cell types of the mouse, loss of the gene by targeted disruption does not lead to a defect in general cell viability (Soriano et al., 1991). The presence of other tyrosine kinases related to *src* may make pp60^{c-src} function unessential except in unusual circumstances.

Most tyrosine kinases are localized to the plasma membrane, whether they are integral membrane proteins such as the growth factor receptors, or peripheral membrane proteins that complex with integral plasma membrane proteins. This makes sense for proteins that are signaling receptors themselves or transducers for cell surface receptors. The *c-src* protein is also associated with the plasma membrane. In fibroblasts, for example, pp60^{c-src} is recovered in subcellular fractions enriched in plasma membrane markers (Courtneidge et al., 1980) and it or its substrates may interact with gap junction components (Azarnia and Loewenstein, 1987; Azarnia et al., 1988). In some cell types, pp60^{c-src} is associated with the attachment of the actin-based cytoskeleton to the cell surface, for example at adherens junctions (Tsukita et al., 1991) and at growth cones of extended neurites (Maness

et al., 1988; Sobue, 1990). The location of pp60^{c-src} is not restricted exclusively to the cell surface, however. In platelets and chromaffin cells pp60^{c-src} is associated with secretory granules in addition to the plasma membrane (Ferrell et al., 1990; Parsons and Creutz, 1986; Grandori and Hanafusa, 1988), and in neurons, fractions enriched in synaptic vesicles contain pp60^{c-src} (Hirano et al., 1988; Barnekow et al., 1990). In fibroblasts pp60^{c-src} is recovered from intracellular membranes associated with the nuclear envelope (Resh and Erikson, 1985), and if pp60^{c-src} is overexpressed it shows both plasma membrane localization and accumulation in puncta throughout the cytoplasm (Kaplan et al., 1990; David-Pfeuty and Nouvian-Dooghe, 1990). The colocalization of pp60^{c-src} and endocytosed Con A in fibroblasts overexpressing the *c-src* protein suggests that pp60^{c-src} may be associated with endosomes (David-Pfeuty and Nouvian-Dooghe, 1990). It is possible that the intracellular localization reflects mis-sorting of a protein intended for the cell surface. If, on the contrary, pp60^{c-src} is selectively targeted to the intracellular organelles it becomes less likely that pp60^{c-src} is exclusively part of a conventional signaling receptor.

Specific targeting of pp60^{c-src} to intracellular organelles could require an interaction with a component enriched in that organelle. Membrane association of both the viral and cellular forms of the *src* gene product is dependent in part

on covalent attachment of the 14-carbon fatty acid myristate to the amino terminus of the src proteins (Buss et al., 1986; Garber et al., 1985; Cross et al., 1984; Kamps et al., 1985). However, certain deletions of amino-terminal sequences result in myristylated but nonmembrane-associated pp60^{c-src} (Garber et al., 1985; Krueger et al., 1982; Kaplan et al., 1990). These observations suggest that sequence elements within the amino-terminal portion of the protein function together with the myristyl moiety in membrane association. That the membrane association may be mediated by interaction with a membrane receptor is further suggested by the finding that membrane fractions contain saturable binding sites for myristylated src protein (Resh, 1989). Furthermore, a 32-kD polypeptide present in fibroblast membrane fractions and several proteins present in platelet membrane fractions can be specifically cross-linked to myristylated amino-terminal src peptides (Resh and Ling, 1990; Feder and Bishop, 1991).

To learn if the c-src protein is indeed targeted to intracellular organelles and to estimate the selectivity of targeting, we have quantified the enrichment of pp60^{c-src} in synaptic vesicles isolated from the neuroendocrine cell line, PC12. We chose synaptic vesicles because pp60^{c-src} is naturally enriched in neural tissue (Cotton and Brugge, 1983; Brugge 1985). A previous attempt to examine the subcellular distribution of pp60^{c-src} activity in the brain found similar levels of enzymatic activity associated with all membrane fractions (Hirano et al., 1988). Enrichment was found in crude microsomal and crude synaptic vesicle fractions but the tyrosine kinase activity in these fractions was only 30% greater than the starting homogenate. Brain synaptic vesicles purified by glass bead chromatography contain pp60^{c-src} which causes the *in vitro* tyrosine phosphorylation of the synaptic vesicle protein, synaptophysin (Barnekow et al., 1990). Although the highest kinase activity is reported to be in the synaptic vesicle fraction, no comparison with other membranous organelles was given. If pp60^{c-src} is present on all membranes its receptor must be widely distributed in the cell. If pp60^{c-src} is preferentially targeted to synaptic vesicles, then they should be enriched in a putative receptor for pp60^{c-src} membrane association. Synaptic vesicles have a relatively simple biochemical composition, a feature that could facilitate identification of such a receptor if it is vesicle specific. Our experiments quantitate the distribution of pp60^{c-src} in PC12 cells, a cell line that contains endocrine synaptic vesicles (Navone et al., 1986; Wiedenmann et al., 1988) that have a composition similar to that of brain synaptic vesicles (Clift-O'Grady et al., 1990). We show that these PC12 synaptic vesicles are enriched in pp60^{c-src}, which should facilitate identification of both the receptor and the domains involved in targeting.

Materials and Methods

Immunofluorescence

PC12 cells were grown in media containing nerve growth factor (100 ng/ml; Calbiochem Corp., La Jolla, CA) for at least 5 d before plating on coverslips coated with poly-D-lysine and laminin. The cells were fixed with 3% paraformaldehyde for 30 min, washed two times with PBS, and two times with PBS-glycine (20 mM), and then permeabilized for 20 min with PBS-glycine-saponin (0.1%) (Schweizer et al., 1988). The coverslips were then inverted on 15 μ l PBS-saponin containing either none, one, or both of the primary antibodies for 30 min. The primary antibodies used were 327 (anti-

src mouse mAb) (Lipsich et al., 1983) at 5 μ g/ml and an anti-synaptophysin rabbit polyclonal serum at 1:500 (Linstedt and Kelly, 1991). After five washes with PBS-saponin the coverslips were inverted on 15 μ l PBS-saponin containing one or both of the following antibodies: FITC-labeled sheep anti-rabbit (dilution 1:400) (Cappel Laboratories, West Chester, PA), or biotinylated horse anti-mouse antibody (dilution 1:40) (Vector Laboratories, Burlingame, CA). The biotinylated antibody was detected with streptavidin-rhodamine (dilution 1:100) (Molecular Probes, Inc., Eugene, OR). After five final washes with PBS-saponin the coverslips were rinsed with water and mounted on glass slides.

