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SYNAPTIC VESICLE BIOGENESIS: TARGETING OF SYNAPTIC VESICLE PROTEINS

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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of the

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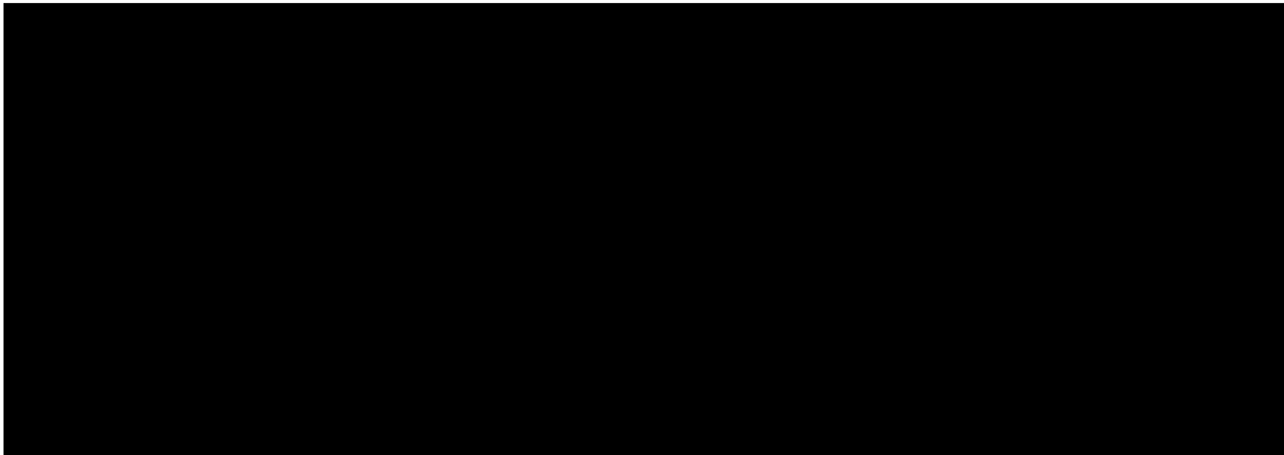
San Francisco



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This thesis is dedicated to my immediate family:

Mom, Dad, Laurel, Pam, Grandma Wanda, and Grandpa Dan.

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Synaptic Vesicle Biogenesis: Targeting of Synaptic Vesicle Proteins.

Adam Dawson Linstedt

ABSTRACT

The experiments presented here characterize the targeting of two membrane proteins, one integral and one peripheral, to synaptic vesicles.

Synaptophysin is a major synaptic vesicle protein that spans the membrane four times. To ask whether synaptic vesicle targeting information is entirely nerve cell specific, I have generated a full length synaptophysin cDNA clone and used it to express synaptophysin in non-neuronal cells. Synaptophysin was localized to endocytotic vesicles that contained early endosome markers and were larger than synaptic vesicles. The neuroendocrine cell line, PC12, contained these vesicles in addition to synaptic vesicle-sized vesicles that excluded an endosome marker. These data clarify conflicting reports (Leube et al., 1989; Johnston et al., 1989, Clift O'Grady et al., 1990) and suggest that synaptophysin contains targeting information for both endosomes and synaptic vesicles.

Endosome targeting of receptor proteins via the clathrin-coated vesicle pathway involves specific sequences within cytoplasmic tail domains. To begin to test whether sequences within the COOH-cytoplasmic domain of synaptophysin mediate its endosomal targeting I have expressed a synaptophysin molecule lacking this domain in 3T3 cells and measured its rate of internalization. Although full length synaptophysin was efficiently endocytosed, I could not detect internalization of the mutant construct. This finding suggests that the COOH-terminal tail is required for targeting of synaptophysin to early endosomes. The presence of an endosomal targeting signal within synaptophysin suggests the involvement of endosomes in synaptic vesicle biogenesis.

The peripheral membrane protein I have studied is $pp60^{c-src}$, the myristylated protein tyrosine kinase product of the proto-oncogene c-src. Although $pp60^{c-src}$ has diverse cellular localizations, I have found that in PC12 cells it is specifically associated with synaptic vesicles since: i) the $pp60^{c-src}$ immunofluorescent pattern overlapped with synaptophysin, ii) 50% of the $pp60^{c-src}$ was recovered in a crude fraction enriched in synaptic vesicles, iii) an antibody against the COOH-cytoplasmic tail of synaptophysin immunodepleted all of the $pp60^{c-src}$ vesicles in this fraction, and iv) $pp60^{c-src}$ co-purified with synaptophysin during a 100-fold purification of PC12 synaptic vesicles.

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INTRODUCTION

Introduction

Synaptic vesicles mediate synaptic transmission by releasing their neurotransmitter content by exocytosis in response to cell stimulation. After fusion with the presynaptic plasma membrane, new synaptic vesicles are formed by endocytosis and eventually refill with neurotransmitter. Prior to exocytosis it is thought that they are first part of a pool clustered near sites of release and then become part of a readily releasable pool actually docked at sites on the presynaptic plasma membrane. These processes, fusion, budding, transmitter uptake, clustering, and docking, and their regulation must be performed by the proteins of the synaptic vesicle alone or together with other components in the nerve terminal. Great progress has taken place recently, and can be expected to continue, in linking each process with the proteins that are involved. This progress is reviewed in chapter 1 of this manuscript, and also in a recently published review by Südhof and Jahn (1991).

Synaptic vesicles are readily recognizable in electron micrographs as electron lucent spherical vesicles accumulated at presynaptic sites in neuronal tissues. Their small and uniform size (40-50nm diameters) make them unique organelles, presumably specialized for the release of packets of neurotransmitter. These physical characteristics have allowed for the purification of synaptic vesicles to near homogeneity and subsequently the characterization of components that comprise synaptic vesicles. In general the protein components of synaptic vesicles are highly concentrated in synaptic vesicles relative to other cellular compartments. The ultimate motivation of the experiments described in this manuscript is to understand how synaptic vesicle specific proteins are targeted to the synaptic vesicle. Since it is targeting that generates the organelle this can restated: how is a synaptic vesicle made?

The protein composition of synaptic vesicles is relatively simple containing less than 50 major protein components (Südhof and Jahn, 1991). The most abundant of these have been isolated, characterized with antibodies, and the amino acid sequence deduced from cDNA clones. Because there is no obvious sequence motif shared by all synaptic vesicle proteins the mechanism mediating their specific localization may be complex. In addition the endocytotic pathway by which synaptic vesicles reform remains controversial (reviewed in Heuser, 1989). Reformation directly from the plasma membrane (Ceccarelli et al., 1973) suggests a mechanism, and presumably targeting signals, novel to neuronal cells. If, on the other hand, synaptic vesicle reformation involves internalization in coated vesicles (Heuser and Reese, 1973) then synaptic vesicle proteins should also have internalization sequences that direct delivery to early endosomes. Chapter 2 and chapter 3 characterize the targeting of a major integral membrane protein of the synaptic vesicle, synaptophysin. In brief synaptophysin appears to contain signals for localization to both endosomes and synaptic vesicles. The endosome targeting signal is present, at least in part, in the COOH-terminal cytoplasmic tail. These results reinforce the model that synaptic vesicle recycling involves an endosome-like intermediate.

Synaptic vesicle proteins include both peripheral and integral membrane proteins. The mechanisms mediating targeting of peripheral membrane proteins are likely to be distinct. Chapter 4 establishes the targeting of a peripheral membrane protein, $pp60^{c\text{-}src}$, to PC12 synaptic vesicles. This extends previous reports that indicated that $pp60^{c\text{-}src}$ is a component of synaptic vesicles (Hirano et al., 1988; Barnekow et al., 1990) and establishes a system for addressing several key issues regarding the targeting of the c-src protein. Unlike other synaptic vesicle proteins, $pp60^{c\text{-}src}$ is expressed in cells that do not contain synaptic vesicles and it appears to be localized to a variety of cellular compartments. This diverse targeting could be explained by the presence of multiple src receptors (Resh, 1989; Resh and Ling, 1990; Feder et al., 1991), that may interact with unique domains in

the src molecule (Kaplan et al., 1990). The relative simplicity of the PC12 synaptic vesicle protein composition may allow identification of a synaptic vesicle src receptor. Furthermore, domains within src that mediate localization to synaptic vesicles may be mapped since the targeting of mutagenized src constructs can be quantitated after transfection of PC12 cells.

CHAPTER 1

Molecular Architecture of the Nerve Terminal

Current Opinion in Neurobiology, in press.

Summary

The synaptic vesicle has been the focus of considerable progress in a molecular description of the nerve terminal. Both the functioning and the molecular composition of the synaptic vesicle are well characterized, in part due to their relative simplicity. This review concentrates on recent work that identifies the specific proteins involved in some of the functions and their mechanism of action.

Introduction

Synaptic vesicles contain neurotransmitter and release their content by exocytosis during synaptic transmission. Of the synaptic vesicles clustered at sites of release, a subset are docked at the active zone, the region of plasma membrane that contacts the postsynaptic cell. Because neurotransmission is so fast, only the docked synaptic vesicles can mediate the earliest release of neurotransmitter. The empty sites are then refilled from the reservoir of clustered vesicles. Synaptic vesicles are reformed by endocytosis at the terminal and refilled by active transport of neurotransmitter. Paralleling the release of neurotransmitter from synaptic vesicles is the release of neuropeptide from large dense core secretory vesicles, which are not constrained to dock and fuse at the active zone. Large dense core secretory vesicles are formed at the Golgi complex and transported on microtubules to sites of release. The exact nature of the transport vesicle that carries synaptic vesicle components is not known.

Recent research has helped us to understand how synaptic vesicle membranes are recovered by endocytosis and why nerve terminals might have two mechanisms of paracrine communication in parallel, the neurotransmitter and the neuropeptide. The components of the synaptic vesicles have recently been reviewed (Südhof and Jahn, 1991). That review emphasized reasonable predictions about the functions of synaptic vesicle membrane proteins, given their amino acid sequence and their structure. In this review we

focus more on the predictions cell biology makes for the function and targeting of nerve terminal, especially synaptic vesicle, proteins.

Synaptic Vesicle Clustering and Docking

We have a much clearer picture of the molecules involved in vesicle clustering because of the work of Greengard and his colleagues on the synapsins (DeCamilli et al., 1990). The synapsins are a family of closely related phosphoproteins that interact with synaptic vesicles and actin filaments. Because of their location, their physical properties and their phosphorylation during neurotransmitter release they are likely to play a role in vesicle clustering. A recent paper by Han et al. (1991) beautifully supported that conjecture by showing that increased expression of one of the synapsins, synapsin IIb, in a neuroblastoma-glioma hybrid NG-108, induced both synaptic vesicle-filled varicosities and synapses between these cells (for review see Kelly, 1991). The mechanism by which the cluster is localized to the active zone is unknown, but is likely to involve a link with a presynaptic plasma membrane component.

Synaptic vesicles may be restrained to the active zone region by cytoskeletal elements, but if this pool of synaptic vesicles is to replace vesicles emptied by exocytosis, the restraints must be transiently removed during exocytosis. The synapsin-actin network has excellent credentials for a restraining role since actin disassembly correlates with increased norepinephrine release from synaptosomes (Bernstien and Bamburg, 1989), and synapsin association with synaptic vesicles and actin is diminished by the types of phosphorylation events that occur during exocytosis (DeCamilli et al., 1990).

Since docking involves localization of synaptic vesicles to sites along the plasma membrane opposite the synaptic cleft it must involve components in both the synaptic vesicle and the plasma membrane. Filamentous elements associated with the plasma membrane in the frog neuromuscular junction, or the elements of the ribbon synapse are obvious candidates for participation in docking. Although the molecular components

involved have not been identified the interaction of synaptophysin with physophillin, a protein that appears to be present in presynaptic membranes, is consistent with the type of protein-protein interaction likely to mediate docking (Thomas and Betz, 1990).

The fidelity of synaptic vesicle-plasma membrane interactions may be increased by the functioning of rab3A (also called smg p25A), a synaptic vesicle associated member of the family of low molecular weight GTPases. Strong evidence implicates the rab GTPases in intraorganelle transport, and the localization of different rab proteins to different subcompartments suggests that they may play a role in targeting fusion events. An insightful model frequently used to explain how they might function is based on the function of another GTPase, EF-Tu (Bourne et al., 1990). EF-Tu inhibits an essentially irreversible step in protein synthesis, peptide-bond formation, until it is reasonably likely that the molecule bound to the synthetic machinery is the correct one and not an inappropriate one. Correct interactions are high affinity ones, and will be of longer duration than incorrect, low affinity ones. To measure the affinity of the interaction, its duration is measured by initiating the GTPase activity of the GTP-protein on contact. If the interaction lasts until GTP is hydrolyzed to GDP, inhibition is removed and the reaction proceeds. By analogy, the only vesicle that will go to an irreversibly docked state on the cell surface will be those that attach for a sufficiently long time for the GTP to hydrolyse (see Figure 1.1). Obviously, if a timing mechanism with a single GTP-binding protein improves the accuracy of the docking events, the accuracy of the timing mechanism is enhanced if two or more GTP-binding proteins have to be converted to the GDP form. As yet there is no evidence for the involvement of more than one rab protein in docking.

It is important, however, not to accept the Bourne model of GTP-binding protein function as dogma. It very much needs to have its predictions put to experimental test. One such test has been to explore the effect on rab3A distribution of stimulating neurotransmitter release from synaptosomes. Immediately after depolarization of synaptosomes rab3A is no longer present in the synaptic vesicle containing fraction, even

though the amount of the vesicle membrane protein, synaptophysin, remains constant (Fischer von Mollard et al., 1990). This result fits exactly with the prediction of the model if we assume that all the synaptic vesicles in the synaptosome dock and fuse with the plasma membrane during the depolarization, that the vesicle membrane recycles quickly after exocytosis, and that the recycled membranes bind rab3A very poorly. If any of these assumptions proves to be incorrect then alternative models may be necessary.

What could be inducing rab3A dissociation, assuming it is occuring? The cytoplasmic protein, GDP dissociation inhibitor, interacts with the GDP form of rab3A causing dissociation and preventing reassociation of rab3A-GDP with synaptic membranes (Araki et al., 1990). Alternatively, stimulation dependent phosphorylations could be important since two other rab proteins are known to be phosphorylated by the protein kinase cdc2 and their membrane association changes during the cell cycle (Baily et al., 1991). This kinase is known to block fusion between endosomes (Tuomikoski et al., 1989). When another low molecular weight GTPase, smg p21B, is phosphorylated by protein kinase A, it binds a regulatory protein, smg p21 GDP dissociation stimulator, and is translocated from membrane to cytoplasm (Hata et al., 1991).

Synaptic Vesicle Fusion

Formally one can distinguish machinery involved in clustering, docking and fusion, but in practice the same molecules may be involved in these functions. The interaction that underlies docking could play a crucial role in fusion. Synaptic transmission can occur in such a brief time interval that one must propose little more than a single event (e.g. conformational change) taking place prior to fusion of docked vesicles. Almers has proposed a specific mechanism of fusion in which the initial step is a change in a preassembled pore from a closed to an open state forming a conductance channel (Almers and Tse, 1990). As stated above the Bourne model predicts that Rab3A dissociation would be coupled to an irreversible step in docking. One such step could be assembly of the pore

in a closed configuration. Opening the pore, to allow neurotransmitter release, would be triggered by interaction of calcium with the molecular components of the preassembled pore. Possible molecular components contributed by the synaptic vesicle to formation of such a pore include synaptophysin and synaptotagmin. Synaptophysin has channel forming ability (Thomas et al., 1988), has a calcium binding activity in its COOH-cytoplasmic tail (Rehm et al., 1986), and as mentioned above can interact with a plasma membrane component (Thomas and Betz, 1990). The cytoplasmic tail of synaptotagmin has an acidic phospholipid binding activity and a putative calcium binding domain (Perrin et al., 1990).

During extensive stimulation synaptic vesicle membrane is rapidly added to the plasma membrane, to the point of synaptic vesicle depletion (Torri-Tarrelli et al., 1990). This creates two problems for the docking/fusion pore model. First, one must understand at a molecular level how to proceed from a proteinaceous pore to fused membranes. Almers presents an ingenious solution that involves lipid flow between subunits (Almers and Tse, 1990). However, if taken too literally this model requires the subunits to take up an unprecedented topology in the membrane, in which, for example, the walls of the channel become completely extracellular, even regions that were intracellular during biosynthesis. Second, a plasma membrane docking protein involved in fusion must either be in vast excess or recycle back to an active state much more rapidly than the synaptic vesicle components.

An alternative might be that the membrane of a docked vesicle fuses with the plasma membrane by a phospholipid rearrangement. Highly localized rearrangements in pure phospholipid bilayers driven by an attractive hydrophobic force allow fusion without having to overcome repulsive forces such as hydration (Helm et al., 1989). The involvement of proteins (fusase) could regulate and stabilize phospholipid rearrangements between the membranes. It is difficult, however, to see how a lipid lined pore can be

formed in the time required, and give the relatively stable conductances observed by Almers and his colleagues (Almers and Tse, 1990).

Recycling of Synaptic Vesicles

It is well-established that synaptic vesicle membranes recycle at the nerve terminal. A clue to the mechanism of recycling comes from expression of the synaptic vesicle membrane protein, synaptophysin, in fibroblast cells. By immunofluorescence (Johnston et al., 1989) or by biochemical analysis (Linstedt and Kelly, 1991), synaptophysin is targeted to the endosomal pathway, by information residing in its carboxyterminal cytoplasmic tail (Kaneda & Kelly, unpublished results). It seems likely, therefore, that the synaptic vesicle is generated as a branch of the endosomal recycling pathway. A similar proposal has been made for the transcytotic pathway in epithelial cells, although in this case Mostov and his colleagues have managed to identify the targeting signal and show that it is a site of phosphorylation (Casanova et al., 1990).

Sorting of the polymeric immunoglobulin receptor out of the endosomal cycle requires a phosphorylation event (Casanova et al., 1990). Diversion of the EGF receptor into the degradative pathway requires an active kinase and autophosphorylation (Helin and Beguinot, 1991; Sorkin et al., 1991). When a carboxyterminal region of the mannose 6-phosphate receptor is deleted it remains in the endosome cycle and is targeted inefficiently to the prelysosomal compartment (Lobel et al., 1989). These examples of multistage targeting strongly suggest that synaptic vesicle membrane proteins will also have two sorting elements, one targeting to the endosome and a second targeting from the endosome into the synaptic vesicle. The machinery for reading the synaptic vesicle level of targeting is apparently not present in fibroblasts since no synaptophysin-rich, synaptic vesicle-like structures are seen in transfected fibroblasts (Linstedt and Kelly, 1991; see Leube et al., 1989 for opposing view). A plausible model incorporating what is known of synaptic vesicle biogenesis is shown in Figure 1.2. Again we do not know what specific features of

neuroendocrine cells might allow synaptic vesicle formation, there are likely candidates, for example the neural specific light chain of clathrin (Acton and Brodsky, 1990) or the neural specific alpha- adaptin (Robinson 1989).