Differential Centrifugation

Cells were removed from the plates by scraping in buffer A (150 mM NaCl, 1 mM EGTA, 1 mM MgCl₂ and 10 mM Hepes, pH 7.4) and collected by centrifugation at 300 *g* for 7 min. Homogenization was in buffer A, usually 0.8 ml, containing a protease inhibitor cocktail (pepstatin, chymostatin, leupeptin, and aprotinin at 10 ng/ml; 1 mM PMSF; 1 μ g/ml *o*-phenanthroline; 10 μ M benzamidine) using a Cell Cracker (European Molecular Biology Laboratory) with 10 strokes and a 12- μ m clearance. The homogenate was separated into a nuclear pellet (P1) and postnuclear supernatant (S1) by centrifugation at 1,000 *g* for 5 min in a SS34 rotor (Sorvall Instruments, Newtown, CT). The S1 was centrifuged at 27,000 *g* for 35 min in the SS34 rotor to obtain a pellet of large membranes (P2) and a high speed supernatant (S2). The S2 was fractionated into small membranes (P3) and cytosol (S3) by centrifugation at 127,000 *g* for 60 min in an air centrifuge (Beckman Instruments, Palo Alto, CA). Pellets were resuspended and assayed for pp60^{c-src} and synaptophysin by immunoblotting, protein content by Pierce assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard, and membrane protein content by Pierce assay after extraction with Triton X-114 as described (Bordier, 1981).

Organelle Immunoisolation

Aliquots of 20 mg of Dynabeads M-450 (DYNAL, Inc., Great Neck, NY), magnetic polystyrene beads coated with sheep anti-mouse IgG1, were incubated with 20 μ g of either anti-synaptophysin (SY38) or mouse γ -globulin (Pel-Freez Biologicals, Rogers, AR) overnight at 4°C then rinsed with buffer A. Three aliquots of the S2 fraction prepared from two 15-cm plates of PC12 cells were used. One aliquot was added to the SY38 beads, one was added to the control beads, and the final was left untreated. Each sample was rotated for 60 min at 4°C. Using a magnet to retain the beads, the S2 fractions were removed and centrifuged for 60 min at 127,000 *g* in the air centrifuge. The resulting pellets (P3) were lysed and analyzed by immunoblotting for pp60^{c-src} and synaptophysin. The isolated beads were subjected to sequential washes in buffer A and after each wash a fraction was extracted with 1% SDS and the extract was assayed for the presence of pp60^{c-src} by immunoblotting.

Velocity and Flotation Gradients

For velocity gradient analysis, the S1 fraction from either unlabeled or metabolically labeled cells was layered on 4.4 ml linear 5–20% sucrose, or 5–25% glycerol gradients in buffer A underlayered with a 0.4 ml 50% sucrose pad and centrifuged in a SW55 rotor (Beckman Instruments) at 4°C for 60 min at 48,000 rpm. For flotation gradient analysis samples pooled from the sucrose velocity gradients were adjusted to 50% sucrose with a 70% sucrose solution and underlayered on 4 ml linear 20–40% sucrose gradients containing 10 mM Hepes, pH 7.4, and 1 mM EGTA and centrifuged in the SW55 rotor at 4°C overnight at 48,000 rpm. All gradients contained the protease inhibitor cocktail (see above). Fractions were collected from the bottom of the tube. Protein content in fractions from unlabeled cells was determined with BSA as a standard using either the Pierce assay or the Quantigold assay (Diversified Biotech, Newton Centre, MA). Scintillation counting was used to determine protein content in fractions from labeled cells. Antigen content was assayed by immunoblotting from unlabeled cells or immunoprecipitation from labeled cells.

Labeling and Immunoprecipitation

For metabolic labeling, cells were incubated overnight in DME-H21 media depleted of cysteine and methionine but supplemented with 2% FCS and [³⁵S]Translabel (ICN K&K Laboratories Inc., Irvine, CA) at 100 μ Ci/ml. Synaptophysin immunoprecipitations were carried out in buffer containing 1% NP-40, 0.4% deoxycholate, 0.3% SDS, 66 mM EDTA, 10 mM Tris, pH 7.4, with an anti-rat brain synaptic vesicle serum that recognizes synap-

tophysin (Clift-O'Grady et al., 1990). Immunoprecipitation of pp60^{c-src} was in RIPA buffer with mAb 327 as described previously (Kaplan et al., 1990).

Immunoblotting

Proteins were separated by SDS-PAGE (10% gels) and transferred to nitrocellulose using a semi-dry electrotransfer apparatus (E&K, Saratoga, CA). Proteins were visualized by staining in Ponceau S for several minutes followed by rinses with water. Blocking was for 60 min followed by incubation with SY38 (20 ng/ml) and 327 (3 μ g/ml) for 60 min, three 5-min washes, incubation with ¹²⁵I-goat anti-mouse IgG (Cappel Laboratories) for 60 min and three final washes. All incubations were in PBS containing 5% non-fat dry milk and 0.05% Tween 20. The nitrocellulose was exposed to x-ray film at -70°C with an enhancing screen. Autoradiograms were quantitated using a phosphorimager (Molecular Dynamics).

Results

pp60^{c-src} and Synaptophysin Immunofluorescent Patterns Overlap in PC12 Cells

Analysis of the intracellular localization of pp60^{c-src} in PC12 cells by immunofluorescence microscopy revealed a punctate distribution of immunoreactivity. The cells shown in Fig. 1 were grown for 5 d in nerve growth factor (to promote neurite extension) and plated on poly-D-lysine and laminin-coated coverslips, then fixed with formaldehyde, saponin permeabilized and double stained for pp60^{c-src} (Fig. 1 B) and synaptophysin (Fig. 1 C). Puncta of pp60^{c-src} immunoreactivity were scattered throughout the cytoplasm (Fig. 1 B). Intense staining of the plasma membrane was not apparent, which contrasts with the staining pattern seen in fibroblasts overexpressing pp60^{c-src} (Kaplan et al., 1990; David-Pfeuty and Nouvian-Dooghe, 1990). Staining was present in neuritic processes, particularly in the growth cone area, consistent with previous reports of pp60^{c-src} localization to growth cones (Sobue, 1990). This pattern was very similar in overall appearance to the synaptophysin pattern (Fig. 1 C). Synaptophysin staining was more intense overall, and slightly more intense throughout the processes. Many pp60^{c-src} puncta, particularly in the processes and growth cone areas, were in alignment with synaptophysin puncta. Similar antibody-specific distributions were obtained using other monoclonal or polyclonal antibodies directed against either antigen. The coincidence of pp60^{c-src} and synaptophysin staining suggested that pp60^{c-src} may be localized to membranes that participate in synaptic vesicle recycling (such as endosomes or synaptic vesicles or both).