Generation of synaptic vesicles from endosomes is also consistent with the morphological data on photoreceptors (Sulzer and Holtzman, 1989) and the temperature-sensitive Drosophila mutant, shibire (Koenig and Ikeda, 1989). What is still unclear is how synaptic vesicle proteins get from plasma membrane to endosome. Some data argue for clathrin-coated vesicle involvement, others for invagination mechanisms and indeed both pathways might operate in parallel and be used under different physiological conditions. The exact route may not be important since in non-neuronal cells, both the fast clathrin-coated mechanism and the slower non-clathrin coated one both deliver to the early endosome (Tran et al., 1987). It is likely that the shibire mutation, now known to induce a defect in the protein dynamin (van der Bliek and Meyerowitz, 1991; Chen et al., 1991), blocks internalization (step 1, Figure 1.2). Although shibire oocytes accumulate coated pits at the restrictive temperature (Tsuruhara et al., 1990), shibire synapses do not, and synaptic vesicle reformation upon return to the permissive temperature is mediated by non-coated structures (Koenig and Ikeda, 1989). Therefore, if there are two routes to the early endosome, dynamin must be involved in both.

Biogenesis of Synaptic Vesicles

Although synaptic vesicles can be generated by endocytosis, the membrane proteins must somehow get to the plasma membrane from its synthesis site in the cell body. Either there is a unique mechanism for transporting synaptic vesicle proteins down the axon or they are transported down with other nerve terminal components. It now seems clear that the proposed transport of synaptic vesicle proteins to the nerve terminal in the membranes of dense core secretory granules (Lowe et al., 1988) does not fit the data from biosynthesis studies in PC12 cells (Cutler and Cramer, 1990). This leaves two possibilities: synaptic

vesicle components are not sorted from constitutively secreted material, or there exists a novel pathway out of the Golgi in cells with synaptic vesicles.

It does not seem likely that synaptic vesicles are transported down to the nerve terminal. The unc-104 mutant of C. elegans has few if any synaptic vesicles in its synaptic boutons. Correlating with the loss of synaptic vesicles is the accumulation of vesicles in the cytoplasm of the cell body (Hall and Hedgecock, 1991). These putative precursor vesicles, however, have a dense content, unlike the clear synaptic vesicles. On the other hand all transport to the nerve terminals cannot be defective for axons have formed. Since the product of the unc-104 gene is a kinesin-like molecule, the mutation may affect a subclass of membranous vesicles that are transported down the axon. It would be exciting to know the molecular components of the vesicles that accumulate in the cell bodies of unc104 mutants.

Two Exocytotic Pathways

The evidence is very strong that the neurotransmitter and the neuropeptide release pathways differ in many ways, including their biosynthesis, the sites of release and the triggers for release. A recent paper, however, that highlighted the differences between the two release pathways, also helped suggest why there might be two pathways and how they are related.

Synaptosomes have little spontaneous, calcium-dependent release of the neurotransmitter glutamate. In contrast, calcium-dependent basal secretion of the neuropeptide CCK in the absence of stimulation is quite high (Verhage et al., 1991). When the intrasynaptosomal calcium level is raised, CCK release occurs at lower calcium levels than glutamate. In contrast, opening the calcium channels preferentially releases glutamate over CCK. The proposed hypothesis is that the synaptic vesicle release sites are located near the calcium channels and so sense a transiently high local calcium concentration. The advantage of such a regulation is that calcium concentrations near release sites will be

elevated only very briefly, because reduction of calcium levels by diffusion is rapid, more rapid than ATP-dependent Ca^{2+} removal from the cytoplasm. Diffusion control, therefore, allows very rapid firing rates.

We have proposed earlier that hormones and presumably neuropeptides have a significant basal secretion rate, since this allows the circulating level of hormone to be depressed, as well as elevated (Matsuuchi and Kelly, 1991). Endocrine cells, therefore, behave physiologically like rod cells in the retina, which can respond normally to both light and darkness. The data of Verhage et al. (1991) show that nerve terminals can also have basal secretion of the neuropeptide CCK, at rest, presumably because neuropeptide secretion occurs at lower cytoplasmic calcium concentrations. The higher calcium concentrations required for neurotransmitter release ensure that neurotransmitter release is highly phasic, with little or no tonic or basal release in the absence of stimulation, and highly localized.

FIGURE 1.1. Model of rab protein function. 1) A vesicle has the rab protein associated with a part of its T-shaped docking apparatus. 2) The vesicle attaches to a docking site in the plasma membrane, making a reversible complex, and does not dock because the rab protein is inhibitory. The docking site on the plasma membrane acts as a GTPase activating protein (GAP) and increases dramatically the GTP-hydrolysis rate of the rab protein. 3) If the association between the vesicle and the plasma membrane is low affinity, the vesicle will dissociate before the GTP is hydrolysed and no permanent docking is achieved. The GTP form of the rab protein is converted to the GDP form, which is then replaced by an exchange factor. 4) After an average time that depends on the hydrolysis rate of the activated rab protein, the GTP form is converted to the GDP form and rab no longer prevents docking. Fusion can come about only when the vesicle is docked.

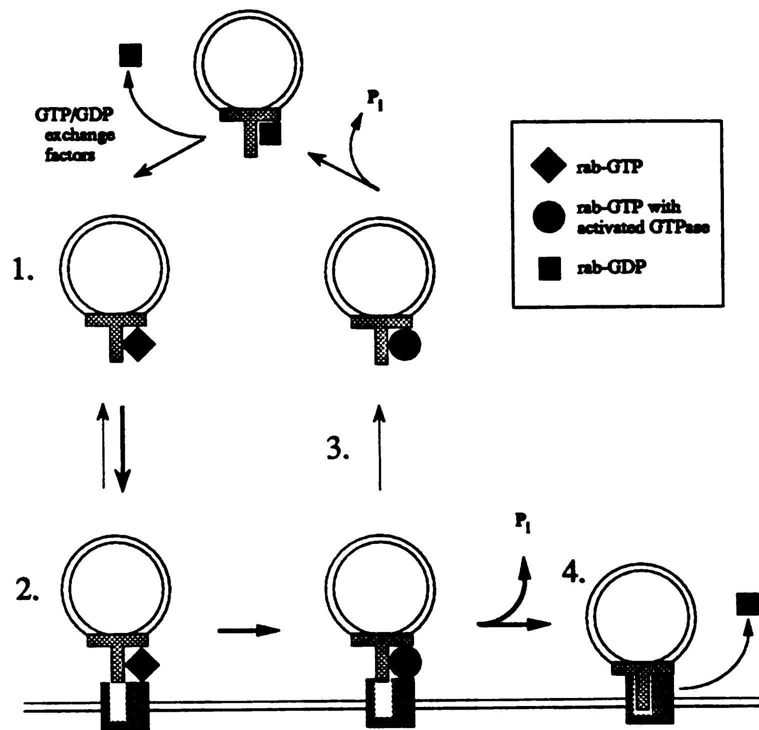
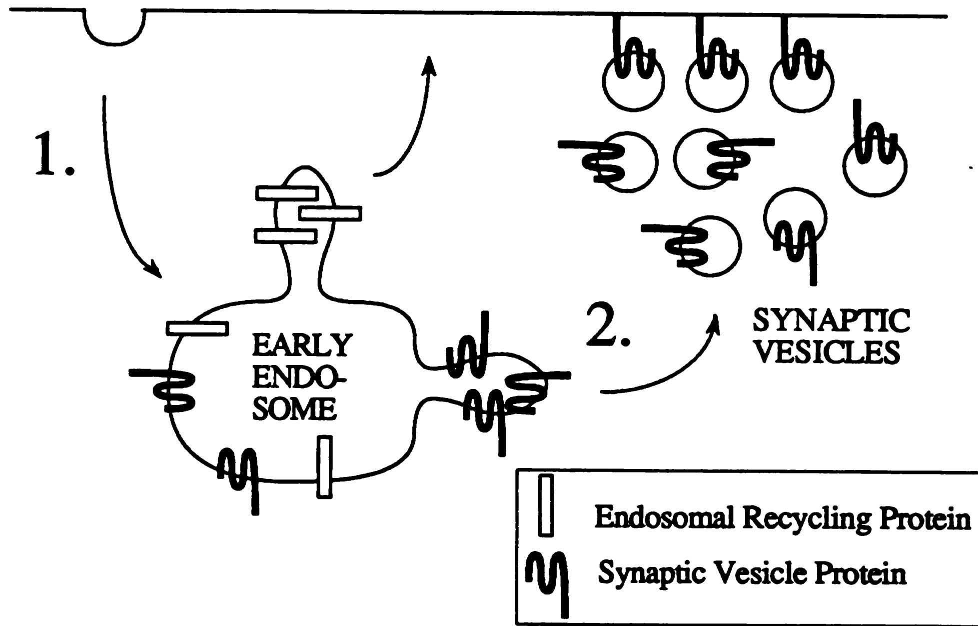

GTP/GDP exchange factors
P_i
rab-GTP
rab-GTP with activated GTPase
rab-GDP
1.
2.
3.
4.
P_i

FIGURE 1.2. Model of synaptic vesicle recycling. 1) Synaptic vesicle proteins are internalized from the plasma membrane and targeted to early endosomes with other endocytosed proteins. 2) Synaptic vesicles are formed in a step that involves sorting of synaptic vesicle components from other proteins in the early endosome.


1.
EARLY
ENDO-
SOME
2.
SYNAPTIC
VESICLES
Endosomal Recycling Protein
Synaptic Vesicle Protein

CHAPTER 2

Synaptophysin is Sorted from Endocytotic Markers in Neuroendocrine PC12 Cells but not Transfected Fibroblasts

Neuron, Vol. 7, 309-317 (1991)

SUMMARY

The targeting of synaptophysin, a major synaptic vesicle protein, in transfected non-neuronal cells has important implications for synaptic vesicle biogenesis, but has proved controversial. We have analyzed four transfected cell types by differential centrifugation and velocity gradient sedimentation to determine whether synaptophysin is targeted to endosomes or to synaptic vesicle-like structures. Synaptophysin was recovered only in vesicles that sedimented more rapidly than synaptic vesicles. The synaptophysin-containing vesicles were labeled if a surface labeled cell was warmed to 37°C, co-migrated with transferrin receptor-containing vesicles on velocity and density gradients, and could be completely immunoadsorbed by anti-LDL receptor tail antibodies. These data demonstrate that synaptophysin was targeted to the early endocytotic pathway in the transfected cells and are inconsistent with the suggestion that synaptophysin expression induces a novel population of vesicles (Leube et al., 1991). Targeting of synaptophysin to early endosomes implicates their role in synaptic vesicle biogenesis.

INTRODUCTION

Synaptic transmission involves fusion of synaptic vesicles with the presynaptic plasma membrane and release of their neurotransmitter content. To replace those lost, new synaptic vesicles are formed in the nerve terminal via an endocytotic pathway and loaded with neurotransmitter by active transport. Recycling of synaptic vesicle membrane components at the nerve terminal obviates a requirement for transport of newly synthesized proteins from the cell body and allows rapid reformation of synaptic vesicles.

Since synaptic vesicles are known to have unique protein components some mechanism must exist for selective inclusion of the unique proteins in synaptic vesicles and the exclusion of other membrane proteins during recycling. At least two explanations have been suggested (for review see Heuser, 1989). One is that a transient opening forms on contact between plasma and synaptic vesicle membranes allowing contents to escape (Ceccarelli et al., 1973; Ceccarelli and Hurlbut, 1980; Torri-Tarelli et al., 1987). In this case, no significant mixing of membranes need occur and synaptic vesicles reform directly from the plasma membrane. Analysis of transient currents associated with mast cell exocytosis indicate the existence of fusion pores between vesicle and plasma membrane (Breckenridge and Almers, 1987). If a similar mechanism operates for synaptic vesicle exocytosis most transmitter may be released through fusion pores (Almers and Tse, 1990). Although closure of mast cell fusion pores has been observed, a more frequent outcome is dilation of the pore with vesicle addition to the plasma membrane (Spruce et al., 1990). That synaptic vesicle and plasma membrane coalesce in the nerve terminal, if only transiently, is indicated by increases in both synaptic vesicle components in the terminal plasma membrane (von Wedel et al., 1981; Valtorta et al., 1988) and total amount of terminal membrane (Heuser and Reese, 1973; Wiley et al., 1987) after extensive stimulation of exocytosis in conditions preventing membrane retrieval.

A second explanation is that segregation takes place by a conventional clathrin-coated pit mechanism (Heuser and Reese, 1973; Miller and Heuser, 1984). Like coated vesicles in all cell types, coated vesicles in the synaptic terminal appear to fuse with endosomes (Heuser, 1989; Sulzer and Holtzman, 1989), and it is from endosomes that new synaptic vesicles are predicted to bud. In support of this model, both coated vesicle mediated endocytosis (Kosaka and Ikeda, 1983; Tsuruhara et al, 1990) and synaptic vesicle reformation (Koenig et al., 1989; Koenig and Ikeda, 1989) are blocked at the restrictive

temperature in the temperature sensitive shibire mutant of Drosophila. However, the mutation may also affect non-coated vesicle endocytosis (Koenig and Ikeda, 1989).

The mechanism by which synaptic vesicle proteins segregate into new synaptic vesicles presumably involves targeting information within synaptic vesicle proteins. The presence of synaptic vesicle membrane proteins in cell lines, neuroendocrine cells such as PC12, or transfected non-neuronal cells, allows easy analysis of what sort of targeting information synaptic vesicle membrane proteins might have and whether the ability to decipher the targeting information might be cell type specific. The data obtained so far have been exciting, but controversial. Immunoelectron micrographs localize transfected synaptophysin to similar synaptic vesicle-size structures in PC12 cells and transfected cells (Leube et al.,1989; Johnston et al.,1989). The coincidence of synaptophysin and transferrin fluorescence patterns in transfected cells suggests that the vesicles might be part of the ubiquitous local recycling pathway containing transferrin receptor (Johnston et al.,1989). However, light microscopy does not have the resolution to prove colocalization to the same membrane, and double labeling studies at the electron microscopic level have not been performed. Indeed, velocity sedimentation revealed that the synaptic vesicle-size vesicles in PC12 cells exclude endosomal markers (Clift-O'Grady et al., 1990). That synaptophysin appears to be the sole protein found after immunoisolation of the synaptophysin-containing vesicles from transfected cells implies that transfected cells might also contain synaptic vesicle-like structures devoid of endosomal proteins (Leube et al.,1989). This finding suggests that the immunoisolated membranes are not pre-existing vesicles to which synaptophysin is targeted but are induced by synaptophysin alone. If synaptophysin can by itself exclude other membrane proteins then we have a simple molecular mechanism explaining synaptic vesicle formation.

To determine whether transfected synaptophysin is targeted to endosomal vesicles or to vesicles that exclude endosomal markers we studied the localization of synaptophysin in non-neuronal cells and compared it to early endosome markers. In contrast to the results with the synaptic vesicle-like vesicles of PC12 cells (Clift-O'Grady et al., 1990), we find that in transfected cells synaptophysin does not accumulate in vesicles with the dimensions of synaptic vesicles, and that synaptophysin co-localizes with both the transferrin and LDL receptors in even the smallest sized vesicles we can identify. These data demonstrate that in the cells we studied, synaptophysin is not inducing a vesicle that excludes other membrane proteins. An explanation of the results of Leube et al. (1989) could be that the other proteins present in synaptophysin-containing vesicles were too sparse to be identified by metabolic labeling. Furthermore, the efficient internalization of synaptophysin and its codistribution with endosomal markers strongly suggest that it is targeted to the early endosome pathway. Since we find that in the neuroendocrine PC12 cells, synaptophysin is targeted to both synaptic vesicles and vesicles that contain the LDL receptor it must have sorting information specifying both these addresses. Our data are therefore very consistent with the involvement of the endosomal pathway in synaptic vesicle biogenesis, supporting the data from morphological studies (Heuser and Reese, 1973; Sulzer and Holtzman, 1989).

METHODS

CELL CULTURES AND TRANSFECTIONS. PC12 growth media was DME H-21 supplemented with 10% horse serum and 5% fetal calf serum. CHO cells were grown in F-12 containing 10% fetal calf serum. COS, 3T3 (including Psi-2 and PA-12), and MDCK cells were grown in DME H-21 containing 10% fetal calf serum. All growth media contained penicillin (100U/ml) and streptomycin (100U/ml). All cell types were grown in humidified incubators at 37°C with 5% CO_2 except PC12 at 10% CO_2. As described

previously (Clift-O'Grady et al., 1990), the pSM expression vector containing SV40 early promoter and synaptophysin cDNA was transiently introduced into COS cells using lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, Maryland). For stable cell lines expressing synaptophysin a retrovirus production and infection protocol was followed (Perkins et al., 1983). The synaptophysin cDNA was cloned into the pMV7 vector (Kirschmeier et al., 1988) at the Hind III cloning site under the regulation of the Moloney murine sarcoma virus 5' LTR. Drug resistance was conferred by the neo gene on the same vector under the regulation of the herpes simplex virus thymidine kinase promoter. The vector was introduced into the Psi-2 packaging cell line by calcium phosphate (Chen and Okayama, 1988). Supernatants were used to infect the PA-12 cell line. These were selected with G418 (400μg/ml) and propagated. Supernatants from these cells were used to transfect MDCK, CHO, and 3T3 cells. After selection with G418 (400μg/ml) individual clones were picked and stable cell lines were screened for synaptophysin expression by immunofluorescence and immunoprecipitation (see below).

IMMUNOFLUORESCENCE. Cells plated on poly-D-lysine coated coverslips were fixed with 3% paraformaldehyde for 30 min., washed two times with PBS, and two times with PBS-glycine (20mM), and then permeabilized for 20 min with PBS-glycine-saponin (0.1%) (Schweizer et al., 1988). The coverslips were then inverted on 15μl PBS-saponin containing SY38 (anti-synaptophysin mAb, Boehringer Mannheim Biochemicals, Mannheim, FRG) at 5μg/ml for 30 min. After five washes with PBS-saponin the coverslips were inverted on 15μl PBS-saponin containing FITC-labeled goat anti-mouse IGG1 (Fisher Biotech, Pittsburgh, PA) diluted two-fold for 30 min. After five final washes with PBS-saponin the coverslips were rinsed with water and mounted on glass slides with DABCO (Sigma, St. Louis, MO).

LABELING AND IMMUNOPRECIPITATION. For metabolic labeling, cells were incubated overnight in DME-H21 media depleted of cysteine and methionine but supplemented with 2% fetal calf serum and [^{35}S]Translabel (ICN K & K Laboratories Inc., Irving, CA) at 100µCi/ml. In some experiments the cells were transferred to normal growth medium for various periods of chase. Surface labeling was achieved with either iodination using ^{125}I sulfo-SHPP (Thompson et al., 1987) or biotinylation (see below). After surface iodination the cells were either maintained on ice or warmed to 37°C for 30 min. Labeled cells (metabolic or surface) were either lysed directly or separated into subcellular fractions (see below) and then lysed. Immunoprecipitations were carried out in buffer containing 1% NP-40, 0.4% deoxycholate, 0.3% SDS, 66mM EDTA, 10mM Tris (pH 7.4) as described previously (Clift-O'Grady et al., 1990). The antibodies used were: an anti-rat brain synaptic vesicle rabbit serum that recognizes synaptophysin, a rat mAb in cell culture supernatant (ATCC TIB 219) against transferrin receptor, a purified rabbit IgG against mannose-6-phosphate receptor (kindly provided by Dr. Bill Brown, Cornell University), and a mouse mAb in cell culture supernatant (X31) against influenza hemagglutinin. In some experiments, the immunoprecipitates were incubated with recombinant Strep plicatus endoglycosidase H (Boehringer Mannheim Biochemicals) overnight prior to SDS-PAGE.