pp60^{c-src} Is Highly Enriched in Fractions Containing Small Vesicles

To begin to test whether pp60^{c-src} associates with PC12 synaptic vesicles we used a simple differential centrifugation scheme that has proved useful for studying the distribution of synaptophysin-containing membranes (Clift-O'Grady et al., 1990; Linstedt and Kelly, 1991). Synaptophysin is recovered in both the pellet (P2) and supernatant of a 27,000-g centrifugation of PC12 lysates. The synaptophysin in the supernatant, which can be concentrated in a 127,000-g pellet (P3), is mostly in synaptic vesicles, while that in the P2 is present in larger membranes (Clift-O'Grady et al., 1990; Linstedt and Kelly, 1991). In contrast, an endosome marker, the low density lipoprotein receptor, is recovered in the P2,

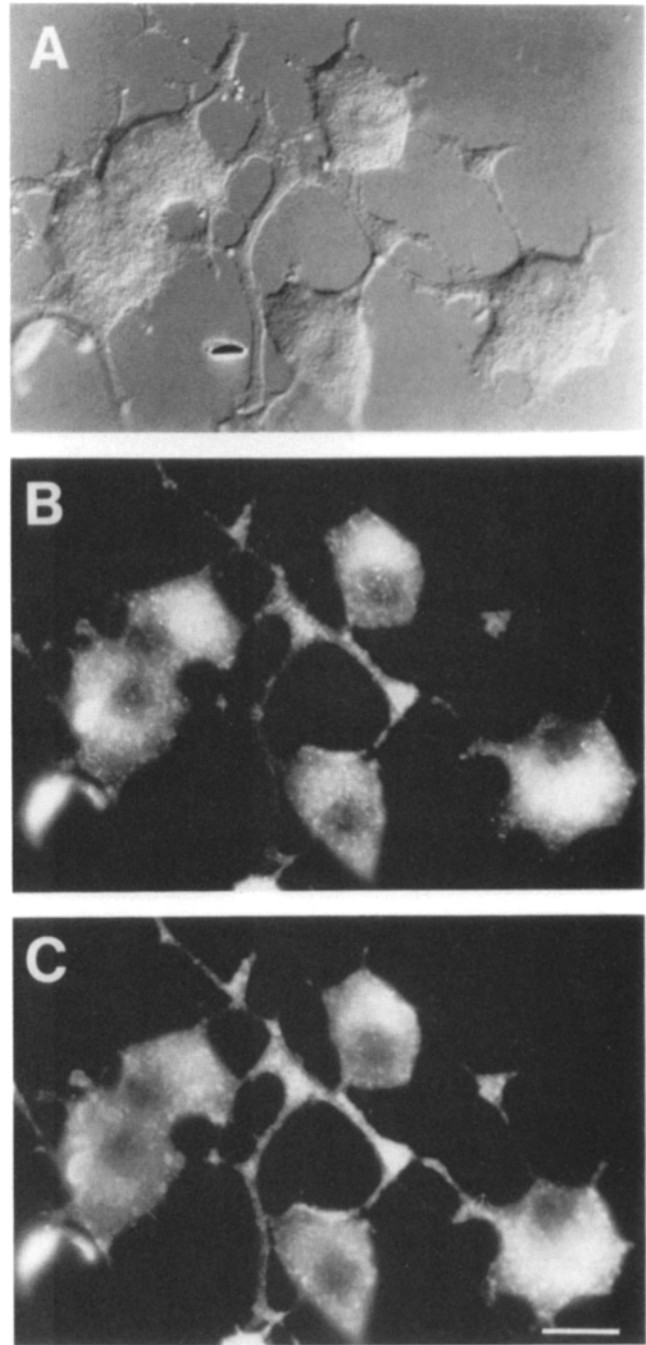


Figure 1. Localization of pp60^{c-src} (B) and synaptophysin (C) in PC12 cells by double label immunofluorescence. Both pp60^{c-src} and synaptophysin immunoreactivity are dispersed throughout the cytoplasm and processes in very fine puncta. A Nomarski image of the stained cells is also presented (A).

with only minor amounts remaining in the supernatant (Linstedt and Kelly, 1991).

Paralleling the distribution of synaptophysin, a substantial amount of the sedimentable pp60^{c-src} present in the post-nuclear supernatant was recovered in both the P2 and P3 fractions (Fig. 2 A). The pp60^{c-src} recovered in the P3 fraction accounted for 44 \pm 9% while in the same fraction 40 \pm 10% of the synaptophysin was recovered ($n = 4$). In