IMMUNOBLOTTING. Proteins were separated by SDS-PAGE (7% or 10% gels) and transferred to nitrocellulose using a semi-dry electrotransfer apparatus (E & K, Saratoga, CA). Blocking and antibody incubations were for 60 min each, separated by 3 five minute washes, in phosphate buffered saline containing 5% non-fat dry milk and 0.05% Tween 20. The detection antibodies , ^{125}I-goat anti-mouse IgG (Cappel, West Chester, PA) or ^{125}I-protein A (ICN) were used at 0.1µCi/ml. The nitrocellulose was exposed to X-ray film at -70°C with an enhancing screen. Autoradiograms were quantitated by optical densitometry. The primary antibodies used were: anti-synaptophysin mouse mAb SY38 at

20ng/ml, anti-gel purified synaptophysin polyclonal rabbit serum at 1:500, and anti-transferrin receptor rat mAb TIB 219 at 1:10. Detection of transferrin receptor required a rabbit anti-rat IgG secondary antibody (Cappel). For the simultaneous detection of synaptophysin and transferrin receptor the nitrocellulose was cut just above the 46kD molecular weight marker (Rainbow markers, Amersham Corp., Arlington Heights, IL) prior to antibody incubations.

DIFFERENTIAL CENTRIFUGATION. Cells were removed from the plates by scraping in buffer A (150mM NaCl, 1mM EGTA, 1mM MgCl2 and 10mM Hepes pH 7.4) and collected by centrifugation at 300xg for 7 min. Homogenization was in 0.8ml buffer A containing protease inhibitors (pepstatin, chymostatin, leupeptin, and aprotinin at 10ng/ml; 1mM PMSF; 1μg/ml o-phenanthroline; 10μM benzamidine) using a Cell Cracker (European Molecular Biology Laboratory) with 10 strokes and a 12μm clearance. The homogenate was separated into a nuclear pellet (P1) and post-nuclear supernatant (S1) by centrifugation at 1,000xg for 5 min in a SS34 rotor (Sorvall, New Town, CN). The S1 was centrifuged at 27,000xg for 30 min in a 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 127,000xg for 60 min in an air centrifuge (Beckman Instruments, Palo Alto, CA). Pellets were resuspended in buffer containing 1% NP40 and protease inhibitors, insoluble material was removed in a microfuge, and protein content was determined (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard. Antigens were assayed by either immunoblotting or immunoprecipitation.

VELOCITY AND DENSITY GRADIENT ANALYSIS. For velocity gradient analysis, samples (200-250μl S1 or S2) were layered on 4.4ml linear 5-25% glycerol gradients in buffer A underlayered with a 0.4ml 50% sucrose pad and centrifuged in a SW55 rotor

(Beckman Instruments) at 4°C for 60 min at either 48,000 or 25,000 rpm (see text). For density gradient analysis samples were layered on 4.8ml linear 10-50% sucrose gradients in buffer A and centrifuged in a SW55 rotor at 4°C overnight at 48,000 rpm. Fractions were collected from the bottom of the tube. Prior to immunoblot analysis, proteins in each fraction were concentrated by precipitation with 10% trichloro-acetic acid in the presence of deoxycholate (0.2mg/ml) as carrier, washed with acetone, and solubilized in 50µl 7M urea.

ENDOCYTOSIS ASSAY. Synaptophysin- or influenza hemagglutinin-transfected cells grown on 10cm plates were washed with PBS five times and once with biotinylation buffer (250mM sucrose, 2mM $CaCl_2$, 10mM triethanolamine-acetic acid (pH 7.4)(Matter et al., 1990)). All steps were carried out on ice except where indicated. The cell surface was then biotinylated by two successive 15min incubations with 500µg/ml NHS-SS-Biotin (Pierce Chemical Co.) in biotinylation buffer. After two five minute washes in DME containing 0.2% BSA, the plates were incubated at 37°C for various times (0, 10, and 30 minutes) to allow endocytosis. The label remaining on the surface was removed by two washes in PBS containing 10% fetal calf serum, two successive 20 min incubations in reducing solution (50mM glutathione, 90mM NaCl, 60mM NaOH, and 10% fetal calf serum), and a 15 min incubation in PBS containing 5mg/ml iodoacetamide and 1% BSA. The antigens were recovered by immunoprecipitation and transferred to nitrocellulose according to the descriptions above. After blocking in TGG (PBS containing 0.5% Tween 20, 1M glucose, 10% glycerol) with 3% BSA and 1% non-fat dry milk, the blot was incubated with ^{125}I-labeled streptavidin (1µCi/ml) in TGG with 0.3% BSA and washed three times in PBS containing 0.5% Tween 20 (Lisanti et al., 1988). For each experiment one plate was maintained on ice and not reduced to allow determination of the total amount of antigen labeled.

ORGANELLE IMMUNOISOLATION. Aliquots of 20mg of Dynabeads M-450, magnetic polystyrene beads coated with sheep anti-mouse IgG1 (Dynal Inc., Great Neck, NY), were incubated with 20μg of either anti-synaptophysin (SY38), anti-LDL receptor (4A4, kindly provided by Dr. Yk Ho, University of Texas), or mouse gamma globulin (Pel-Freez Biologicals, Rogers, AR) overnight at 4°C then rinsed with buffer A. The S2 fraction from two 15cm plates of either transfected 3T3 cells or PC12 cells was divided into four equal . One aliquot was added to the SY38 beads, one aliquot was added to the 4A4 beads, one aliquot 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 127,000xg in an air centrifuge (Beckman Instruments). The resulting pellets and the isolated beads (after 3 five minute washes in buffer A) were lysed and analyzed by immunoblotting.

RESULTS

SYNAPTOPHYSIN EXPRESSION IN NON-NEUROENDOCRINE CELLS. We studied the localization of synaptophysin in transfected cells to determine whether it was targeted to endosomes or to synaptic vesicle-like structures. PC12 cells were used as positive controls because PC12 cells have a population of synaptic vesicle-size vesicles rich in synaptophysin (Leube et al., 1989; Clift-O'Grady et al., 1990). Stable cell lines expressing synaptophysin were generated from two fibroblast cell lines (NIH 3T3 and CHO) and one epithelial cell line (MDCK) by retroviral infection using the MLV LTR promoter. Transient expression of synaptophysin in COS cells was achieved with an SV40 promoter expression construct. Synaptophysin was easily detected in all transfected cell types by immunoprecipitation, immunoblotting or immunofluorescence and was not detected in non-transfected controls. The stably transfected cell lines expressed

synaptophysin at a 5-10 fold lower level than in PC12 cells when detected by immunoblotting and normalized by total protein, whereas transiently transfected COS cells expressed synaptophysin at a 1-2 fold higher level than PC12 cells on a per cell basis (data not shown). Pulse-chase experiments using the transfected 3T3 cells indicated that the half-life of transfected synaptophysin was greater than 24 hours. Furthermore, neither non-glycosylated nor endoglycosidase H sensitive synaptophysin was detectable at steady state (data not shown). All transfected cell types exhibited punctate cytoplasmic staining and strong perinuclear staining when processed by immunofluorescence analysis using antibodies that recognize synaptophysin (data not shown). This pattern was similar to that found for identically processed PC12 cells and to previously published micrographs (Leube et al.,1989; Johnston et al.,1989). Therefore, consistent with previous reports (Leube et al.,1989; Johnston et al.,1989), transfected synaptophysin appeared to be stable, processed normally, and targeted to an intracellular location in non-neuronal cells.

LACK OF SYNAPTIC VESICLE-SIZE VESICLES IN TRANSFECTED CELLS. Although no striking morphological differences were found, synaptophysin in transfected cells and PC12 cells was distributed differently when analyzed using differential centrifugation. Synaptophysin recovery in each of four fractions (P1, P2, P3, S3) was determined for PC12 cells and each of the transfected cell types by immunoblotting (Fig 2.1). Centrifugation at 27,000xg has been shown to separate synaptic vesicle-size vesicles which remain in the supernatant (S2) from larger membranes which sediment (P2)(Clift-O'Grady et al., 1990). The membranes in the S2 were collected as pellets (P3) after high speed centrifugation. While a substantial amount of synaptophysin was recovered in the P3 fraction from PC12 cells, very little was recovered in the P3 fraction from transfected cells (Fig 2.1). Since equal amounts of protein were loaded in each lane the specific activity (cpm/μg protein) of synaptophysin in the P3 fraction of PC12 cells was much greater than for transfected cells. Of the total synaptophysin in the post nuclear

supernatant, 3% (± 3%, n=5) was recovered in the P3 of transfected 3T3 cells compared to 40% (± 5%, n=5) in the P3 of PC12 cells. It appeared that very little if any synaptophysin in transfected cells was in membranes the size of synaptic vesicles. This was confirmed by comparison of samples from PC12 and transfected cells on velocity gradients (Fig 2.2; see also (Clift-O'Grady et al., 1990)). When the post nuclear supernatant (S1) of PC12 cells was analyzed, synaptic vesicle-size membranes were clearly detectable as a peak of synaptophysin in the middle of the gradient in addition to the larger membranes that collected on a pad at the bottom of the tube. No such peak was found for transfected CHO cells; only the larger membranes on the pad were detected (Fig 2.2A). Analysis of 27,000xg supernatants (S2) from PC12 and transfected CHO cells showed that even the small amount of synaptophysin recovered in the S2 fraction from transfected cells contained few synaptic vesicle-size structures (Fig 2.2B). In PC12 cells most of the synaptophysin in the S2 was in synaptic vesicle-size vesicles in the middle of the gradients, whereas the synaptophysin in the S2 fraction from transfected cells was mostly recovered in large membranes that accumulate on a dense pad at the bottom of the gradient. These results agree with those described for transfected COS cells by Clift-O'Grady et al. (1990). The amount of synaptophysin present in membranes of synaptic vesicle size (pool of fractions 5-7) was less than 1% of that present in the S1 and the membranes had a continuous rather than discrete size distribution. Similar results were obtained after homogenization in a low salt buffer used by Leube et al. (1989) and with all other transfected cell types including the epithelial cell line, MDCK. These data are inconsistent with the induction of synaptic vesicle-size vesicles by synaptophysin in our transfected cells.

ENDOCYTOSIS OF SYNAPTOPHYSIN IN TRANSFECTED CELLS. Although synaptophysin was not targeted to synaptic vesicle-size structures in transfected cells it did undergo endocytosis. To compare the endocytosis of synaptophysin with that of the

transferrin receptor, a membrane protein enriched in the early endocytotic pathway, cell surface proteins were labeled by biotinylation using NHS-SS-biotin, a sulfated biotin derivative that reacts with primary amino groups and allows reversible derivatization. After biotinylation, antigens were recovered by immunoprecipitation, transferred to nitrocellulose and detected by blotting with iodinated streptavidin (Lisanti et al., 1988). For the endocytosis assay the cells were warmed to 37°C for 10 or 30 minutes to allow endocytosis of surface labeled proteins, and then the label remaining on the surface was removed by reduction of the disulfide prior to lysis (Le Bivic et al., 1990). Significant amounts of both synaptophysin and transferrin receptor were recovered in a pool resistant to disulfide reduction after 10 minutes of internalization (Fig 2.3). In contrast the same analysis for a marker that is not efficiently endocytosed, influenza hemagglutinin, resulted in no detectable internalization (Fig 2.3). These data demonstrate that synaptophysin was efficiently endocytosed.

ISOLATION OF AN ENDOSOME ENRICHED VESICULAR FRACTION. We next compared the distribution of surface labeled synaptophysin and transferrin receptor in the subcellular fractions P1, P2, S2 to determine whether the synaptophysin-containing vesicles in the S2 fraction were derived by endocytosis. In these experiments cell surface proteins were labeled by cell surface iodination using a membrane impermeant reagent, ^{125}I-sulfo-SHPP (Thompson et al., 1987). After iodination on ice to prevent endocytosis the cells were fractionated and antigens were recovered from each fraction by immunoprecipitation. The majority of synaptophysin (80%) was recovered in the P1 fraction, while the remainder was present in the P2 fraction (Fig 2.4A). Since only synaptophysin present in the plasma membrane would be labeled under these conditions, this experiment showed that the S2 fraction was devoid of plasma membrane. This conclusion was further supported by the finding that the S2 fraction also lacked iodinated transferrin and mannose-6-phosphate receptors (Fig 2.4A). If the cells were warmed to

37°C for 30 minutes to allow endocytosis prior to fractionation, some synaptophysin redistributed into the S2 fraction (Fig 2.4B). Thus the synaptophysin-containing vesicles present in the S2 fraction were derived, at least in part, by endocytosis. Both labeled transferrin and mannose-6-phosphate receptors were also recovered in the S2 fraction after warming (Fig 2.4B) confirming the presence of endocytotic vesicles. To test whether the S2 fraction also contained synaptophysin-vesicles derived from early steps in the biosynthetic pathway, the amount of metabolically labeled synaptophysin recovered in the S2 fraction was determined after overnight labeling with or without a six hour chase. The synaptophysin in the S2 did not appear to be predominantly derived from biosynthetic compartments since the chase did not diminish the amount of synaptophysin recovered (data not shown). Also the synaptophysin in the S2 was not endoglycosidase H sensitive. Taken together these results indicated that synaptophysin-containing vesicles in the S2 fraction were derived largely from endosomal compartments and not from biosynthetic compartments or the cell surface.

CO-SEDIMENTATION OF SYNAPTOPHYSIN AND TRANSFERRIN RECEPTOR VESICLES. We next sought to determine whether the synaptophysin-containing vesicles in the S2 fraction were derived from the early endosome pathway that recycles the transferrin receptor. Although the S2 fraction did not contain a population of synaptic vesicle-size vesicles it was still possible that the synaptophysin-containing vesicles excluded other proteins as suggested by the data of Leube et al. (1989). We first compared the distribution on velocity gradients of synaptophysin and transferrin receptor. Analysis of the same S2 fractions from transfected 3T3 cells for both molecules yielded identical distributions (Fig 2.5A). Even the membranes that sedimented at the position of synaptic vesicle-size membranes (fractions 6-8) showed no relative enrichment of synaptophysin. We next compared the distribution of synaptophysin and transferrin receptor from transfected 3T3 S2s on equilibrium density gradients. Synaptophysin-

containing membranes and transferrin receptor membranes both came to equilibrium at a density of 1.13 g/cc (Fig 2.5B). Therefore, in contrast to what is found in PC12 cells (Clift-O'Grady et al., 1990) synaptophysin in transfected cells is not being sorted into a special class of small vesicles that excludes other endocytotic molecules. These results are consistent with targeting of synaptophysin to small vesicles involved in the local recycling pathway of transferrin receptor.

CO-IMMUNOISOLATION OF SYNAPTOPHYSIN AND ENDOSOME VESICLES. To test directly for colocalization of synaptophysin and markers of the early endocytotic pathway, vesicle immunoisolation experiments were carried out using magnetic immunobeads coated with antibodies against the cytoplasmic tail of an early endosome marker, LDL receptor (4A4, van Driel et al., 1987), or synaptophysin (SY38, Wiedenmann and Franke, 1985). After removal of the immunobeads, membranes remaining in the supernatants were collected by centrifugation and both these membranes and the membranes on the immunobeads were assayed for the presence of synaptophysin by immunoblot. As a control for non-specific adsorption, beads coated with mouse gamma globulin were used. There was neither significant depletion nor detectable binding of synaptophysin membranes when control beads were used to treat S2 supernatants of transfected 3T3 cells (Fig 2.6A, control). In contrast, the anti-synaptophysin beads depleted all synaptophysin-containing membranes and these membranes were recovered on the immunobeads (Fig 2.6A, synaptophysin). The treated supernatants and corresponding immunobeads were also assayed for transferrin receptor by immunoblot. The anti-synaptophysin immunobeads completely depleted transferrin receptor membranes (not detectable in four experiments), and these were recovered (110+15%, n=4) on the immunobeads (data not shown). Therefore membranes containing the early endosome marker, transferrin receptor, contained sufficient synaptophysin to allow immunoisolation. To test whether all synaptophysin membranes contained endocytotic markers, the

transfected 3T3 supernatants were treated with the anti-LDL receptor immunobeads. This treatment resulted in depletion of transferrin receptor as expected (not shown), but also depletion of synaptophysin-containing membranes (Fig 2.6A, LDLR). The synaptophysin was recovered on the isolated beads. This result demonstrates that there was no significant population of vesicles enriched in synaptophysin but devoid of early endosome molecules in the transfected cells.

In contrast to the results presented above for transfected cells, we have evidence in PC12 cells for a synaptophysin-containing vesicle type that excludes transferrin and the LDL receptor (Clift O'Grady et al., 1990; Kaneda, Y. and Kelly, R. B., in preparation). That some synaptophysin in PC12 cell supernatants was indeed in vesicles devoid of LDL receptor was confirmed by treatment with anti-LDL receptor beads. This treatment did not result in depletion of synaptophysin-containing membranes (Fig 2.6B, LDLR). Doubling the amount of the anti-LDL receptor beads did not result in further depletion of synaptophysin suggesting that immunobeads were not limiting. The same amount of anti-synaptophysin immunobeads removed all synaptophysin membranes (Fig 2.6B, synaptophysin), while control beads yielded no significant binding (Fig 2.6B, control). Note, however, that significant amounts of synaptophysin were recovered on the anti-LDL receptor immunobeads (Fig 2.6B, LDLR). This is evidence that even in PC12 cells there is overlap between the endosome pathway and the synaptic vesicle one. We suggest that synaptophysin is targeted to early endosomes but the ability to sort it away from endocytotic markers is only found in neurons and endocrine cells. In the non-neuronal cell lines examined here, there was no evidence that synaptophysin might be capable of inducing a new vesicle type.

DISCUSSION

We have focussed on two properties of synaptic vesicle biogenesis: the generation of a vesicle of homogeneous size, and the exclusion from synaptic vesicles of other membrane markers. Our results demonstrate that in transfected non-neuronal cells synaptophysin did not accumulate in synaptic vesicle-size vesicles. Whereas 30 - 40% of the synaptophysin in PC12 cells is recovered in synaptic vesicle-sized structures, less than 1% is recovered in such vesicles in our transfected cells. Exclusion of other membrane proteins from synaptophysin-containing vesicles has been controversial. One set of data has suggested that transfected synaptophysin is targeted to a pre-existing pathway (Johnston et al.,1989), while another suggests that it induces a novel organelle (Leube et al.,1989) in non-neuronal cells. Each model has important implications for synaptic vesicle biogenesis, one suggesting a modification of the early endosome pathway and the other that synaptophysin by itself can induce a microvesicle that excludes non-synaptic vesicle proteins. In our biochemical experiments synaptophysin was endocytosed with the efficiency of the transferrin receptor, recovered from a vesicle fraction enriched in endosomal markers, and synaptophysin-containing vesicles in this fraction could not be purified away from the transferrin receptor and the LDL receptor either by size separation or by immunopurification. PC12 cells were used as a positive control, because sorting of synaptophysin into a unique population of synaptic vesicle-size vesicles has been clearly demonstrated (Clift-O'Grady et al., 1990; Kaneda, Y. and Kelly, R.B., in preparation; this paper). Our data are not easily consistent, therefore, with induction of a synaptic vesicle-like structure by synaptophysin expression.

The colocalization of synaptophysin and transferrin and LDL receptors to the same vesicles suggests that synaptophysin is targeted to the early endocytotic pathway. This

finding extends the observation of overlapping peri-nuclear and punctate cytoplasmic immunofluorescence patterns of transferrin and synaptophysin (Johnston et al., 1989). One prominent feature of early endosomes is tubular extensions that contain membrane proteins destined for return to the plasma membrane (Geuze et al., 1984). The synaptic vesicle-size structures positive for synaptophysin seen in electron micrographs (Leube et al.,1989; Johnston et al.,1989) of transfected cells may be these tubular structures in cross section. We found no evidence for a homogeneous population of synaptic vesicle-size organelles by velocity gradient analysis. The synaptophysin-containing membranes recovered on the sucrose cushions at the bottom of the velocity gradients may be intact endosomes. The small amount of synaptophysin-containing membranes smeared towards the central fractions of the gradients may represent small vesicles that are part of the local recycling pathway or endosomal tubules that vesicularized during homogenization creating a continuum of small vesicle sizes.