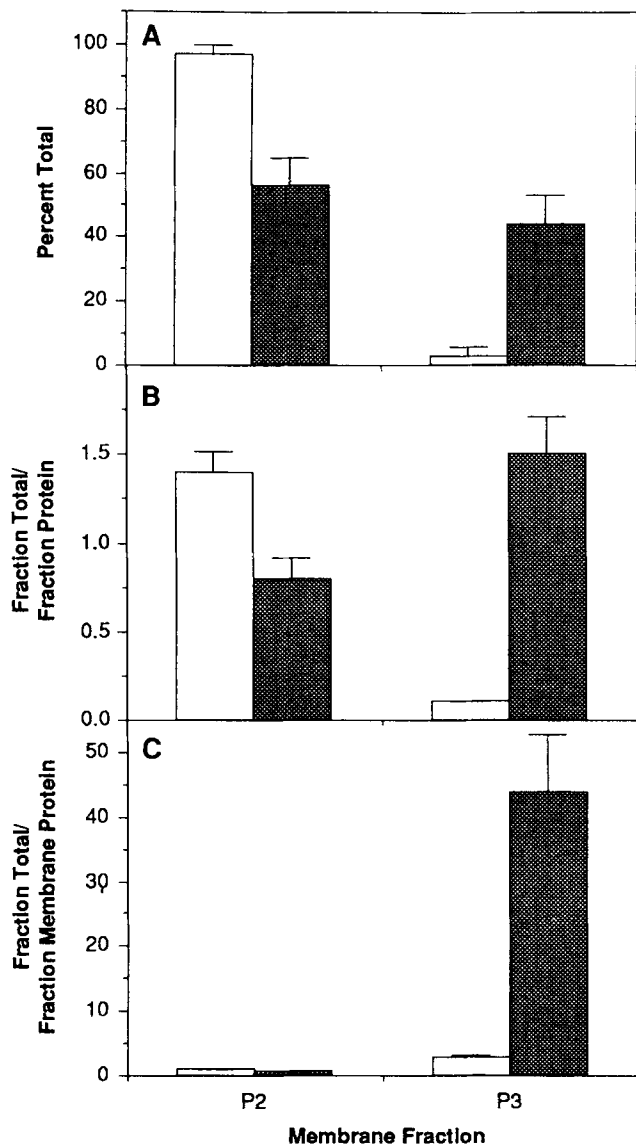


Figure 2. Presence of pp60^{c-src} in a slowly sedimenting fraction from PC12 but not CHO cells. PC12 (▣) and CHO (□) cells were separated by differential centrifugation and each fraction was assayed for pp60^{c-src} by immunoblotting. The recovery of pp60^{c-src} in the P2 and P3 fractions is shown: (A) as percentage of the total sedimentable pp60^{c-src} in the post nuclear supernatant (P2 + P3); (B) as recovery when normalized by the total amount of protein in each fraction; and (C) as recovery when normalized by the amount of membrane protein in each fraction. The data are averages ± SD, n = 3 for CHO, n = 4 for PC12. P1, P2, and P3 centrifugations were 1,000 g for 5 min, 27,000 g for 30 min, and 125,000 g for 60 min, respectively. Soluble pp60^{c-src}, and pp60^{c-src} that was recovered in the nuclear pellet are not shown. Membrane protein was determined by recovery in the detergent phase of Triton X-114.

marked contrast, only an insignificant amount of pp60^{c-src}, 3 ± 3% (n = 3), was recovered in the equivalent P3 fraction from CHO fibroblast cells (Fig. 2 A). The absence of small, pp60^{c-src}-containing vesicles in fibroblasts correlates with their lack of synaptic vesicles. Synaptophysin in transfected fibroblasts is targeted to endosomes and recovered primarily in the P2 fraction (Linstedt and Kelly, 1991). The distribu-

tion of pp60^{c-src} between P2 and P3 fractions was no different in CHO cells transfected with synaptophysin. Therefore, a substantial portion of pp60^{c-src} cosediments with a slowly sedimenting membrane population present in PC12 cells, but presumably absent in CHO cells.

The enrichment of pp60^{c-src} in the PC12 P3 fraction was twice that of the P2 fraction when compared with total protein present in each fraction (Fig. 2 B). Consistent with the absence of synaptic vesicles in CHO cells, pp60^{c-src} was not enriched in the CHO P3 fraction after normalization to protein content. When the PC12 P3 fraction was analyzed on a flotation gradient very little protein (<5%) was associated with membrane fractions. The amounts of membrane protein in the P2 and P3 fractions were compared directly by measuring the amount of protein that could be extracted into the detergent phase of Triton X-114. Since almost all of the total membrane protein in the postnuclear supernatant was recovered in the P2 fraction (data not shown), the protein in the P3 fraction must be due largely to nonmembranous complexes. When normalized to membrane protein rather than total protein, the pp60^{c-src} associated with the P3 fraction was ~75-fold more enriched than the pp60^{c-src} in the P2 fraction (Fig. 2 C).

Isolation of pp60^{c-src} Vesicles with an Anti-synaptophysin Antibody

To test directly for association of pp60^{c-src} with PC12 synaptic vesicles, vesicle immunoisolation experiments were carried out using magnetic immunobeads, coated with a mAb that recognizes an epitope in the cytoplasmic tail of synaptophysin, at concentrations sufficient to deplete the membranes containing synaptophysin (Fig. 3). After removal of the immunobeads, membranes remaining in the supernatants were collected by centrifugation and assayed for the presence of pp60^{c-src} and synaptophysin by immunoblot. As a control for nonspecific adsorption, beads coated with mouse gamma globulin were used. A comparison of the recoveries of pp60^{c-src} or synaptophysin membranes in nontreated (N) and control bead-treated (C) supernatants indicated that there was no significant depletion by the control beads (Fig. 3, compare lanes 1 and 2). In contrast, treatment of a PC12 27,000 g supernatant with the anti-synaptophysin beads (S) depleted all synaptophysin-containing membranes (Fig. 3, lane 3). Immunoblots of this same material with antibodies against pp60^{c-src} demonstrated that removal of synaptophysin membranes depleted membrane associated pp60^{c-src} (Fig. 3, lane 3). The isolated beads were extracted under conditions that allowed recovery of pp60^{c-src} but prevented solubilization of the mouse antibodies coating the beads. This allowed an immunoblot assay of the bead fraction using a mouse mAb against pp60^{c-src} that demonstrated that pp60^{c-src} was recovered from the isolated anti-synaptophysin beads (Fig. 3, lane 5), but not from the control beads (Fig. 3, lane 4). Analysis of synaptophysin recovery in the bead fraction required solubilization conditions that disrupted antibody binding and thus also solubilized the mouse antibodies that coat the magnetic beads. In parallel experiments using a rabbit polyclonal antibody that recognizes synaptophysin, the bead fraction was assayed by immunoblot and synaptophysin recovery was shown to be quantitative and specific (Linstedt and Kelly, 1991).