Synaptic vesicle-size vesicles containing only synaptophysin were immunopurified from a synaptophysin-transfected hepatocellular carcinoma cell line homogenized in a low salt buffer (Leube et al.,1989). We transfected a variety of non-neuroendocrine cell types and, in some experiments homogenized using the same buffer, but always recovered synaptophysin in vesicles that co-sedimented with transferrin receptor and sedimented faster than synaptic vesicles. For transfected 3T3 cells we showed that the synaptophysin-containing vesicles also contained LDL receptor and transferrin receptor by immunopurification and immunoblotting. Perhaps hepatocellular carcinoma cells are unusual in their ability to generate small vesicles that contain only synaptophysin. Alternatively, transferrin receptor and LDL receptor may have been missed previously because they were not assayed directly. In the experiments of Leube et al. (1989) synaptophysin was the only radiolabeled species detected after vesicle immunoisolation from metabolically labeled cells. Under these conditions high levels of expression of

transfected synaptophysin may obscure the presence of other proteins. A prediction of our experiments is that immunoblots of the immunoisolated vesicles of Leube et al. (1989) with antibodies against early endosome markers would indicate their presence.

The finding that synaptophysin is targeted to endosomes in both transfected non-neuronal cells and the synaptic vesicle-containing PC12 cells suggests that the pathway for synaptic vesicle biogenesis may be a specialization of the endosomal recycling pathway (Fig 2.7). Specific signals in the synaptophysin molecule may underlie the targeting of synaptophysin. It seems likely that the sequence information that allows endocytosis and trafficking to endosomes in fibroblasts would serve a similar function in neural and neuroendocrine cells. This may be considered the primary sorting signal for synaptic vesicle proteins and would be expected to share properties with signals of other proteins in the endosomal recycling pathway. Although a linear consensus sequence for internalization is not known, the secondary structure surrounding specific tyrosine residues in cytoplasmic tails appears crucial (Ktistakis et al., 1990; Collawn et al., 1990). Synaptophysin contains several tyrosine residues in its carboxy terminal cytoplasmic tail, but their function has not been studied.

Synaptophysin and other synaptic vesicle proteins must then be subjected to a second sorting step that is specific to neural and neuroendocrine cells. The secondary sorting signal would direct synaptic vesicle proteins away from non-synaptic vesicle proteins in the endosome and allow synaptic vesicle formation (Fig 2.7). This sorting step may be mediated by an unknown sequence feature shared among synaptic vesicle proteins. The efficiency of this sorting step could in principal be determined by comparing the ratio of endosomal synaptophysin targeted to synaptic vesicles and that returning to the plasma membrane. The exclusion of the LDL receptor from PC12 synaptic vesicles suggests that

even if sorting of synaptophysin into synaptic vesicles is less than perfect, there is tight control preventing incorporation of non-synaptic vesicle proteins into synaptic vesicles.

The identification of primary and secondary sorting signals is now possible by transfection of cDNAs encoding mutated synaptophysin molecules. Endosomal targeting can be studied in transfected fibroblasts, and synaptic vesicle targeting can be studied in transfected PC12 cells. As the true complexity of membrane protein trafficking emerges it is more apparent that sequential sorting signals must be used for proper targeting. Synaptic vesicle biogenesis offers an excellent system to study such signals.

FIGURE 2.1. Presence of synaptophysin in high speed supernatants of PC12, and transfected cell lines. Equal amounts of protein from each subcellular fraction from the indicated cell types were assayed for synaptophysin by immunoblotting. P1, P2 and P3 centrifugations were 1,000xg for 5 min., 27,000xg for 30min., and 125,000xg for 60 min. respectively. The autoradiogram shown was exposed for 24 hours.

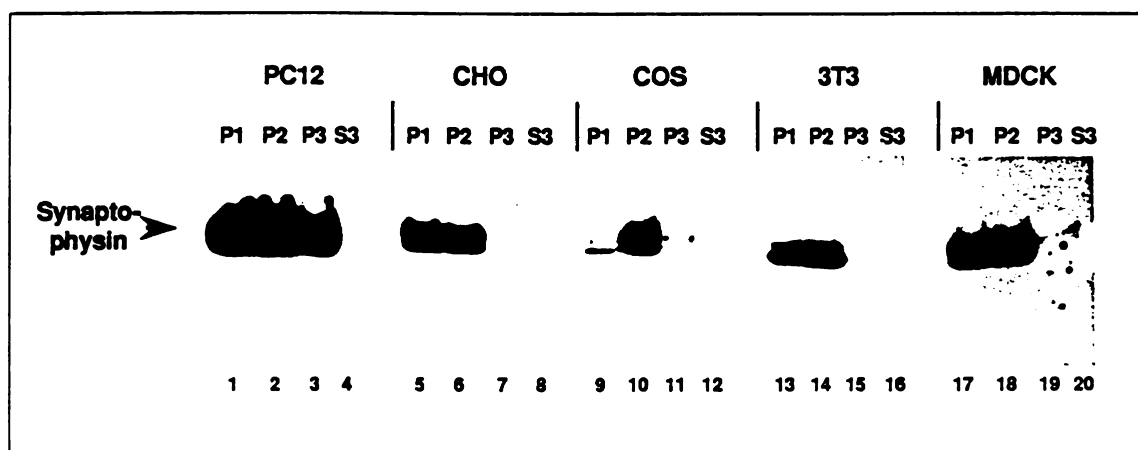

PC12
CHO
COS
3T3
MDCK
P1 P2 P3 S3
P1 P2 P3 S3
P1 P2 P3 S3
P1 P2 P3 S3
P1 P2 P3 S3
Synapto-physin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIGURE 2.2. Velocity gradient analysis of PC12 and transfected cells. PC12 and transfected CHO S1 fractions (A) and S2 fractions (B) were separated on linear 5-25% glycerol gradients underlayed with sucrose cushions, centrifuged at 48K in an SW55 rotor for 60 min., fractionated, and each fraction assayed for synaptophysin by densitometry of immunoblots. The fraction of the total in each fraction is plotted where total equaled the sum of all fractions in the gradient. Fractions are numbered from the bottom.

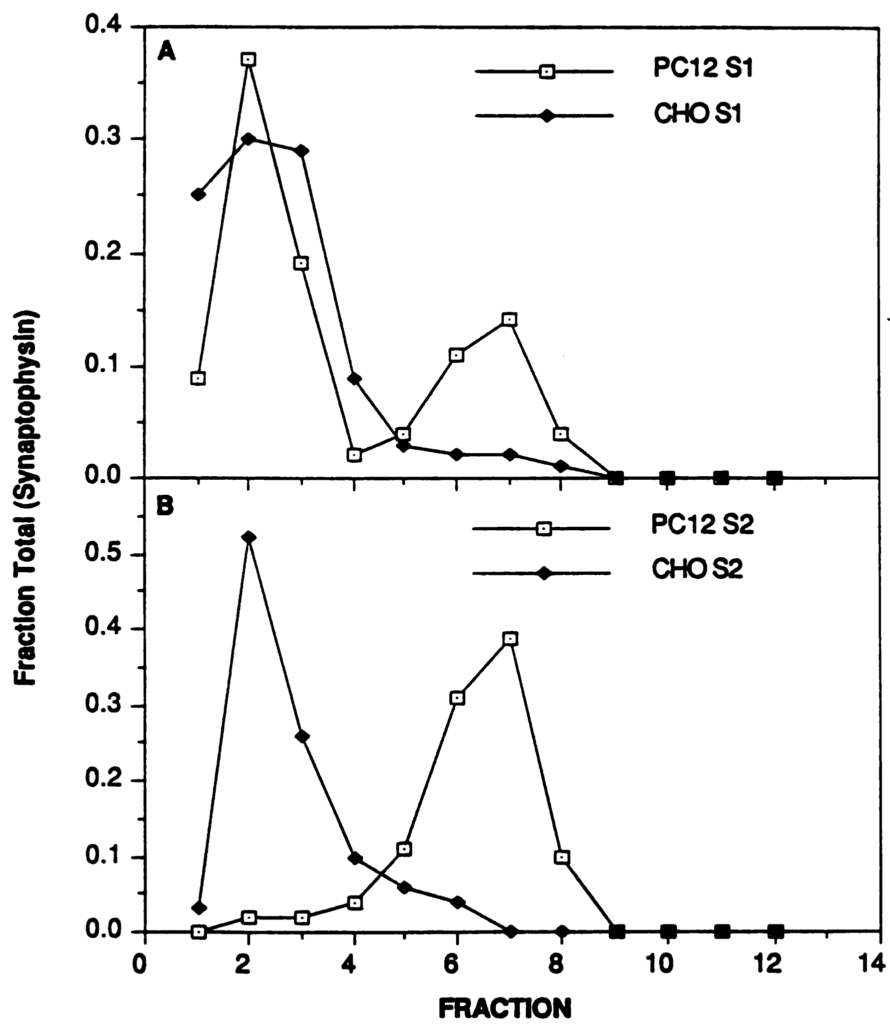

A
PC12 S1
CHO S1
B
PC12 S2
CHO S2
Fraction Total (Synaptophysin)
FRACTION
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
12
14

FIGURE 2.3. Internalization of synaptophysin in transfected 3T3 cells. After surface biotinylation the cells were incubated at 37°C for the times indicated to allow endocytosis. The label remaining on the surface was removed and the amount of labeled synaptophysin, transferrin receptor, or influenza hemagglutinin was determined after immunoprecipitation and blotting with ^{125}I-streptavidin. The data are plotted as the fraction of the amount recovered from cells not treated to remove surface label. The range of the results are shown for synaptophysin (n=2) and transferrin receptor (n=4).

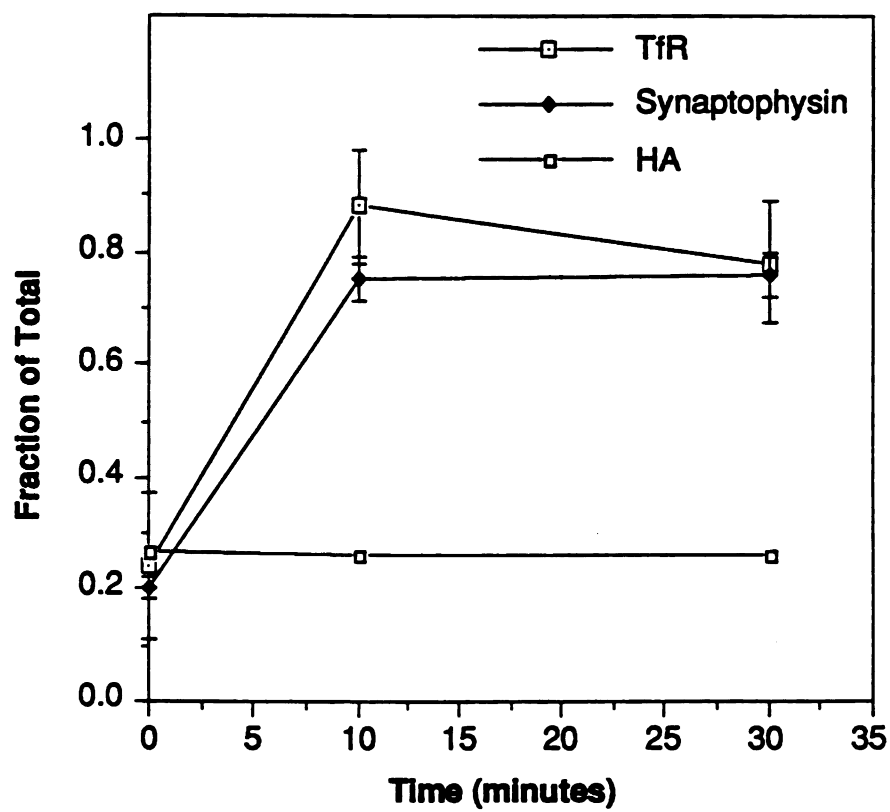
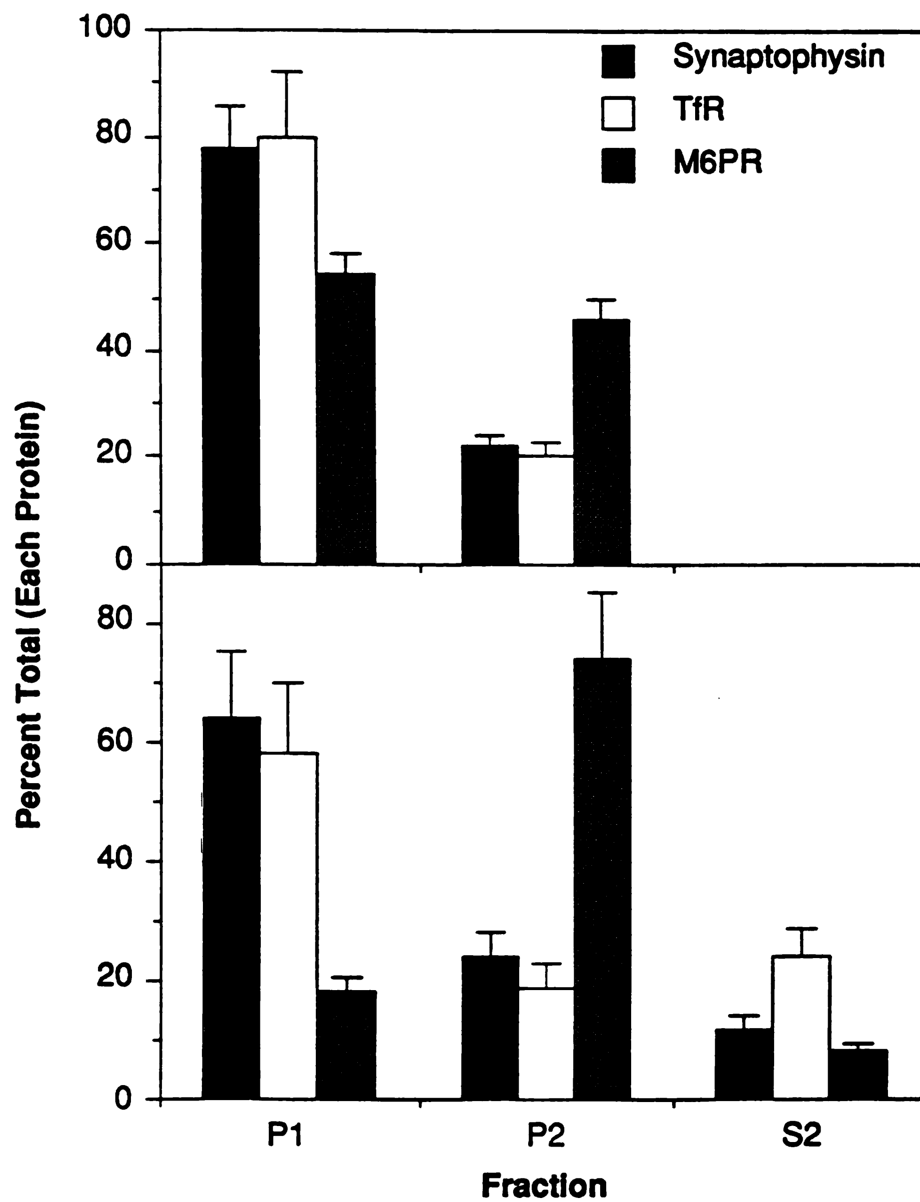
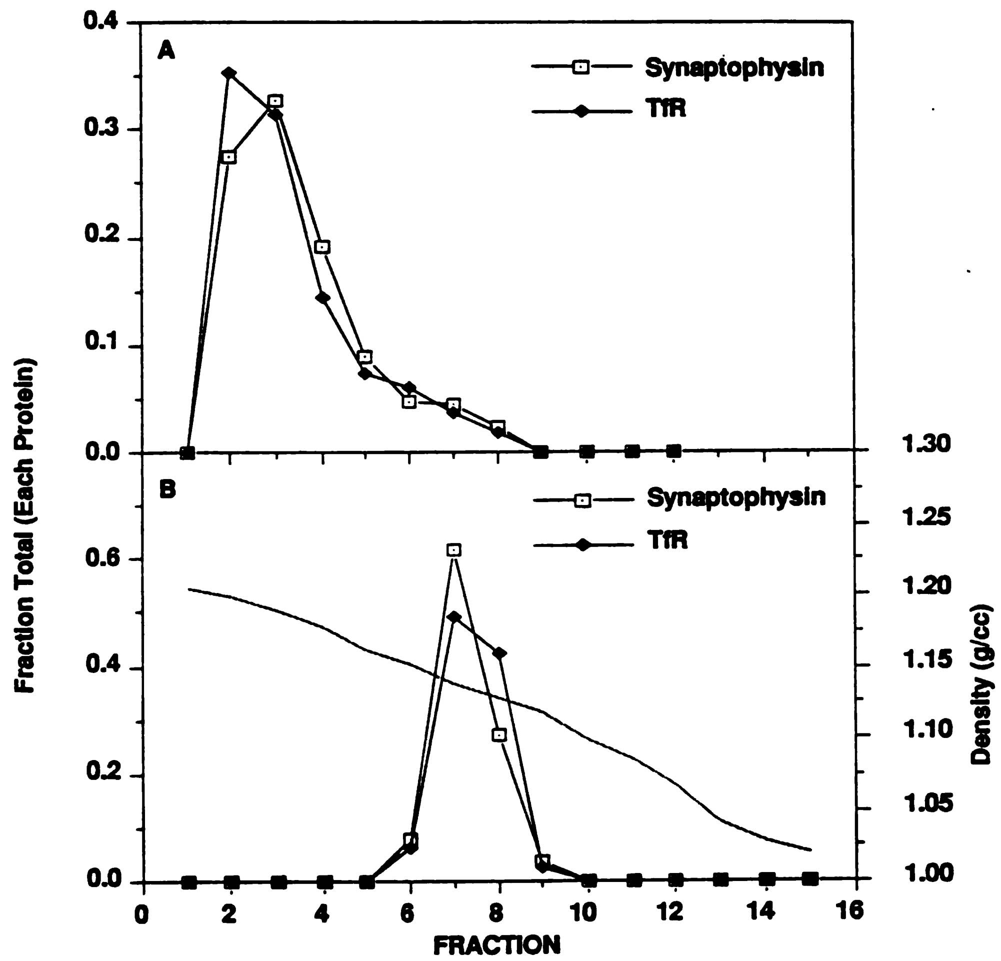

TfR
Synaptophysin
HA
1.0
0.8
0.6
0.4
0.2
0.0
Fraction of Total
0
5
10
15
20
25
30
35
Time (minutes)

FIGURE 2.4. Appearance of surface labeled proteins in the S2 fraction. Surface iodinated cells were fractionated (A) or warmed to 37°C for 30 min and then fractionated (B).Each fraction was assayed for synaptophysin, transferrin receptor, and mannose-6-phosphate receptor by immunoprecipitation. The amount in each fraction (average and range) is plotted as fraction of the total recovered (n=2).



Synaptophysin
TfR
M6PR
100
80
60
40
20
0
80
60
40
20
0
Percent Total (Each Protein)
P1
P2
S2
Fraction

FIGURE 2.5. Comparison of synaptophysin and transferrin receptor by velocity and density gradient analysis. A. Transfected 3T3 S2 fraction separated on a linear 5-25% glycerol gradient underlayed with a sucrose cushion and centrifuged at 48K in an SW55 rotor for 60 min. B. Transfected 3T3 S2 fraction separated on a linear 10-50% sucrose gradient centrifuged at 48K for 18 hr. Each fraction was assayed for synaptophysin and transferrin receptor by densitometry of immunoblots. Fractions are numbered from the bottom.



A
Synaptophysin
TfR
B
Synaptophysin
TfR
Fraction Total (Each Protein)
Density (g/cc)
FRACTION
0.4
0.3
0.2
0.1
0.0
0.6
0.4
0.2
0.0
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0
2
4
6
8
10
12
14
16

FIGURE 2.6. Immunodepletion of synaptophysin and LDL receptor containing membranes from the S2 fraction of transfected 3T3 cells (A) or PC12 cells (B). The S2 fractions were either non-treated, treated with control antibody beads, treated with anti-synaptophysin beads, or treated with anti-LDL receptor beads. After removal of the beads the membranes remaining in each supernatant were collected by centrifugation and analyzed for synaptophysin by immunoblotting (supernatants). The isolated immunobeads were extracted in sample buffer and these extracts were also analyzed for synaptophysin by immunoblotting (immunobeads). The amount of synaptophysin recovered for each treatment is plotted as the fraction of synaptophysin recovered in the non-treated controls. The error bars indicate the range of the results (n=4 for transfected cells, n=2 for PC12 cells). nd= not detected.

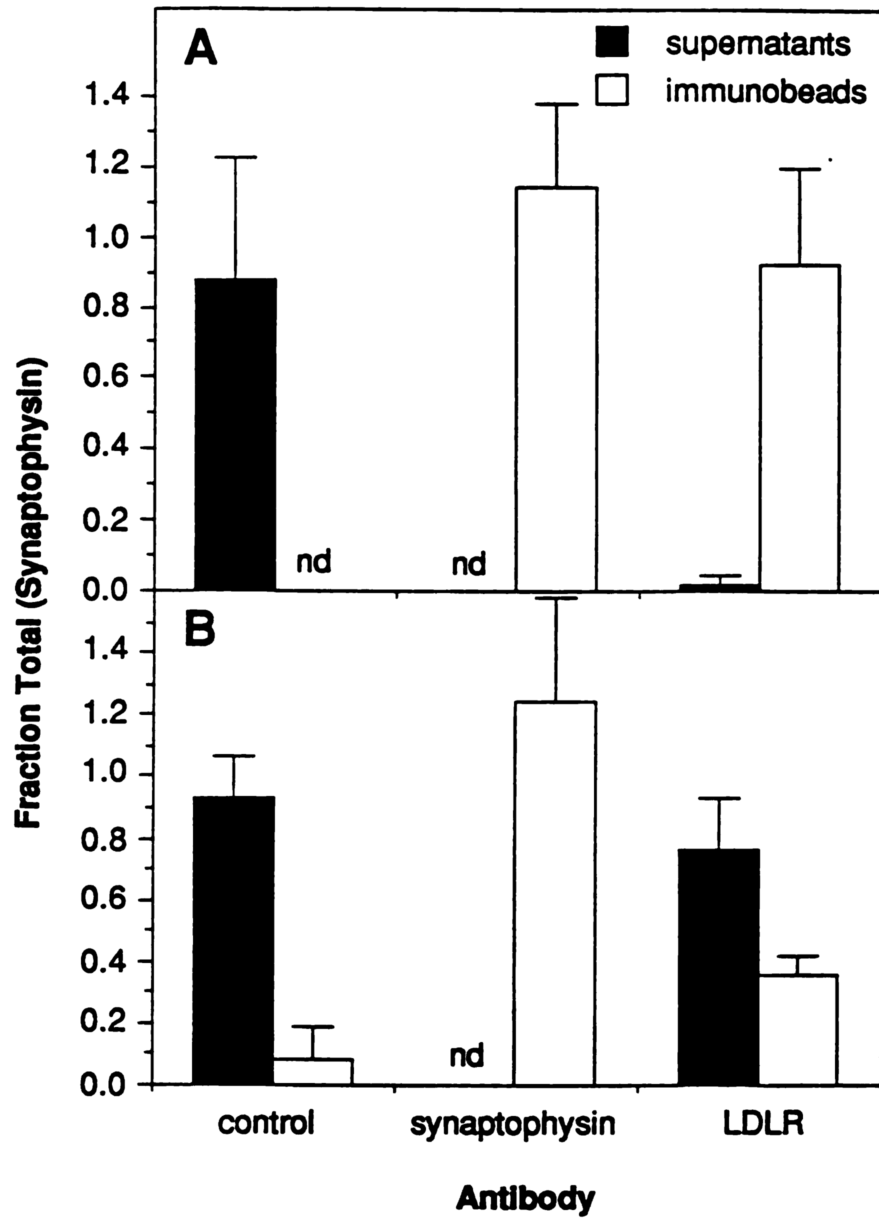
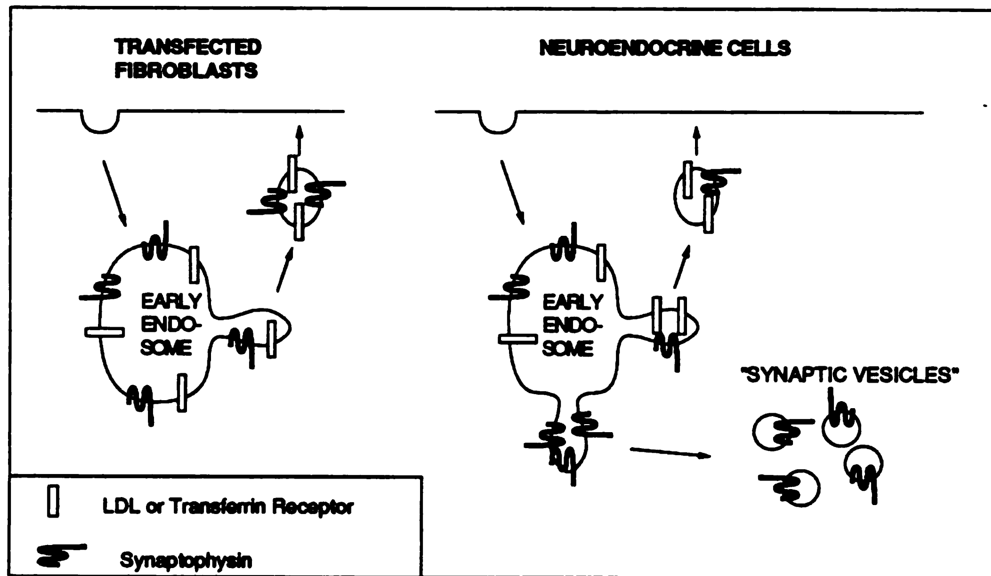

A
B
supernatants
immunobeads
nd
nd
nd
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fraction Total (Synaptophysin)
control
synaptophysin
LDLR
Antibody

FIGURE 2.7. Model for synaptophysin targeting in transfected non-neuronal cells and neuroendocrine cells. Synaptophysin participates in the early endosome recycling pathway in both cell types . Neuroendocrine and neuronal cells have a unique pathway allowing synaptic vesicle formation from endosomes.


TRANSFECTED
FIBROBLASTS
NEUROENDOCRINE CELLS
EARLY
ENDO-
SOME
EARLY
ENDO-
SOME
"SYNAPTIC VESICLES"
LDL or Transferrin Receptor
Synaptophysin

CHAPTER 3

Endocytosis of the Synaptic Vesicle Protein, Synaptophysin, Requires the COOH-terminal Tail

J. Physiol., Paris, Vol. 85, 1-7

SUMMARY

Synaptic vesicles participate in a cycle of fusion with the plasma membrane and reformation by endocytosis. Endocytosis of membrane proteins by the well studied clathrin-coated vesicle pathway has been shown to involve specific sequences within the cytoplasmic tail domain. Proteins taken up by clathrin-coated vesicles are directed to early endosomes from which they may return to plasma membrane. Recent evidence suggests that the synaptic vesicle protein synaptophysin is targeted to early endosomes in transfected fibroblasts and in neuroendocrine cells. To begin to test whether sequences within the COOH-cytoplasmic domain are required for internalization we have expressed a synaptophysin molecule lacking this domain in 3T3 cells and measured its rate of internalization. While a full length synaptophysin was internalized efficiently, we could not detect internalization of the mutant construct. These data are consistent with a model in which the COOH-terminal tail is required for coated-pit localization and hence targeting of synaptophysin to early endosomes.

INTRODUCTION

Synaptic vesicles release their neurotransmitter content during synaptic transmission by fusion with the plasma membrane. The pathway by which synaptic vesicles reform remains controversial (Heuser, 1989). One model is that synaptic vesicle fusion with the plasma membrane is transient. A pore forms between the membranes and then closes allowing release of neurotransmitter without mixing of membrane components (Ceccarelli et al., 1973). Another model is that synaptic vesicle components are internalized from the plasma membrane by a clathrin-coated pit mechanism, and delivered to endosomes (Heuser and Reese, 1973). New synaptic vesicles then form from the endosomes.

The majority of experiments testing these models have been morphological. The recent availability of cDNA clones encoding synaptic vesicle membrane proteins has made possible a different type of experiment. Membrane proteins that are internalized to endosomes by the coated vesicle pathway contain specific sequences which are required for their internalization (Goldstein et al., 1985). If synaptic vesicle reformation involves internalization in coated vesicles then synaptic vesicle proteins should also have internalization sequences that direct their delivery to early endosomes. Support for this model has come from experiments where a cloned synaptic vesicle protein, synaptophysin, is expressed in fibroblasts by transfection and found to localize to the early endosome pathway (Johnston et al., 1989 b; Linstedt and Kelly, 1991). Synaptophysin was also present in early endosomes of the neuroendocrine cell line PC12, in addition to its presence in synaptic vesicles (Linstedt and Kelly, 1991). Together these finding imply that synaptophysin contains targeting information for both early endosome and synaptic vesicle localization.

The identity of the sequence elements that underlie membrane protein targeting can by obtained by studies of mutated and chimeric molecules. Such studies have established that the cytoplasmic tails of low-density lipoprotein (Anderson et al., 1977; Lehrman et al., 1985; Davis et al., 1986, 1987), transferrin (Rothenberger et al., 1987; Iacopetta et al.; Jing et al., 1990), mannose-6-phosphate (Lobel et al., 1989), polymeric immunoglobulin (Mostov et al., 1986), and immunoglobulin G (Miettinen et al., 1989) receptors contain structural determinants required for clustering in coated pits. Although no "internalization" consensus sequence is shared by all these cytoplasmic tails, putative structural and sequence motifs have been identified (Chen et al., 1990; Collawn et al., 1990; Ktistakis et al., 1990; Canfield et al., 1991; McGraw et al., 1991). Unlike these receptors which have single membrane spanning domains, synaptophysin contains four transmembrane domains

and is oriented so that the NH_2-terminal, the COOH-terminal, and a small loop between the second and third transmembrane domains are in the cytoplasm (Johnston et al., 1989 a). However, the COOH-terminal domain is a prominent feature of the protein comprising 25% of the molecular weight and containing a repeat structure of unknown function (Südhof et al., 1987; Leube et al., 1987; Buckley et al., 1987). The addition of this domain to a tailless low-density lipoprotein receptor is sufficient to allow the chimera to undergo endocytosis, suggesting that the synaptophysin COOH-terminal contains an internalization signal (Kaneda, Y. and Kelly R.B., submitted).

To test whether the COOH-terminal cytoplasmic domain is necessary for internalization we have constructed a mutant synaptophysin lacking all but twelve amino acids of the COOH-terminal tail. This construct as well as the full length synaptophysin was tested for appearance on the cell surface and internalization.

MATERIALS AND METHODS

SYNAPTOPHYSIN cDNAs. The truncated-synaptophysin cDNA was generated as follows. A full length rat brain synaptophysin cDNA (Clift-O'Grady et al., 1990) was inserted into Bluescript (Stratagene, La Jolla, CA) at the Hind III cloning site. This plasmid was digested with BssH II, and then incubated with the Klenow enzyme to blunt the ends. After extraction and precipitation the DNA was incubated with the phosphorylated linker 5'-CATAAAGCTTTATG-3' and DNA ligase. After extraction and precipitation the ligated products were digested with Hind III to isolate the truncated-synaptophysin insert. This insert was then cloned into the Hind III site of the pMV7 vector (Kirschmeier et al., 1988) under the regulation of the Moloney murine sarcoma virus 5' LTR, and sequenced to confirm the correctness of the construction. Drug resistance was

conferred by the neo gene on the same vector under the regulation of the herpes simplex virus thymidine kinase promoter.

CELL CULTURES AND TRANSFECTIONS. NIH 3T3 (including Psi-2 and PA-12) cells were grown in DME H-21 containing 10% fetal calf serum, penicillin (100U/ml) and streptomycin (100U/ml) in humidified incubators at 37°C with 5% CO_2. For transfections a retrovirus production and infection protocol was followed (Perkins et al., 1983). The pMV7 vectors containing either full length or truncated-synaptophysin were introduced into the Psi-2 packaging cell line by calcium phosphate (Chen and Okayama, 1988). Supernatants were used to infect the PA-12 cell line. These were selected with G418 (400μg/ml) and propagated. Supernatants from these cells were used to transfect 3T3 cells. After selection with G418 (400μg/ml) individual clones were picked and propagated.

LABELING AND IMMUNOPRECIPITATION. For metabolic labeling, cells were incubated overnight in DME-H21 media depleted of cysteine and methionine but supplemented with 2% fetal calf serum and [^{35}S]Translabel (ICN K & K Laboratories Inc., Irving, CA) at 100μCi/ml. Surface labeling was achieved with either iodination using ^{125}I sulfo-SHPP (Thompson et al., 1987) or biotinylation (see below). Labeled cells (metabolic or surface) were lysed and immunoprecipitations were carried out in buffer containing 1% NP-40, 0.4% deoxycholate, 0.3% SDS, 66mM EDTA, 10mM Tris (pH 7.4) as described previously (Clift-O'Grady et al., 1990). The antibodies used were: an anti-rat brain synaptic vesicle rabbit serum that recognizes synaptophysin, a rat mAb in cell culture supernatant (ATCC TIB 219) against transferrin receptor, and a mouse mAb in cell culture supernatant (X31) against influenza hemagglutinin. In some experiments, the immunoprecipitates were incubated with recombinant *Strep plicatus* endoglycosidase H (Boehringer Mannheim Biochemicals, Indianapolis, IN) overnight prior to SDS-PAGE.

ENDOCYTOSIS ASSAY. Transfected cells expressing synaptophysin, truncated synaptophysin, or influenza hemagglutinin were grown on 10cm plates then washed five times with PBS and once with biotinylation buffer (250mM sucrose, 2mM $CaCl_2$, 10mM triethanolamine-acetic acid (pH 7.4)(Matter et al., 1990)). All steps were carried out on ice except where indicated. The cell surface was then biotinylated by two successive 15 min incubations with 500μg/ml NHS-SS-Biotin (Pierce Chemical Co.) in biotinylation buffer. After two, five minute washes in DME containing 0.2% BSA, the plates were incubated at 37°C for various times (0, 10, and 30 minutes) to allow endocytosis. The label remaining on the surface was removed by two washes in PBS containing 10% fetal calf serum, two successive 20 min incubations in reducing solution (50mM glutathione, 90mM NaCl, 60mM NaOH, and 10% fetal calf serum), and a 15 min incubation in PBS containing 5mg/ml iodoacetamide and 1% BSA. The antigens were recovered by immunoprecipitation, as described above, and transferred to nitrocellulose. After blocking in TGG (PBS containing 0.5% Tween 20, 1M glucose, 10% glycerol) with 3% BSA and 1% non-fat dry milk, the blot was incubated with ^{125}I-labeled streptavidin (1μCi/ml) in TGG with 0.3% BSA and washed three times in PBS containing 0.5% Tween 20 (Lisanti et al., 1988). For each experiment one plate was maintained on ice without disulfide reduction to allow determination of the total amount of labeled antigen.

RESULTS

CONSTRUCTION OF COOH-TERMINAL TAIL DELETED SYNAPTOPHYSIN. We have generated a full length rat synaptophysin cDNA and cloned it into various expression vectors. Transfection of several non-neuronal cell lines with these vectors leads to the production of stable and glycosylated synaptophysin molecules (Clift O'Grady et al, 1990;

Linstedt and Kelly, 1991). To generate a synaptophysin molecule lacking the COOH-terminal tail we took advantage of a unique restriction site on the 3' side of the fourth transmembrane domain (Fig 3.1). After digestion, the ends of the DNA were made blunt, and a linker designed to place an in-frame stop codon was attached (Fig 3.1). This construct was cloned into a retrovirus vector, pMV7, that also contained the neo gene for drug resistance (Fig 3.1, inset). For comparison wild type synaptophysin was also cloned into the pMV7 vector. Both constructs were carried through two packaging cell lines to generate the virus used to infect NIH 3T3 cells. Stable drug resistant clones were propagated for screening.

EXPRESSION OF MUTATED AND WILD-TYPE SYNAPTOPHYSIN IN 3T3 FIBROBLASTS. Cells expressing full length synaptophysin were easily identified by either immunofluorescence staining or immunoblotting using a monoclonal antibody that recognizes an epitope within the synaptophysin COOH-terminal tail (Linstedt and Kelly, 1991). Synaptophysin could also be detected by immunoprecipitation with a rabbit serum generated against rat brain synaptic vesicles (Fig 3.2). Synaptophysin from transfected 3T3 cells was found to be a triplet on SDS-PAGE of approximately 40kD (Fig 3.2, lane 5). Assuming glycosylation on at least one of the two potential glycosylation sites present in the lumenal domains the migration is consistent with the calculated molecular weight of 33,273 from the cDNA clone. The 3T3 species also co-migrated with synaptophysin from PC12 cells (Fig 2, lane 1) and transfected MDCK cells (Fig 3.2, lane 3), but was absent in untransfected cells (Fig 3.2, lanes 2 and 4). The three synaptophysin bands from 3T3 cells do not represent immature or precursor species since treatment with endoglycosidase H had no affect on their migration (Fig 3.2, compare lanes 7 and 8). When the same construct is used to express synaptophysin in a number of cell types, the glycosylation is heterogeneous and the pattern of glycosylation is different for each cell type (Linstedt and

Kelly, 1991). A comparison of endogenous PC12 and rat brain synaptophysin also reveals a striking difference in glycosylation (Navone et al., 1986).

When the anti-synaptic vesicle polyclonal antibody was also used to immunoprecipitate labeled extracts of cells transfected with truncated synaptophysin, no specific bands were detected (Fig 3.2, lane 6). Assuming that the antibodies in this serum only recognized the COOH-terminal cytoplasmic tail (the deleted domain) we tried a serum that recognized an epitope within the second lumenal domain. This serum was generated by injection of a peptide corresponding to this domain (Lowe and Kelly, unpublished). Judged by staining of untransfected cells the anti-peptide serum gave high backgrounds. This prevented immunofluorescence analysis of the cells transfected with truncated-synaptophysin. Although the anti-peptide serum immunoprecipitated a number of background bands from 3T3 cells, both full length and truncated synaptophysin were detectable as species immunoprecipitated from their respective transfected cell lines (Fig 3.3, lanes 2 and 3) but not untransfected cells (Fig 3.3, lane 1). The serum also allowed detection of the truncated synaptophysin by immunoblotting (Fig 3.3, lane 4). The migration of the truncated-synaptophysin was faster than expected for the calculated molecular weight of 25,696 from the cDNA clone. Like full length synaptophysin from 3T3 cells, truncated-synaptophysin migrated as a triplet, suggesting that it was glycosylated in the same manner.