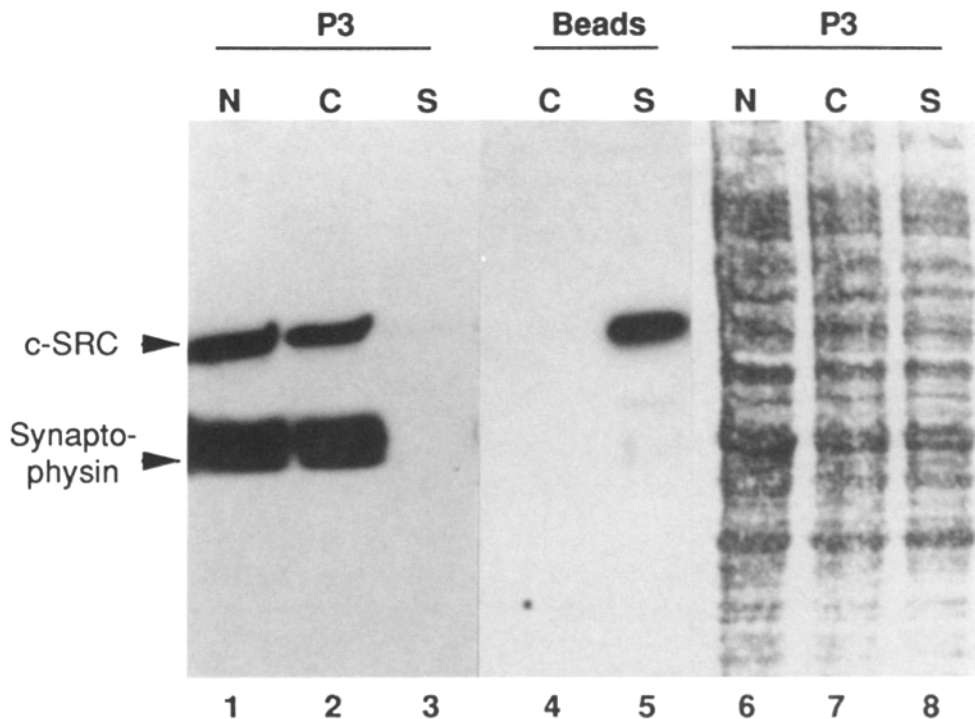


Figure 3. Immunodepletion of pp60^{c-src} containing membranes with anti-synaptophysin immunobeads. S2 fractions were either nontreated (N), treated with control antibody beads (C), or treated with anti-synaptophysin beads (S). After removal of the beads the membranes remaining in each supernatant were collected in a pellet (P3) and analyzed for pp60^{c-src} and synaptophysin by immunoblotting (lanes 1-3). The isolated immunobeads were extracted with 1% SDS and these extracts were analyzed for pp60^{c-src} by immunoblotting (Beads, lanes 4 and 5). The similarity of the protein patterns (lanes 6-7) observed with Ponceau S staining of the nitrocellulose (photocopied before immunoblotting) indicated that immunobead treatment did not significantly deplete major proteins recovered in the P3 fraction.

The Triton X-114 partition assays presented in the previous section indicated that of the total protein in the P3 fraction most was not membrane protein. Consistent with this finding, removal of synaptophysin membranes by immunodepletion did not significantly reduce the amount of total protein recovered in the P3 fraction (Fig. 3, compare lanes 7 and 8). The depletion of pp60^{c-src} by the anti-synaptophysin bead treatment indicated that pp60^{c-src} in the P3 fraction was membrane associated (not part of a cytosolic protein complex) and that the membranes in the P3 were highly enriched in pp60^{c-src}. Furthermore, since a large fraction of the synaptophysin present in the PC12 27,000-g supernatant is in synaptic vesicles, the depletion of pp60^{c-src} by removal of synaptophysin-containing membranes suggested that pp60^{c-src} in this fraction is associated with the synaptic vesicles. These data verify that pp60^{c-src} must be highly enriched in PC12 synaptic vesicles. Based on the enrichment presented above (Fig. 2 C) a unique membrane population (e.g., plasma membrane) in the P2 would have to comprise <2% of the total membranes present to contain pp60^{c-src} with the same enrichment as PC12 synaptic vesicles.

Copurification of pp60^{c-src} with Endocrine Synaptic Vesicles

Since the experiments presented above indicated that the pp60^{c-src} was associated with the PC12 synaptic vesicles we sought to purify the vesicles with the src protein associated. We first analyzed the behavior of pp60^{c-src}-containing vesicles on velocity gradients. When the postnuclear supernatant was analyzed, synaptic vesicle membranes were clearly detectable as a peak of synaptophysin (Fig. 4 A, fraction 8) distinct from the larger membranes that collected on a pad at the bottom of the tube (Fig. 4 A, fraction 2). Coincident with the synaptic vesicles was a peak of pp60^{c-src}. Comparison of

the recovery of pp60^{c-src} associated with small and large membranes after velocity gradient analyses reproducibly indicated that the enrichment of pp60^{c-src} in small vesicles was greater than that for synaptophysin (e.g., compare fractions 2 and 8 in Fig. 4 A). Separation of Rat1A fibroblast postnuclear supernatants yielded a peak of pp60^{c-src} only at the bottom of the gradient (data not shown). This finding, consistent with the results shown in Fig. 2, suggests that the pp60^{c-src} association with synaptic vesicle-sized membranes found in the neuroendocrine PC12 cells does not occur in fibroblasts.

Fractions encompassing the synaptic vesicle-sized membranes on sucrose velocity gradients were pooled and analyzed on sucrose flotation gradients. A peak of pp60^{c-src} and synaptophysin antigenicity co-migrated with a peak of protein at a density of 1.13 gm/cc (Fig. 4 B). It was observed that nearly 100% of the synaptophysin was recovered in the fractions containing buoyant membranes, while only 60-80% of the pp60^{c-src} was similarly recovered. We assume that the pp60^{c-src} that did not float together with the synaptic vesicles was the result of dissociation from the synaptic vesicles for the following reasons. The recovery of pp60^{c-src} with synaptic vesicles on density gradients varied depending on which isolation conditions were used. The pp60^{c-src} did not co-migrate with soluble proteins on the flotation gradients, but trailed from the synaptic vesicle peak toward the position where the sample was applied, suggesting dissociation during sedimentation. Also, up to 50% of the pp60^{c-src} associated with synaptic vesicles isolated on immunobeads slowly dissociated during prolonged buffer washes (data not shown).