SURFACE EXPRESSION AND INTERNALIZATION OF THE SYNAPTOPHYSIN CONSTRUCTS. Synaptophysin can be detected on the cell surface of transfected fibroblasts (Leube et al, 1989; Linstedt and Kelly, 1991). To determine whether synaptophysin lacking the COOH-terminal tail was also present on the plasma membrane we used two techniques. In the first the surface of the transfected and nontransfected 3T3 cells was iodinated using a membrane impermeant reagent, ^{125}I-sulfo-SHPP (Thompson

et al., 1987). After iodination the cells were lysed and immunoprecipitation was carried out with the anti-peptide serum. Consistent with previous results iodinated full length synaptophysin was recovered demonstrating its presence in the plasma membrane (Fig 3.4, lane 3). Analysis of the 3T3 cell line transfected with truncated-synaptophysin demonstrated that surface labeled truncated-synaptophysin could also be recovered (Fig 3.4, lane 2). The presence of truncated-synaptophysin in the plasma membrane indicated that the COOH-terminal cytoplasmic domain was not required for trafficking of synaptophysin to the cell surface.

Surface biotinylation using NHS-SS-biotin, a sulfated biotin derivative that reacts with primary amino groups and allows reversible derivatization, allowed the measurement of internalization of truncated-synaptophysin. After biotinylation, antigens were recovered by immunoprecipitation, transferred to nitrocellulose and detected by blotting with iodinated streptavidin (Lisanti et al., 1988). For the endocytosis assay the cells were warmed to 37°C for 10 or 30 minutes to allow endocytosis of surface labeled proteins, and then the label remaining on the surface was removed by reduction of the disulfide prior to lysis (Le Bivic et al., 1990). As described previously, a significant amount of synaptophysin was recovered in a pool resistant to disulfide reduction after 10 minutes of internalization (Fig 3.5; and Linstedt and Kelly, 1991). In contrast the same analysis for synaptophysin lacking the COOH-terminal tail resulted in no detectable internalization (Fig 3.5). As a positive internal control, transferrin receptor, a protein that is rapidly endocytosed, was immunoprecipitated from the same extracts containing either the tailless or full length synaptophysin. As expected it was efficiently internalized in all experiments (Fig 3.5). For comparison with a marker that is not internalized, a cell line expressing influenza hemagluttinin was also analyzed. The data obtained for hemagluttinin were identical to truncated-synaptophysin (Fig 3.5). Therefore synaptophysin lacking the COOH-terminal cytoplasmic tail was transported to the cell surface but was not internalized

efficiently. These data suggest that the COOH-terminal cytoplasmic tail of synaptophysin is required for its efficient internalization.

DISCUSSION

The colocalization of synaptophysin expressed in fibroblasts with transferrin and LDL receptors, markers of the early endosome pathway, suggests that synaptophysin contains the necessary targeting information for coated pit localization and return from endosomes to the plasma membrane (Johnston et al., 1989 b; Linstedt and Kelly, 1991). Like transferrin receptor, synaptophysin undergoes efficient endocytosis and appears in endosomal fractions (Linstedt and Kelly, 1991). Previous studies of numerous receptors have shown that the determinants for rapid internalization by this pathway are contained within stretches of amino acids in the cytoplasmic domain (see introduction). Unlike these receptors, synaptophysin spans the membrane more than once and therefore has several cytoplasmic domains. We have found that a truncated-synaptophysin, lacking the COOH-terminal cytoplasmic domain, is transported to the cell surface, but not internalized efficiently. Furthermore the addition of the COOH-terminal tail of synaptophysin restores efficient endocytosis to a tailless LDL receptor (Kaneda, Y. and Kelly R.B., submitted). Taken together with the rapid endocytosis and endosomal targeting of the full length synaptophysin, these findings suggest that the COOH-terminal tail, which is the most prominent of synaptophysin's cytoplasmic domains, contains an internalization signal. To our knowledge this is the first demonstration that a cytoplasmic domain of a multi-membrane spanning protein is required for its constitutive endocytosis.

The similarities between the internalization of synaptophysin expressed in fibroblasts and receptors known to localize to coated pits lend further support to the

hypothesis that synaptic vesicle biogenesis involves the conventional coated vesicle pathway (Heuser and Reese, 1973). A prediction of this model is that similar internalization signals exist in synaptic vesicle proteins and the receptors (Linstedt and Kelly, 1991). On the other hand, if synaptic vesicles reform directly from the nerve terminal plasma membrane by a novel mechanism (Ceccarelli, 1973) then the presence of a conventional internalization signal in the synaptophysin tail is less straightforward. It could be coincidence, or it could reflect shared features in the mechanism of internalization.

To understand exactly how similar the synaptophysin internalization signal might be, it will be of interest to define the sequence elements in the synaptophysin COOH-tail that specify internalization. This information could then be compared to the sequence features known to be important in the tails of rapidly endocytosed receptors and proteins. The requirement for a tyrosine residue has been well documented, and it is becoming apparent that the tyrosine must be in the proper context relative to the surrounding amino acids to be functional. Several consensus structural and sequence motifs that describe this context have been described (Chen et al., 1990; Collawn et al., 1990; Ktistakis et al., 1990; Canfield et al., 1991; McGraw et al., 1991), but their predictive value has not been established. The synaptophysin COOH-tail contains nine tyrosine residues (Fig 1), however, further experiments are necessary to implicate any of these as part of an internalization signal.

If synaptic vesicle proteins are internalized in coated vesicles and delivered to endosomes (Sulzer and Holtzman, 1989) in neuronal cells, then synaptic vesicles are likely to form from the endosomes. This model implicates two sorting steps, and therefore the likelihood of two sorting signals, for synaptic vesicle biogenesis. In the first step synaptic vesicle and other rapidly endocytosed proteins are sorted from resident plasma membrane proteins into coated pits. In the second step synaptic vesicle proteins must be sorted from

these proteins and other endosomal proteins into synaptic vesicles. Further mutational analysis should allow disection of these important signals.

FIGURE 3.1. Construction of truncated synaptophysin expression vector. The entire carboxy terminal cytoplasmic domain of synaptophysin is shown (single letter code). Tyrosine residues are marked by an asterisk. This domain was deleted by digestion with BssH II which recognized a unique site 3' of the sequence coding for the fourth transmembrane domain. The linker shown was used to place a stop codon in frame after the alanine (residue #12 in the tail), and provide a Hind III restriction site. Both the full length and truncated synaptophysin cDNAs were cloned into the Hind III site of the expression vector pMV7 (inset).

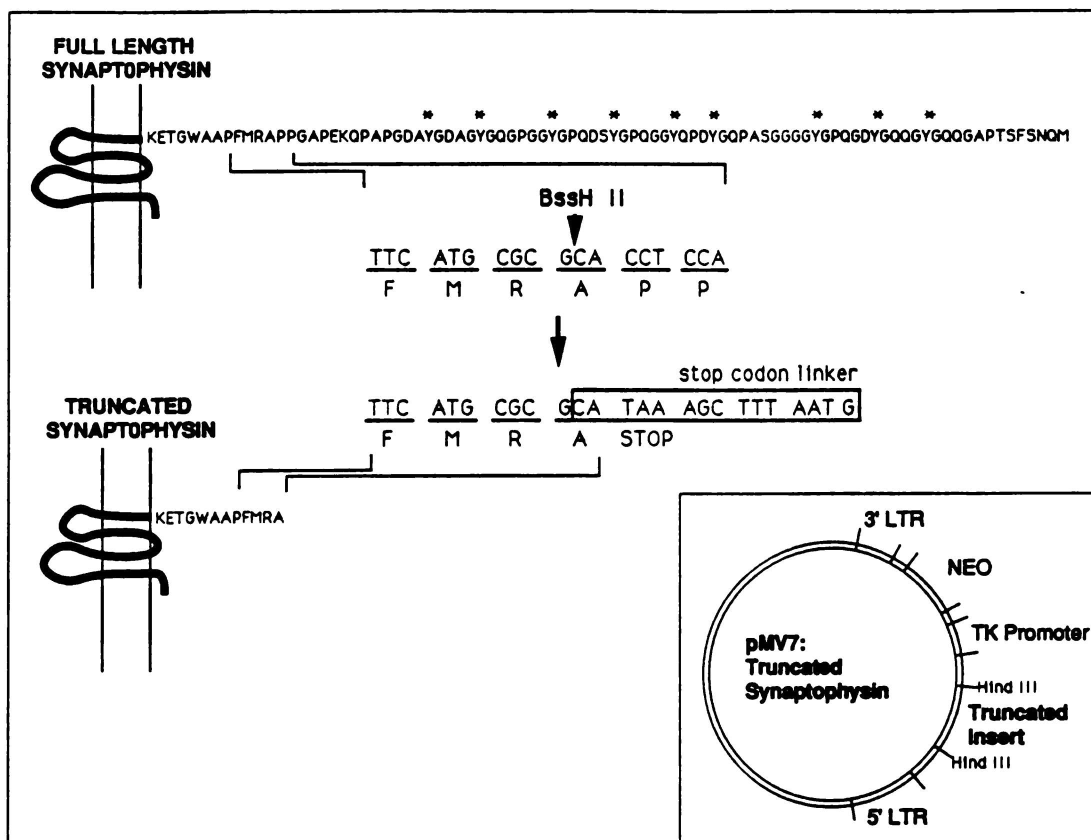

FULL LENGTH SYNAPTOPHYSIN
* * * * * * * * *
KETGWAAPFMRAPPGAPEKQPAPGDAYGDAGYGQGPGGYGPQDSYGPQGGYQPDYGQPASGGGGYGPQGDYGQQGYGQQGAPTSFSNQM
BssH II
TTC ATG CGC GCA CCT CCA
F M R A P P
stop codon linker
TRUNCATED SYNAPTOPHYSIN
TTC ATG CGC GCA TAA AGC TTT AAT G
F M R A STOP
KETGWAAPFMRA
3' LTR
NEO
TK Promoter
pMV7: Truncated Synaptophysin
Hind III
Truncated Insert
Hind III
5' LTR

FIGURE 3.2. Detection of synaptophysin in transfected cells. Metabolically labeled synaptophysin was recovered from various cell lines by immunoprecipitation with an anti-rat brain synaptic vesicle rabbit serum that recognizes synaptophysin. Lanes (1) PC12; (2) MDCK untransfected; (3) MDCK synaptophysin transfected; (4) 3T3 untransfected; (5) 3T3 synaptophysin transfected; (6) 3T3 transfected with truncated-synaptophysin; (7) 3T3 synaptophysin transfected, not treated with endoglycosidase H; (8) same as 7 but treated with endoglycosidase H. Note that truncated-synaptophysin was not immunoprecipitated with this serum (lane 6, see text). The position of molecular weight markers (116, 97, 66, 45, 36, 29, 24, and 20 kilo-Daltons) is indicated.

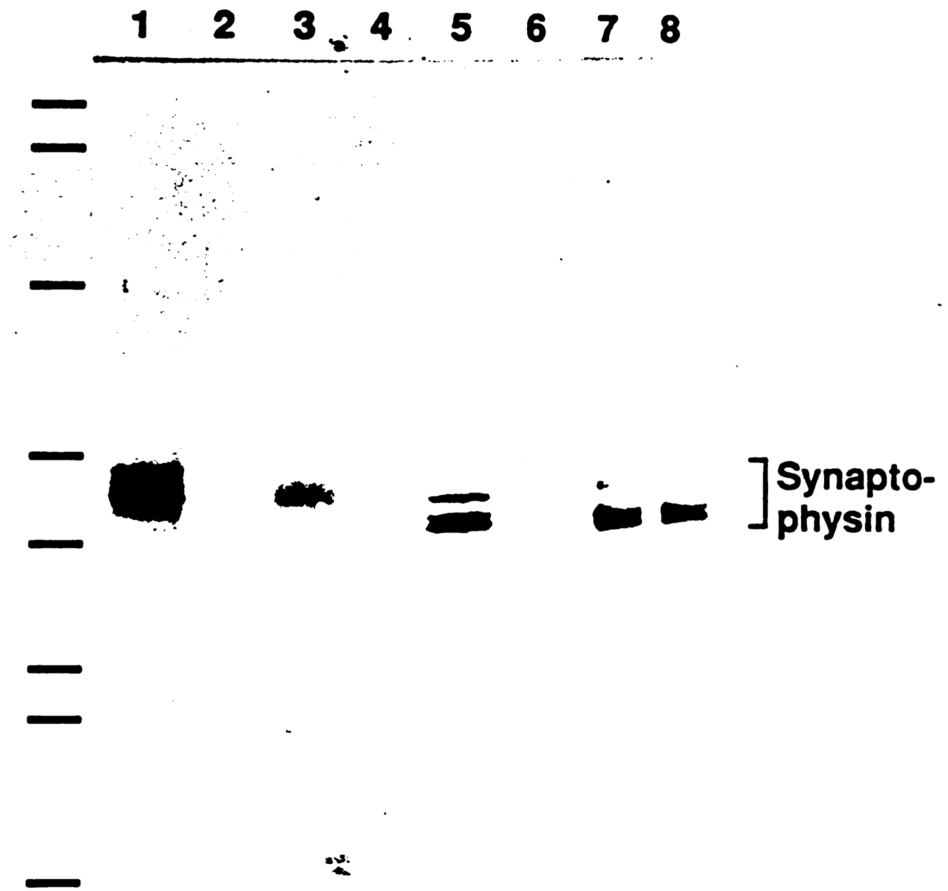

1 2 3 4 5 6 7 8
Synapto-
physin

FIGURE 3.3. Detection of truncated-synaptophysin in transfected cells. Left panel. Immunoprecipitation with an anti-synaptophysin lumenal peptide rabbit serum of untransfected (lane 1), synaptophysin transfected (lane 2), and truncated-synaptophysin transfected (lane 3) 3T3 cells. Molecular weight markers are the same as figure 2. Right panel. Immunoblot analysis with the anti-synaptophysin lumenal peptide serum of extracts from truncated-synaptophysin transfected (lane 4), synaptophysin transfected (lane 5), and untransfected cells (lane 6).

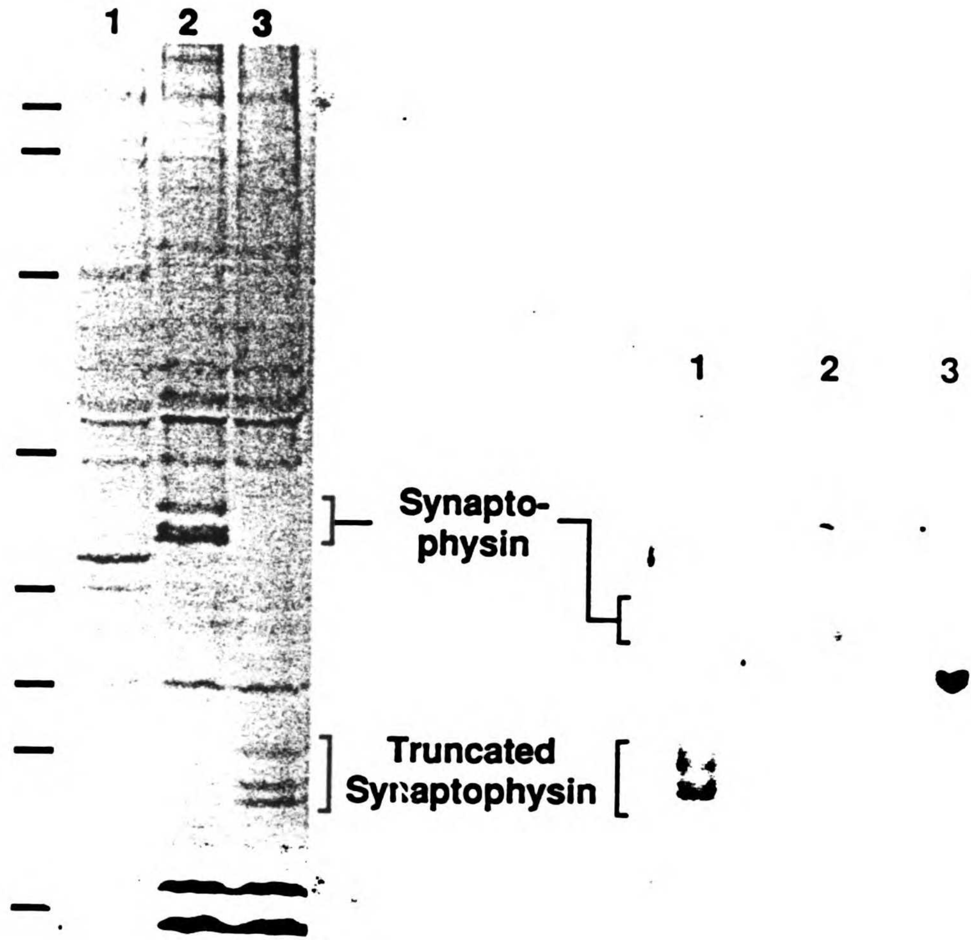

1 2 3
Synapto-
physin
Truncated
Synaptophysin
1 2 3

FIGURE 3.4. Surface labeling of truncated-synaptophysin. The cell surface of untransfected (lane 1), truncated-synaptophysin transfected (lane 2), and synaptophysin transfected (lane 3) 3T3 cells was radio-iodinated. The anti-synaptophysin lumenal peptide rabbit serum was then used for immunoprecipitation analysis of these cells. The position of molecular weight markers (97, 68, 43, 29, and 20 kilo-Daltons) is indicated.

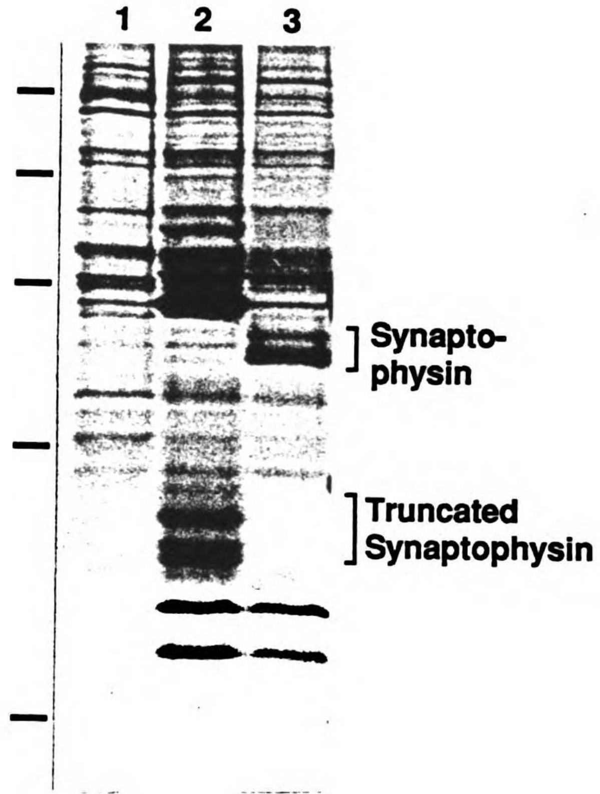
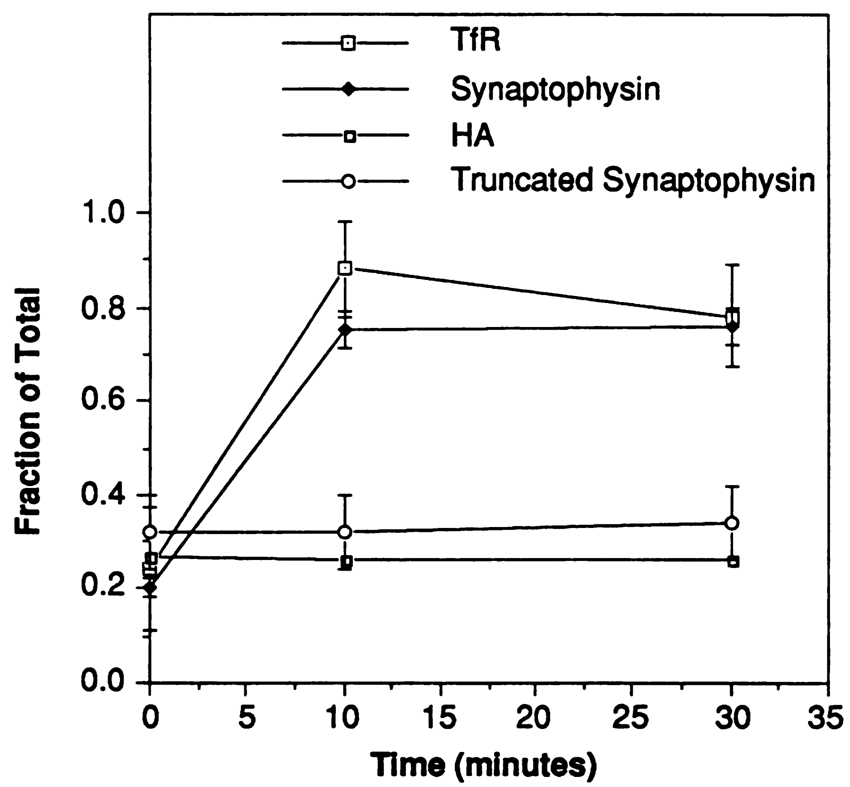

1
2
3
Synapto-
physin
Truncated
Synaptophysin

FIGURE 3.5. Internalization of truncated-synaptophysin in transfected 3T3 cells. After surface biotinylation the cells were incubated at 37°C for the times indicated to allow endocytosis. The label remaining on the surface was removed and the amount of labeled synaptophysin, truncated-synaptophysin, transferrin receptor (TfR), or influenza hemagglutinin (HA) was determined after immunoprecipitation and blotting with ^{125}I-streptavidin. The data are plotted as the fraction of the amount recovered from cells not treated to remove surface label. The range of the results are shown for synaptophysin (n=2), truncated synaptophysin (n=3), and transferrin receptor (n=4).



TfR
Synaptophysin
HA
Truncated Synaptophysin
Fraction of Total
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
Time (minutes)

CHAPTER 4

Specific Association of the Proto-Oncogene Gene Product pp60$^{c\text{-}SRC}$ with an Intracellular Organelle: the PC12 Synaptic Vesicle

ABSTRACT

The protein product of the proto-oncogene c-src is a membrane associated tyrosine kinase of unknown function. The available evidence indicates that $pp60^{c-src}$ associates with distinct membranes within single cell types and has different distributions in different cell types. We have characterized the distribution of the c-src protein in the neuroendocrine cell line PC12. $pp60^{c-src}$ was found to be specifically associated with synaptic vesicles since: i) the $pp60^{c-src}$ immunofluorescent pattern overlapped with a synaptic vesicle marker, synaptophysin, ii) 50% of the $pp60^{c-src}$ was recovered after differential centrifugation in a slowly sedimenting fraction, iii) an anti-synaptophysin tail antibody immunodepleted all of the $pp60^{c-src}$ vesicles in this fraction, and iv) $pp60^{c-src}$ co-purified during a 100-fold purification of PC12 synaptic vesicles.

INTRODUCTION

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 (Veillette et al., 1988). This

makes sense for proteins that are signalling 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 junctions (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 it 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 part of a conventional signalling 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 non-membrane associated pp60^{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 (Resh, 1989). Furthermore, a 32K polypeptide present in membrane fractions can be specifically crosslinked to myristylated amino terminal src peptides (Resh and Ling, 1990).

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$^{c\text{-}src}$ in synaptic vesicles. We chose synaptic vesicles because pp60$^{c\text{-}src}$ is naturally enriched in neural tissue (Cotton and Brugge, 1983; Brugge 1985). Because of their homogeneous physical properties and the availability of antibodies to cytoplasmic domains of synaptic vesicle proteins, synaptic vesicles are easily isolated. Furthermore, a major integral membrane protein of the synaptic vesicle, synaptophysin, has been shown to be an in vitro substrate of pp60$^{c\text{-}src}$ tyrosine kinase activity (Barnekow et al., 1990). If pp60$^{c\text{-}src}$ is preferentially targeted to synaptic vesicles, the vesicles should be enriched in a putative receptor for pp60$^{c\text{-}src}$ 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. However, it was not previously shown that association of pp60$^{c\text{-}src}$ with synaptic vesicles was specific. Our experiments quantitate the distribution of pp60$^{c\text{-}src}$ in PC12 cells, a cell line that contains endocrine synaptic vesicles (Navone et al, 1986; Wiedenmann et al, 1988; Clift-O'Grady et al., 1990).

METHODS

Immunofluorescence. PC12 cells were grown in media containing nerve growth factor (100 ng/ml; Calbiochem, La Jolla, CA) for at least five days prior to plating on poly-D-lysine coated coverslips. The cells were fixed with 3% paraformaldehyde for 30 min., washed two times with PBS, and two times with PBS-glycine (20mM), and then permeabilized for 20 min with PBS-glycine-saponin (0.1%) (Schweizer et al., 1988). The coverslips were then inverted on 15µl 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 5µg/ml and an anti-synaptophysin rabbit polyclonal at 1:500 (Linstedt and Kelly, 1991). After five washes with PBS-saponin the coverslips were inverted on 15µl PBS-saponin containing the appropriate combinations of secondary antibody. Rabbit polyclonals were detected with either rhodamine-labeled or FITC labled sheep anti-rabbit (Jackson) at 1:400. For 327 either FITC- or rhodamine-labeled sheep anti-mouse (Jackson) was used at 1:400. After five final washes with PBS-saponin the coverslips were rinsed with water and mounted on glass slides with DABCO (Sigma, St. Louis, MO).

Differential Centrifugation. Cells were removed from the plates by scraping in buffer A (150mM NaCl, 1mM EGTA, 1mM MgCl2 and 10mM Hepes pH 7.4) and collected by centrifugation at 300xg for 7 min. Homogenization was in buffer A, usually 0.8ml, containing a protease inhibitor cocktail (pepstatin, chymostatin, leupeptin, and aprotinin at 10ng/ml; 1mM PMSF; 1µg/ml o-phenanthroline; 10µM benzamidine) using a Cell Cracker (European Molecular Biology Laboratory) with 10 strokes and a 12µm clearance. The homogenate was separated into a nuclear pellet (P1) and post-nuclear supernatant (S1) by

centrifugation at 1,000xg for 5 min in a SS34 rotor (Sorvall, New Town, CN). The S1 was centrifuged at 27,000xg for 35 min in a 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 127,000xg for 60 min in an air centrifuge (Beckman Instruments, Palo Alto, CA). Pellets were resuspended and assayed for pp60$^{c\text{-}src}$ and synaptophysin by immunoblotting, protein content by Pierce assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard, and membrane protein content by Pierce assay after extraction with Triton X 114.

Organelle Immunoisolation. Aliquots of 20mg of Dynabeads M-450, magnetic polystyrene beads coated with sheep anti-mouse IgG1 (Dynal Inc., Great Neck, NY), were incubated with 20µg of either anti-synaptophysin (SY38) or mouse gamma globulin (Pel-Freez Biologicals, Rogers, AR) overnight at 4°C then rinsed with buffer A. Three aliquots of the S2 fraction prepared from two 15cm plates of PC12 cells were used. One aliquot was added to the SY38 beads, one aliquot 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 127,000xg in an air centrifuge (Beckman Instruments). The resulting pellets (P3) were lysed and analyzed by immunoblotting for pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ by immunoblotting.

Velocity and Flotation Gradients. For velocity gradient analysis, the S1 fraction from either unlabeled or metabolically labeled cells was layered on 4.4ml linear 5-20% sucrose, or 5-25% glycerol gradients in buffer A underlayered with a 0.4ml 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 4ml linear 20-40% sucrose gradients containing 10mM Hepes (pH 7.4) and 1mM EGTA and centrifuged in a 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 bovine serum albumin as a standard using either the Pierce assay (Pierce Chemical Co.) 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.

Lableling and Immunoprecipitation. For metabolic labeling, cells were incubated overnight in DME-H21 media depleted of cysteine and methionine but supplemented with 2% fetal calf serum and [^{35}S]Translabel (ICN K & K Laboratories Inc., Irving, CA) at 100μCi/ml. Synaptophysin immunoprecipitations were carried out in buffer containing 1% NP-40, 0.4% deoxycholate, 0.3% SDS, 66mM EDTA, 10mM Tris (pH 7.4) with an anti-rat brain synaptic vesicle rabbit serum that recognizes synaptophysin (Clift O'Grady et al., 1990). Immunoprecipitation of pp60$^{c\text{-}src}$ was in RIPA buffer with mAb 327 and 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 (20ng/ml) and 327 (3μg/ml) for 60 min, 3 five minute washes, incubation with ^{125}I-goat anti-mouse IgG (Cappel, West Chester, PA) for 60 min and 3 final washes. All incubations were in phosphate buffered saline 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, Sunnyvale, CA).

RESULTS

pp60$^{c\text{-}src}$ and synaptophysin immunofluorescent patterns overlap in PC12 cells. Analysis of the intracellular localization of pp60$^{c\text{-}src}$ in PC12 cells by immunofluorescence microscopy revealed a punctate distribution of immunoreactivity. The cells shown in Figure 4.1 were grown for five days in nerve growth factor (to promote neurite extension) and plated on poly-D lysine then fixed with formaldehyde, saponin permeabilized and double stained for pp60$^{c\text{-}src}$ (A & C) and synaptophysin (B & D). Puncta of pp60$^{c\text{-}src}$ immunoreactivity were scattered throughout the cytoplasm but were present at higher concentrations in the perinuclear region. Intense staining of the plasma membrane was not apparent. Staining was present in neuritic processes, particularly in the growth cone area (Fig 4.1C) consistent with previous reports of pp60$^{c\text{-}src}$ localization to growth cones (Sobue, 1990). This pattern was very similar in overall appearance to the synaptophysin pattern (Fig 4.1, B &D). Synaptophysin staining was slightly less intense in the perinuclear region and more intense throughout the processes. In addition the overall intensity of the pp60$^{c\text{-}src}$ staining was less than synaptophysin. Most, but not all, pp60$^{c\text{-}src}$ puncta 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$^{c\text{-}src}$ and synaptophysin staining suggested that pp60$^{c\text{-}src}$ may be localized to membranes that participate in synaptic vesicle recycling (such as endosomes or synaptic vesicles or both).

pp60$^{c\text{-}src}$ is highly enriched in fractions containing small vesicles. To begin to test whether pp60$^{c\text{-}src}$ 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 27,000xg centrifugation of PC12 lysates. The synaptophysin in the supernatant, which can be concentrated in a 127,000xg 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 LDL-receptor, is recovered in the P2, with only minor amounts remaining in the supernatant (Linstedt and Kelly, 1991).

Paralleling the distribution of synaptophysin, a substantial amount of the sedimentable pp60$^{c\text{-}src}$ present in the post nuclear supernatant was recovered in the both the P2 and P3 fractions (Fig 4.2A). The pp60$^{c\text{-}src}$ recovered in the P3 fraction accounted for 44$\pm$9% while in the same fraction 40$\pm$10% of the synaptophysin was recovered (n=4). In marked contrast, only an insignificant amount of pp60$^{c\text{-}src}$, 3$\pm$3% (n=3), was recovered in the equivalent P3 fraction from CHO fibroblast cells (Fig 4.2A). The absence of small, pp60$^{c\text{-}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 distribution of pp60$^{c\text{-}src}$ between P2 and P3 fractions was no different in CHO cells transfected with synaptophysin. Therefore, a substantial portion of pp60$^{c\text{-}src}$ associates with a slowly sedimenting membrane population present in PC12 cells, but presumably absent in CHO cells.

The enrichment of pp60$^{c\text{-}src}$ in the PC12 P3 fraction was twice that of the P2 fraction when compared to total protein present in each fraction (Fig 4.2B). pp60$^{c\text{-}src}$ was not enriched in the CHO P3 fraction. Because most membrane markers are recovered in the P2 fraction and not the P3 fraction, we expected that the pp60$^{c\text{-}src}$ enrichment in the P3

would be considerably greater if normalized by membrane protein. Indeed, using partitioning into the detergent phase of Triton X-114 as an assay for membrane protein, greater than 99% of the total membrane protein in the post nuclear supernatant was recovered in the P2 fraction. In addition to slowly sedimenting membranes, the P3 fraction contains rapidly-sedimenting protein complexes from the cytosol. Therefore the $pp60^{c\text{-}src}$ associated with the P3 fraction was approximately 75 fold more enriched than the $pp60^{c\text{-}src}$ in the P2 fraction when compared to the membrane protein present in each fraction (Fig 4.2C). For a unique membrane population (eg plasma membrane) in the P2 to contain $pp60^{c\text{-}src}$ with the same enrichment it would have to comprise less than 2% of the total membranes present.

Isolation of $pp60^{c\text{-}src}$ vesicles with an anti-synaptophysin antibody.

To test directly for association of $pp60^{c\text{-}src}$ with PC12 synaptic vesicles, vesicle immunoisolation experiments were carried out using magnetic immunobeads, coated with a monoclonal antibody that recognizes an epitope in the cytoplasmic tail of synaptophysin, at concentrations sufficient to deplete the membranes containing synaptophysin. After removal of the immunobeads, membranes remaining in the supernatants were collected by centrifugation and assayed for the presence of $pp60^{c\text{-}src}$ and synaptophysin by immunoblot. As a control for non-specific adsorption, beads coated with mouse gamma globulin were used. A comparison of the recoveries of $pp60^{c\text{-}src}$ or synaptophysin membranes in non-treated (N) and control bead-treated (C) supernatants indicated that there was no significant depletion by the control beads (Fig 4.3A, compare lanes 1 and 2). In contrast, treatment of a PC12 27,000xg supernatant with the anti-synaptophysin beads (S) depleted all synaptophysin-containing membranes (Fig 4.3A, lane 3). Immunoblots of this same material with antibodies against $pp60^{c\text{-}src}$ demonstrated that removal of synaptophysin membranes depleted membrane associated $pp60^{c\text{-}src}$ (Fig 4.3A, lane 3).

pp60^{c-src} was recovered in extracts from the isolated anti-synaptophysin beads (Fig 4.3A, lane 5), but not from the control beads (Fig 4.3A, lane 4).

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 4.3, compare lanes 7 & 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 an insoluble 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 27,000xg 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.

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 post nuclear supernatant was analysed, synaptic vesicle membranes were clearly detectable as a peak of synaptophysin (Fig 4.4A, fraction 8) distinct from the larger membranes that collected on a pad at the bottom of the tube (Fig 4.4A, 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 (for example compare fractions 2 & 8 in Fig 4.4A). Separation of Rat1A fibroblast post nuclear 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 figure 4.2, suggests that the pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ and synaptophysin antigenicity co-migrated with a peak of protein at a density of 1.13 gm/cc (Fig4.4B). It was observed that nearly 100% of the synaptophysin was recovered in the fractions containing buoyant membranes, while only 60-80% of the pp60$^{c\text{-}src}$ was similarly recovered. We assume that the pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ with synaptic vesicles on density gradients varied depending on which isolation conditions were used. The pp60$^{c\text{-}src}$ 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$^{c\text{-}src}$ associated with synaptic vesicles isolated on immunobeads slowly dissociated during prolonged buffer washes (data not shown).

Quantitation of the recovery of total protein, pp60$^{c\text{-}src}$ and synaptophysin in the post-nuclear supernatant, velocity peak and flotation peak for four independent experiments is presented in Table 1. 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$^{c\text{-}src}$ calculated for purified synaptic vesicles was similar to that found in the P3 fraction when normalized by membrane protein (compare fig 4.2C with table 1). Analysis of the proteins in the peak fractions from the flotation gradients by either fluorography or silver staining indicated that proteins co-migrating with synaptophysin and pp60$^{c\text{-}src}$ were identifiable (data not shown).

DISCUSSION

We have studied the distribution of pp60$^{c\text{-}src}$ in PC12 cells and compared it to that of a synaptic vesicle membrane protein, synaptophysin. In PC12 cells pp60$^{c\text{-}src}$ was found to be specifically associated with the endocrine synaptic vesicles. In cells simultaneously stained with antibodies that recognize either pp60$^{c\text{-}src}$ or synaptophysin the pp60$^{c\text{-}src}$ immunofluorescence pattern extensively overlapped with the synaptophysin pattern. Nearly 50% of the membrane associated pp60$^{c\text{-}src}$ was recovered in a slowly sedimenting fraction enriched in synaptic vesicles and containing very little membrane protein. When this fraction was prepared from a fibroblast cell line a significant amount of pp60$^{c\text{-}src}$ was not present. All of the pp60$^{c\text{-}src}$ recovered in this fraction was removed by immunodepletion of the endocrine synaptic vesicles using antibodies directed against synaptophysin. Furthermore, pp60$^{c\text{-}src}$ co-purified with the endocrine synaptic vesicles through a 100-fold purification.

Our experiments demonstrate targeting of pp60$^{c\text{-}src}$ 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 et al. (personal communication) on platelets is that the targeting of pp60$^{c\text{-}src}$ to PC12 synaptic vesicles is mediated by a specific membrane protein. Cross-linking and co-immunoprecipitation experiments using that purified synaptic vesicle fraction may identify such proteins. Since pp60$^{c\text{-}src}$ is associated with other membranes (including endosomes) in addition to synaptic vesicles in PC12 cells 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$^{c\text{-}src}$ domains.

Synaptic vesicles contain about 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. It is less obvious why a tyrosine kinase should be associated with an intracellular organelle, since tyrosine kinase activity is conventionally associated with plasma membrane receptors and cell signalling. 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.

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 c-AMP dependent and calcium-calmodulin dependent protein kinases, are phosphorylated during exocytosis, which causes their dissociation (Schiebler et al., 1986; Sihra et al, 1989). If exocytosis is stimulated by the venom alpha-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 $pp60^{c\text{-}src}$ and synaptophysin distribution across a velocity gradient of the post nuclear supernatant (see Figure 4.4A) suggested that $pp60^{c\text{-}src}$ 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 $pp60^{c\text{-}src}$ as they cycle through the endosome. At present, therefore, it is plausible to suggest that association and dissociation of synaptic vesicle enriched peripheral membrane proteins is regulated by, or regulate the exocytotic cycle.

TABLE I : Copurification of PC12 Synaptic Vesicles and pp60$^{c\text{-}src}$

	Protein	Synaptophysin		pp60c-src	
	% total	% total	Enrichment	% total	Enrichment
Post Nuclear Sup.	100	100	1	100	1
Velocity Pool	5 ± 1	34 ± 8	6.8	36 ± 3	7.2
Flotation Pool	0.2 ± 0.1	24 ± 6	120	19 ± 2	95

FIGURE 4.1. Localization of pp60^{c-src} (a &c) and synaptophysin (b & d) in PC12 cells by double-label immunofluorescence. Both pp60^{c-src} and synaptophysin immunoreactivity are dispersed throughout the cytoplasm (a & b) and processes with growth cones (c & d) in very fine puncta.