Comparative SDS-PAGE of the pooled fractions at each purification step is presented in Fig. 5. The position of pp60^{c-src} and synaptophysin was determined by immuno-

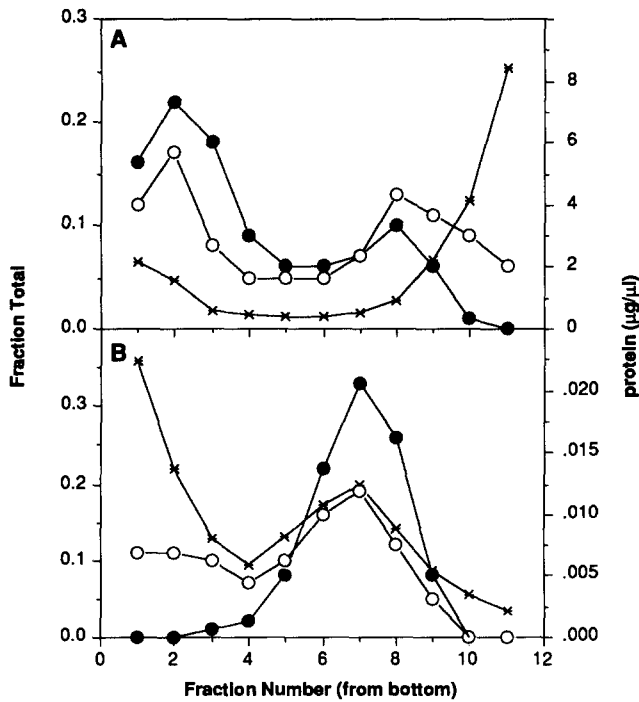


Figure 4. Cosedimentation of pp60^{c-src} and PC12 synaptic vesicles. (A) PC12 postnuclear supernatant separated on a linear 5–20% sucrose gradient underlaid with a dense sucrose cushion and centrifuged at 48,000 rpm for 60 min. (B) Material from the peak of the sucrose velocity gradient (fractions 7 and 8 in A) was adjusted to 50% sucrose and applied under a linear 20–40% sucrose gradient and centrifuged at 48,000 rpm for 12 h. Each fraction was assayed for protein (x), synaptophysin (●), and pp60^{c-src} (○) as described in Materials and Methods.

blotting a separate lane from the same gel (Fig. 5, lane 5). Several bands showed enrichment in the flotation peak relative to the starting material including bands at the same position as pp60^{c-src} and synaptophysin (compare lanes 1 and 4).

Quantitation of the recovery of total protein, pp60^{c-src} and synaptophysin in the postnuclear supernatant, velocity peak, and flotation peak for four independent experiments is presented in Table I. Quantitation of experiments carried out on unlabeled material using protein assays and immunoblots was in agreement with quantitation of experiments carried out on labeled material using scintillation counting and immunoprecipitation. The enrichment of pp60^{c-src} calculated for purified synaptic vesicles was similar to that found in the P3 fraction when normalized by membrane protein (compare Fig. 2 C with Table I).

Table I. Copurification of PC12 Synaptic Vesicles and pp60^{c-src}

	Protein		Synaptophysin		pp60 ^{c-src}	
	Percent total	Percent total	Percent total	Enrichment	Percent total	Enrichment
Postnuclear supernatant	100	100	100	1	100	1
Velocity pool	5 ± 1	34 ± 8	36 ± 3	6.8	19 ± 2	7.2
Flotation pool	0.2 ± 0.1	24 ± 6	120	120	95	95

The numbers indicate average ± SD of four independent experiments.

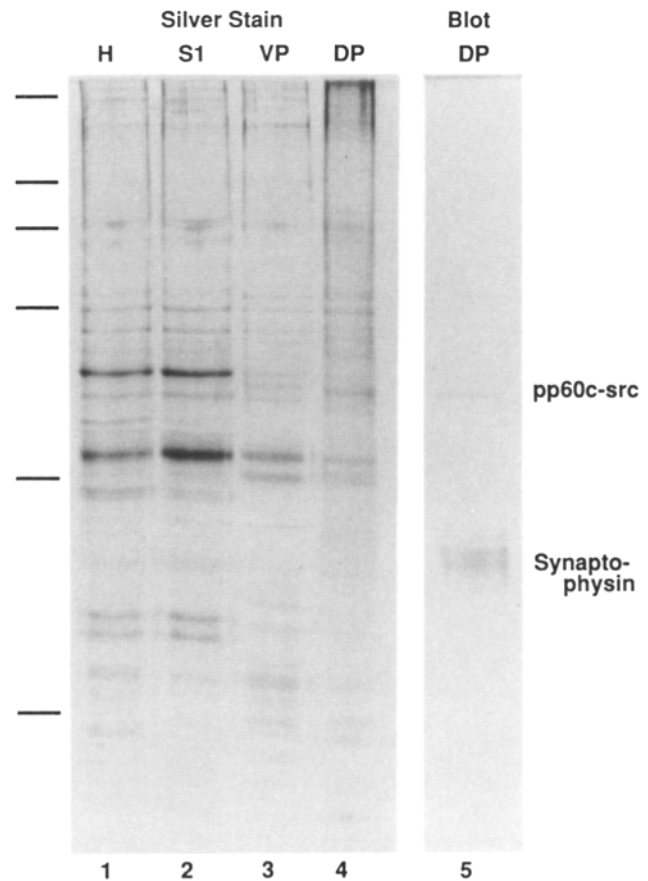


Figure 5. Polypeptide composition of subcellular fractions. Material from the homogenate (lane 1), S1 (lane 2), velocity peak (lane 3), and density peak (lane 4) was subjected to SDS-PAGE (2.4 μg protein/lane; 10% gel) and stained with silver. Part of the gel was subjected to immunoblotting to determine the position of pp60^{c-src} and synaptophysin (lane 5). The position of molecular weight markers (205, 116, 97.4, 66, 45, and 29 kD) is indicated.

Discussion

We have studied the distribution of pp60^{c-src} in PC12 cells and compared it with that of a synaptic vesicle membrane protein, synaptophysin. In PC12 cells pp60^{c-src} was found to be specifically associated with the endocrine synaptic vesicles. In cells simultaneously stained with antibodies that recognize either pp60^{c-src} or synaptophysin the pp60^{c-src} immunofluorescence pattern extensively overlapped with the synaptophysin pattern. Nearly 50% of the membrane-associated pp60^{c-src} was recovered in a slowly sedimenting frac-

tion enriched in synaptic vesicles and containing very little membrane protein. When this fraction was prepared from a fibroblast cell line only an insignificant amount of pp60^{c-src} was present. All of the pp60^{c-src} recovered in the PC12 small vesicle fraction was removed by immunodepletion of the endocrine synaptic vesicles using antibodies directed against synaptophysin. Furthermore, pp60^{c-src} copurified with the endocrine synaptic vesicles through a 100-fold purification.

Our experiments demonstrate targeting of pp60^{c-src} to an isolatable and biochemically identified membrane fraction. A straightforward hypothesis, as suggested by the work of Resh and Ling (1990) on fibroblasts and Feder and Bishop (1991) on platelets is that the targeting of pp60^{c-src} to PC12 synaptic vesicles is mediated by a specific membrane protein. Cross-linking and co-immunoprecipitation experiments using the purified synaptic vesicle fraction may identify such a protein. Since pp60^{c-src} is associated with other membranes, including endosomes, in addition to synaptic vesicles in PC12 cells (our own unpublished observations) it may be that PC12 cells express more than one "src receptor." Kaplan et al. (1990) have suggested that different domains within the src amino terminus may mediate targeting to different cellular compartments. This could be tested in PC12 by comparing the targeting to the synaptic vesicle membrane of transfected proteins lacking or containing different pp60^{c-src} domains.

As the integral membrane proteins cycle from synaptic vesicles through plasma membrane and endosome, synaptic vesicle-specific peripheral membrane proteins can associate and dissociate. The synapsins, major substrates in the nerve terminal for cAMP-dependent and calcium-calmodulin-dependent protein kinases, are phosphorylated during exocytosis, which promotes their dissociation (Schiebler et al., 1986; Sihra et al., 1989). If exocytosis is stimulated by the venom α -latrotoxin in the absence of extracellular calcium, the synapsins are found in association with the plasma membrane (Torri-Tarelli et al., 1990) suggesting that dissociation comes after exocytosis. The small GTP-binding protein, rab 3A, which is restricted to synaptic vesicles (Fischer von Mollard et al., 1990a; Mizoguchi et al., 1990), also dissociates from synaptic vesicles on exocytosis (Fischer von Mollard et al., 1990b).

Although pp60^{c-src} association with PC12 synaptic vesicles was somewhat unstable in vitro, we recovered equal amounts of pp60^{c-src} in synaptic vesicle fractions from cells unstimulated and those stimulated with either high potassium, phorbol esters, or nerve growth factor (data not shown). These experiments are hard to interpret since it is not known what conditions are required to stimulate the exocytosis of PC12 synaptic vesicles. A comparison of pp60^{c-src} and synaptophysin distribution across a velocity gradient of the postnuclear supernatant (Fig. 4 A) suggested that pp60^{c-src} is even more enriched in the synaptic vesicle fractions than synaptophysin. The compartment other than synaptic vesicles in which large amounts of synaptophysin are recovered is the endosome (Johnston et al., 1989; Linstedt and Kelly, 1991). If most of the synaptophysin membranes that collect at the sucrose pad are indeed endosomes, then it would be necessary to postulate that synaptic vesicle membranes lose their pp60^{c-src} as they cycle through the endosome. At present, therefore, it is plausible that association and dissociation of the known synaptic vesicle-enriched periph-

eral membrane proteins, rab3A, synapsin, and pp60^{c-src}, is regulated by, or regulate the exocytotic cycle.

Synaptic vesicles contain ~7% of the protein in the brain (Südhof and Jahn, 1991). If pp60^{c-src} is a major component of brain synaptic vesicles as it is of endocrine synaptic vesicles, then it is easy to explain the enrichment of pp60^{c-src} in neuronal tissues. Tyrosine kinase activity, however, is generally associated with plasma membrane receptors and cell signaling. The conventional view therefore is difficult to reconcile with an association between pp60^{c-src} and an intracellular organelle. One intriguing possibility, given the association between pp60^{c-src} and the actin cytoskeleton mentioned earlier, is that pp60^{c-src} regulates the interaction between membranes, including secretory vesicles, and the actin-based cortical cytoskeleton. Synaptic vesicle membranes are associated with cortical cytoskeleton and serine/threonine kinases are already known to regulate this association during exocytosis. Perhaps tyrosine protein kinases also play a role in disassembling the cortical cytoskeleton to allow exocytosis, or the recovery of membrane by endocytosis.

We are grateful to Louis Reichardt and Aaron Turkewitz (both of the University of California at San Francisco) for their comments on the manuscript, and to Joan Brugge (University of Pennsylvania) for the use of the mAb 327.

M. L. Vetter is a Howard Hughes Medical Institute Predoctoral Fellow. R. B. Kelly was supported by National Institutes of Health (NIH) grants DK33937, NS15927, and NS09878. J. M. Bishop was supported by NIH grant CA44338 and by funds from the G. W. Hooper Foundation.

Received for publication 14 October 1991 and in revised form 16 March 1992.

References

- Azarnia, R., and W. R. Loewenstein. 1987. Polyomavirus middle T antigen down-regulates junctional cell-to-cell communication. *Mol. Cell. Biol.* 7:946-950.
- Azarnia, R., S. Reddy, T. E. Kmiecik, D. Shalloway, and W. R. Loewenstein. 1988. The cellular src gene product regulates junctional cell-to-cell communication. *Science (Wash. DC)*. 239:398-401.
- Barnekow, A., R. Jahn, and M. Schartl. 1990. Synaptophysin: a substrate for the protein tyrosine kinase pp60^{c-src} in intact synaptic vesicles. *Oncogene*. 5:1019-1024.
- Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604-1607.
- Brugge, J. S., P. C. Cotton, A. E. Queral, J. N. Barrett, D. Nonner, and R. W. Keane. 1985. Neurons express high levels of a structurally modified, activated form of pp60^{c-src}. *Nature (Lond.)*. 316:554-557.
- Buss, J. E., M. P. Kamps, K. Gould, and B. M. Sefton. 1986. The absence of myristic acid decreases membrane binding of p60^{src} but does not affect tyrosine protein kinase activity. *J. Virol.* 232:468-474.
- Clift-O'Grady, L., A. D. Linstedt, A. W. Lowe, E. Grote, and R. B. Kelly. 1990. Biogenesis of synaptic vesicle-like structures in a pheochromocytoma cell line PC-12. *J. Cell Biol.* 110:1693-1703.
- Cotton, P. C., and J. S. Brugge. 1983. Neural tissues express high levels of the cellular src gene product pp60^{c-src}. *Mol. Cell. Biol.* 3:1157-1162.
- Courtnidge, S. A., A. D. Levinson, and J. M. Bishop. 1980. The protein encoded by the transforming gene of avian sarcoma virus (pp60^{src}) and a homologous protein in normal cells (pp60^{src-like}) are associated with the plasma membrane. *Proc. Natl. Acad. Sci. USA*. 77:3738-3787.
- Cross, F. R., E. A. Garber, D. Pelman, and H. Hanafusa. 1984. A short sequence in the p60^{src} N terminus is required for p60^{src} myristylation and membrane association and for cell transformation. *Mol. Cell. Biol.* 4:1834-1842.
- David-Pfeuty, T., and Y. Nouvian-Dooche. 1990. Immunolocalization of the cellular src protein in interphase and mitotic NIH c-src overexpresser cells. *J. Cell Biol.* 111:3097-3116.
- Feder, D., and J. M. Bishop. 1991. Identification of platelet membrane proteins that interact with amino-terminal peptides of pp60^{c-src}. *J. Biol. Chem.* 266:19040-19046.
- Ferrell, J. E., J. A. Noble, G. S. Martin, Y. V. Jacques, and D. F. Bainton. 1990. Intracellular localization of pp60^{c-src} in human platelets. *Oncogene*. 5:1033-1036.
- Fischer von Mollard, G., G. A. Mignery, M. Baumert, M. S. Perin, T. J. Han-

- son, P. M. Burger, R. Jahn, and T. C. Südhof. 1990a. rab3 is a small GTP-binding protein exclusively localized to synaptic vesicles. *Proc. Natl. Acad. Sci. USA*. 87:1988-1992.
- Fischer von Mollard, G., T. C. Südhof, and R. Jahn. 1990b. A small GTP-binding protein dissociates from synaptic vesicles during exocytosis. *Nature (Lond.)*. 349:79-81.
- Garber, E. A., F. R. Cross, and H. Hanafusa. 1985. Processing of p60^{src} to its myristylated membrane-bound form. *Mol. Cell. Biol.* 5:2781-2788.
- Grandori, C., and H. Hanafusa. 1988. pp60^{src} is complexed with a cellular protein in subcellular compartments involved in exocytosis. *J. Cell. Biol.* 107:2125-2135.
- Hirano, A. A., P. Greengard, and R. L. Huganir. 1988. Protein tyrosine kinase activity and its endogenous substrates in rat brain: a subcellular and regional survey. *J. Neurochem.* 50:1447-1455.
- Johnston, P. A., P. L. Cameron, H. Stukenbrok, R. Jahn, P. De Camilli, and T. C. Südhof. 1989. Synaptophysin is targeted to similar microvesicles in CHO and PC12 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2863-2872.
- Kamps, M. P., J. E. Buss, and B. M. Sefton. 1985. Mutation of NH₂-terminal glycine of p60^{src} prevents both myristoylation and morphological transformation. *Proc. Natl. Acad. Sci. USA*. 82:4625-4629.
- Kaplan, J. M., H. E. Varmus, and J. M. Bishop. 1990. The src protein contains multiple domains for specific attachment to membranes. *Mol. Cell. Biol.* 10:1000-1009.
- Krueger, J. G., E. A. Garber, A. R. Goldberg, and H. Hanafusa. 1982. Changes in amino-terminal sequences of pp60^{src} lead to decreased membrane association and decreased *in vivo* tumorigenicity. *Cell*. 28:889-896.
- Linstedt, A. D., and R. B. Kelly. 1991. Synaptophysin is sorted from endocytotic markers in neuroendocrine PC12 cells but not transfected fibroblasts. *Neuron*. 7:1-9.
- Lipsich, L. A., A. J. Lewis, and J. S. Brugge. 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. *J. Virol.* 48:352-360.
- Maness, P. F., M. Aubry, C. G. Shores, L. Frame, and K. H. Pfenninger. 1988. c-src gene product in developing rat brain is enriched in nerve growth cone membranes. *Proc. Natl. Acad. Sci. USA*. 85:5001-5005.
- Mizoguchi, A., S. Kim, T. Ueda, A. Kikuchi, H. Yorifuji, N. Hirokawa, and Y. Takai. 1990. Localization and subcellular distribution of smg p25A, a ras p21-like GTP-binding protein, in rat brain. *J. Biol. Chem.* 265:11872-11879.
- Navone, F., R. Jahn, G. D. Gioia, H. Stukenbrok, P. Greengard, and P. De Camilli. 1986. Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J. Cell Biol.* 103:2511-2527.
- Parsons, S. J., and C. E. Creutz. 1986. pp60^{src} activity detected in the chromaffin granule membrane. *Biochem. Biophys. Res. Commun.* 134:736-742.
- Resh, M. D. 1989. Specific and saturable binding of pp60^{v-src} to plasma membranes: evidence for a myristyl-src receptor. *Cell*. 58:281-286.
- Resh, M. D., and R. L. Erikson. 1985. Highly specific antibody to Rous sarcoma virus src gene product recognizes a novel population of pp60^{v-src} and pp60^{c-src} molecules. *J. Cell Biol.* 100:409-417.
- Resh, M. D., and H. Ling. 1990. Identification of a 32K plasma membrane protein that binds to the myristylated amino-terminal sequence of p60^{v-src}. *Nature (Lond.)*. 346:84-86.
- Schiebler, W., R. Jahn, J. P. Doucet, J. Rothlein, and P. Greengard. 1986. Characterization of synapsin I binding to small synaptic vesicles. *J. Biol. Chem.* 261:8383-8390.
- Schweizer, A., J. A. M. Fransen, T. Bächli, L. Ginsel, and H. Hauri. 1988. Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. *J. Cell Biol.* 107:1643-1653.
- Sihra, T. S., J. K. T. Wang, F. S. Gorelick, and P. Greengard. 1989. Translocation of synapsin I in response to depolarization of isolated nerve terminals. *Proc. Natl. Acad. Sci. USA*. 86:8108-8112.
- Sobue, K. 1990. Involvement of the membrane cytoskeletal proteins and the src gene product in growth cone adhesion and movement. *Neurosci. Res. Suppl.* 13:80-91.
- Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell*. 64:693-702.
- Südhof, T. C., and R. Jahn. 1991. Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron*. 6:665-677.
- Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt. 1976. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature (Lond.)*. 260:170-173.
- Torri-Tarelli, F., A. Villa, F. Valtorta, P. De Camilli, P. Greengard, and B. Ceccarelli. 1990. Redistribution of synaptophysin and synapsin I during α -latrotoxin-induced release of neurotransmitter at the neuromuscular junction. *J. Cell Biol.* 110:449-459.
- Tsukita, S., K. Oishi, T. Akiyama, Y. Yamanashi, T. Yamamoto, and S. Tsukita. 1991. Specific proto-oncogenic tyrosine kinases of src family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. *J. Cell Biol.* 113:867-879.
- Wiedenmann, B., H. Rehm, M. Knierim, and C.-M. Becker. 1988. Fractionation of synaptophysin-containing vesicles from rat brain and cultured PC12 pheochromocytoma cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 240:71-77.