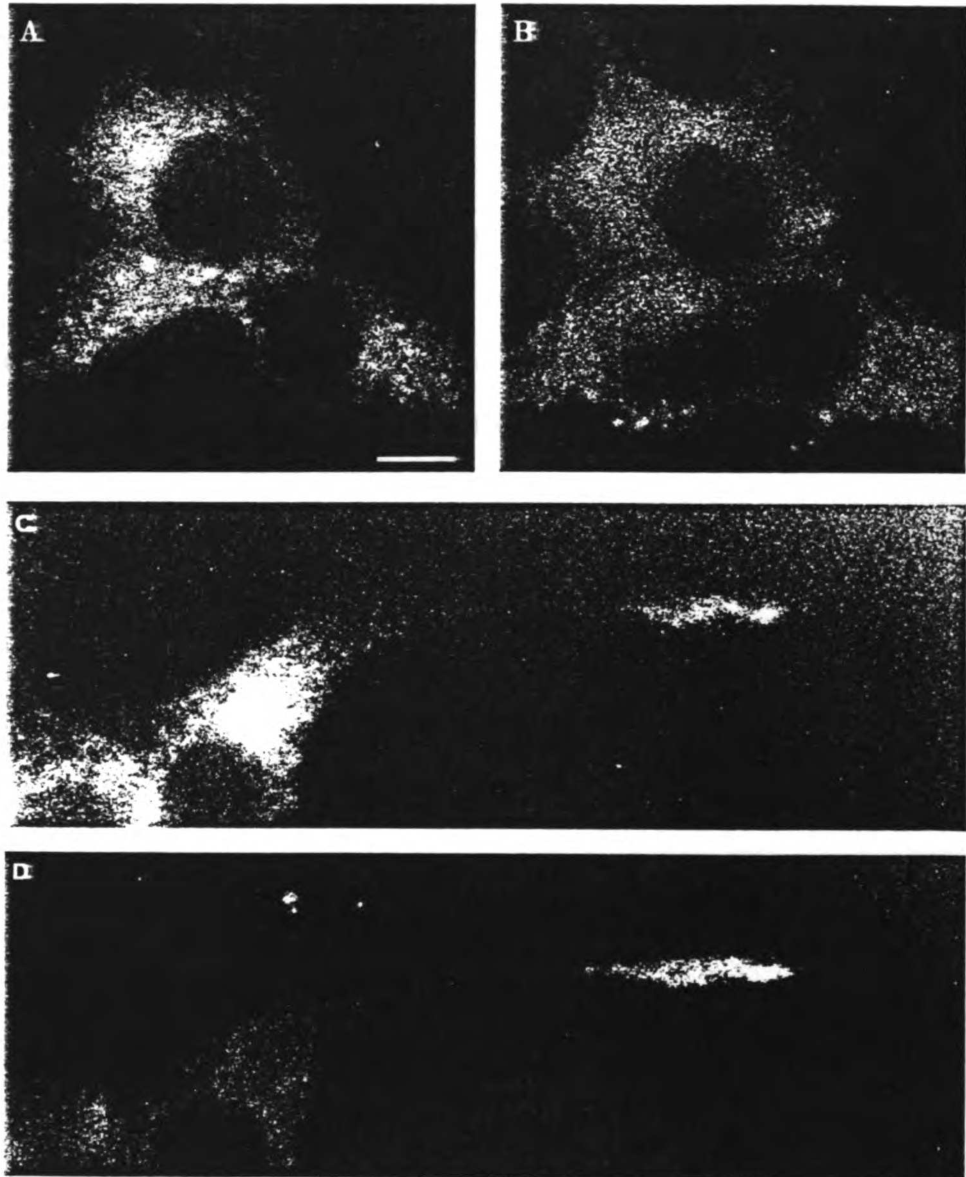

A
B
C
D

FIGURE 4.2. 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 nomalized by the total amount of protein in each fraction; and (C) as recovery when normalized by the amount of membrane protein in each fraction. P1, P2 and P3 centrifugations were 1,000xg for 5 min., 27,000xg for 30min., and 125,000xg 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 TX114.

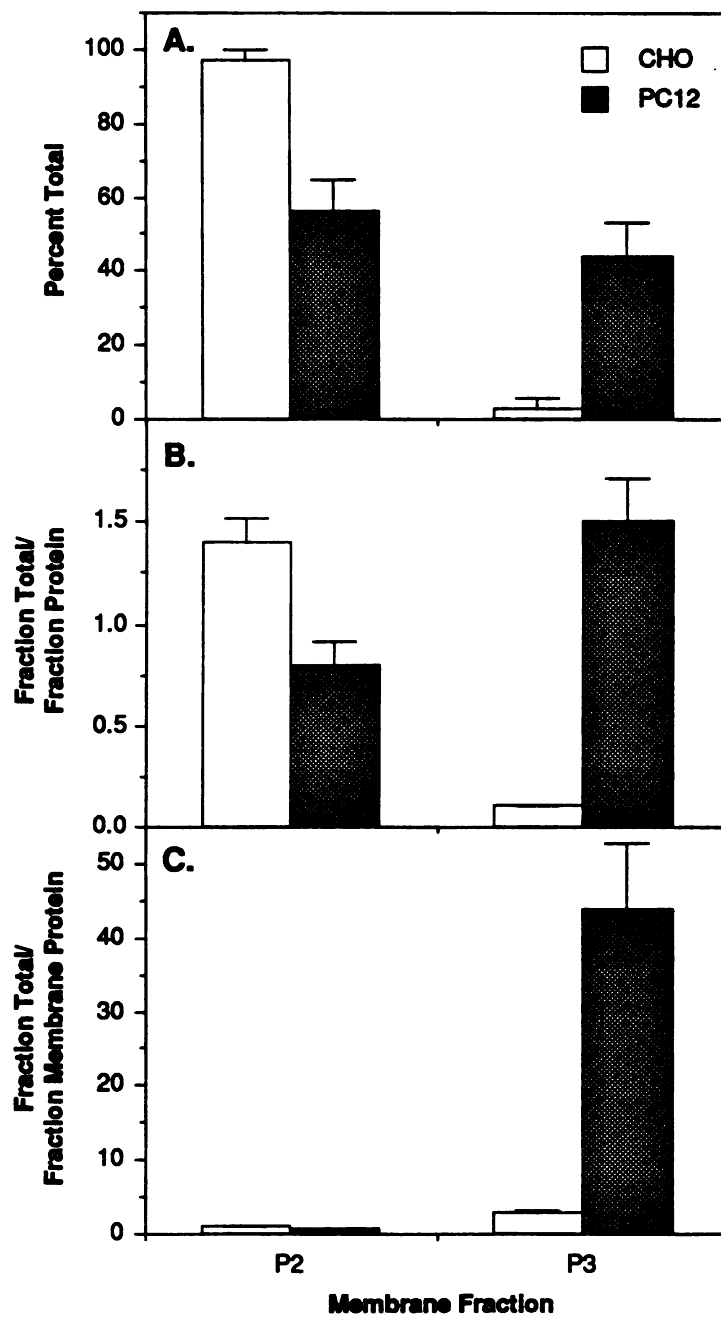

A.
B.
C.
CHO
PC12
Percent Total
Fraction Total/
Fraction Protein
Fraction Total/
Fraction Membrane Protein
100
80
60
40
20
0
1.5
1.0
0.5
0.0
50
40
30
20
10
0
P2
P3
Membrane Fraction

FIGURE 4.3. Immunodepletion of $pp60^{c\text{-}src}$ containing membranes with anti-synaptophysin immunobeads. S2 fractions were either non-treated (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 $pp60^{c\text{-}src}$ and synaptophysin by immunoblotting (lanes 1-3). The isolated immunobeads were extracted in sample buffer and these extracts were analyzed for $pp60^{c\text{-}src}$ by immunoblotting (beads, lanes 4 & 5). The Ponceau S staining pattern of the P3 fractions (lanes 6-7) after treatment show no significant depletion of proteins by the immunobeads.

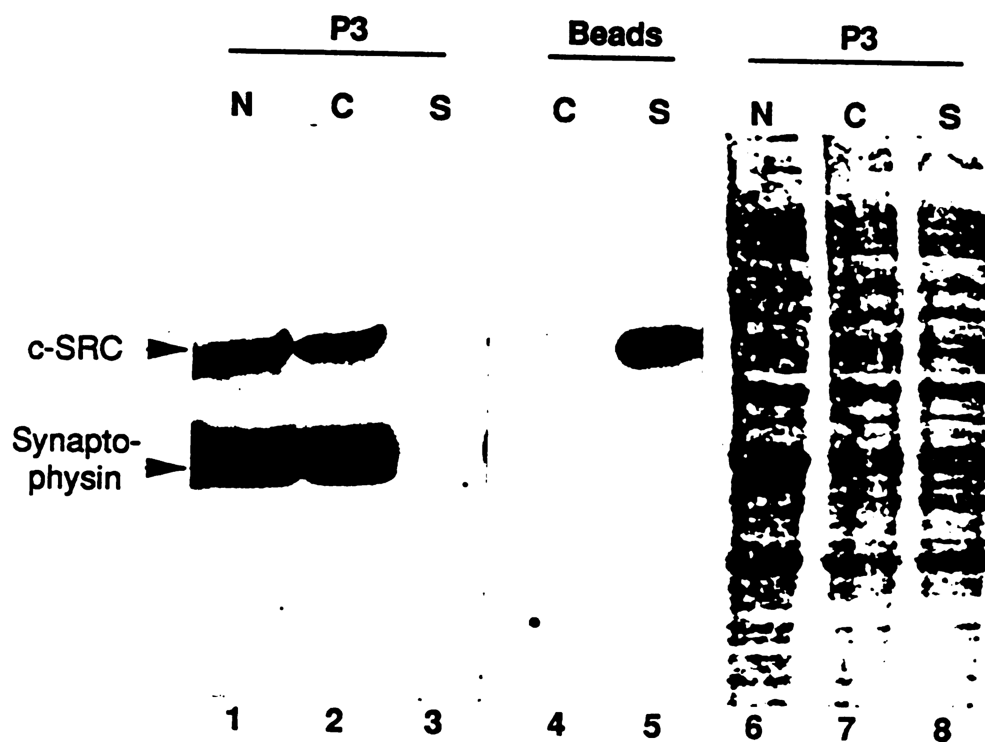
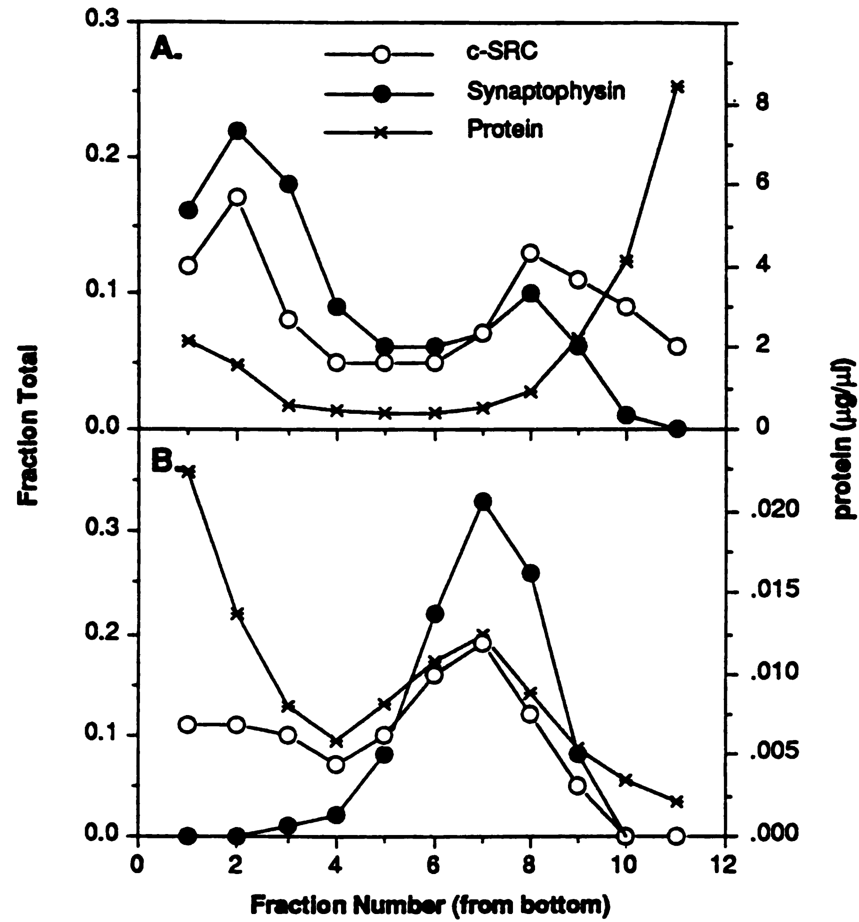

P3
Beads
P3
N
C
S
C
S
N
C
S
c-SRC
Synapto-
physin
1
2
3
4
5
6
7
8

FIGURE 4.4. Cosedimentation of $pp60^{c\text{-}src}$ and PC12 synaptic vesicles. (A) PC12 post nuclear supernatant separated on a linear 5-20% sucrose gradient underlayed with a sucrose cushion and centrifuged at 48,000 rpm for 60 min. (B) Material from the peak of the sucrose velocity gradient (fractions 7 & 8 in A) adjusted to 50% sucrose and applied under a linear 20-40% sucrose gradient and centrifuged at 48,000 rpm for 12hr. Each fraction was assayed for protein, synaptophysin and $pp60^{c\text{-}src}$ as described in the methods section.


A.
B.
c-SRC
Synaptophysin
Protein
Fraction Total
protein (µg/µl)
Fraction Number (from bottom)
0.0
0.1
0.2
0.3
0
2
4
6
8
.000
.005
.010
.015
.020
10
12

SUMMARY

Summary

Synaptophysin expression in transfected fibroblasts does not induce synaptic vesicle formation. Three different groups have expressed synaptophysin in non-neuroendocrine cells (Leube et al.,1989; Johnston et al., 1989; Clift-O'Grady et al., 1990). In each case synaptophysin was found to be localized to a vesicular intracellular compartment. However, conflicting conclusions arose from the comparison of these vesicles with PC12 synaptophysin vesicles and rat brain synaptic vesicles. Leube et al. (1989) concluded that all three vesicle types shared similar properties including size based on immuno-gold labeling with an anti-synaptophysin monoclonal antibody. Using the same technique Johnston et al. (1989) found that the synaptophysin-containing vesicles in transfected fibroblasts and PC12 cells were larger and more heterogeneous than rat brain synaptic vesicles. In my experiments sedimentation on velocity gradients was used to compare sizes (Clift-O'Grady et al., 1990; Chapter 2). PC12 synaptophysin vesicles co-sedimented with rat brain vesicles, but the synaptophysin vesicles recovered from transfected fibroblasts sedimented faster.

How can these conflicting conclusions (summarized in Table II) be understood? One explanation is that the distinction between synaptic vesicles and larger vesicles containing synaptophysin is subtle in immuno-gold micrographs . In contrast, velocity sedimentation clearly resolves the two vesicle classes. Thus the synaptophysin vesicles in PC12 cells, which include both synaptic vesicle-sized and larger vesicles (as indicated by velocity analysis), could either be viewed as similar to, or different from, brain synaptic vesicles depending on whether the smaller or larger vesicles are emphasized. The same argument can be used to explain the varied conclusions for synaptophysin transfected fibroblasts. It follows that the appropriate technique to use for an assay of synaptic vesicle-sized

structures is sedimentation analysis. With this technique transfected fibroblasts have only the larger size class of synaptophysin vesicle (Clift-O'Grady et al., 1990; Chapter 2).

The assumption of the discussion above is that immuno-EM cannot distinguish between the larger synaptophysin vesicles and authentic synaptic vesicles. It must be noted that this is the only technique used to date to identify synaptic vesicles in endocrine tissues. Because these cell types express synaptic vesicle proteins and the proteins are primarily excluded from the dense core granules it seems likely that they do contain synaptic vesicles. However, sedimentation gradients should be used to confirm this view. A note of caution seems appropriate here. Some endocrine tissues (and cell lines) may have only a small percentage of their synaptic vesicle proteins in synaptic vesicle-sized structures. The more dominant vesicle may be endosome vesicles (see below) which primarily sediment more rapidly, but because of heterogeneity in size may partially overlap with synaptic vesicles. Therefore to demonstrate the existence of synaptic vesicles it is important to compare on the same gradient the distribution of an endosome marker, such as transferrin receptor, with a synaptic vesicle protein.

Synaptophysin contains endosomal targeting information. The larger more heterogeneous vesicle population to which synaptophysin is targeted in transfected fibroblasts and PC12 cells is probably part of the early endosome pathway because these vesicles are endocytotically derived and contain transferrin and LDL receptors (Chapter 2). It is likely that synaptophysin recycles through this pathway because its half-life is much greater than its half-time for endocytosis (Chapter 2). These results indicate that in transfected fibroblasts synaptophysin is targeted to the endocytotic pathway. The lack of endocytosis by a synaptophysin mutant lacking the COOH-terminal tail suggests that the targeting information resides, at least in part, in this tail (Chapter 3). In principle the presence of targeting signal for the early endosome pathway in synaptophysin could be a

meaningless coincidence. Alternatively, the presence of such a signal may yield insight into the pathway by which synaptic vesicles reform after fusion with the presynaptic plasma membrane. Specifically, an internalization signal is an almost necessary prediction of the hypothesis of Heuser and Reese (1973) that coated vesicles and early endosomes are intermediates in synaptic vesicle biogenesis. Another interesting idea can be derived from this scheme: a second signal, this one for sorting synaptic vesicle proteins from early endosomes into synaptic vesicles, should be present in synaptophysin. This model is analogous to the sorting of polymeric immunoglobulin out of basolateral endosomes into transcytotic vesicles. Deletion and chimeric constructs containing synaptophysin sequences can be used to test this model.

$pp60^{c\text{-}SRC}$ is a peripheral membrane component of PC12 synaptic vesicles. Approximately 50% of the membrane associated $pp60^{c\text{-}src}$ in PC12 cells was found to be associated with the synaptic vesicle population. In a fraction containing purified synaptic vesicles, $pp60^{c\text{-}src}$ was enriched nearly 100 fold relative to the starting material. Taken together these results suggest that $pp60^{c\text{-}src}$ is specifically targeted to synaptic vesicles (Chapter 4). While it is not obvious what function synaptic vesicle associated $pp60^{c\text{-}src}$ performs this finding opens several avenues for study regarding the targeting of $pp60^{c\text{-}src}$ to the synaptic vesicle membrane. Experiments can address whether specific domains in $pp60^{c\text{-}src}$ mediate synaptic vesicle targeting, whether a " src receptor" exists in the synaptic vesicle membrane, and whether the association of $pp60^{c\text{-}src}$ is regulated.

TABLE II : Presence of Synaptic Vesicles in Synaptophysin Expressing Cell Types

	Brain Neurons	Neuroendocrine Cells (PC12)	Transfected Fibroblasts	Technique
Leube et al., 1989	yes	yes	yes	immuno EM protein composition
Clift-O'Grady et al, 1990 Linstedt and Kelly, 1991	yes	yes	no	sedimentation protein composition
Johnston et al., 1989	yes	no	no	immuno EM immuno fluorescence

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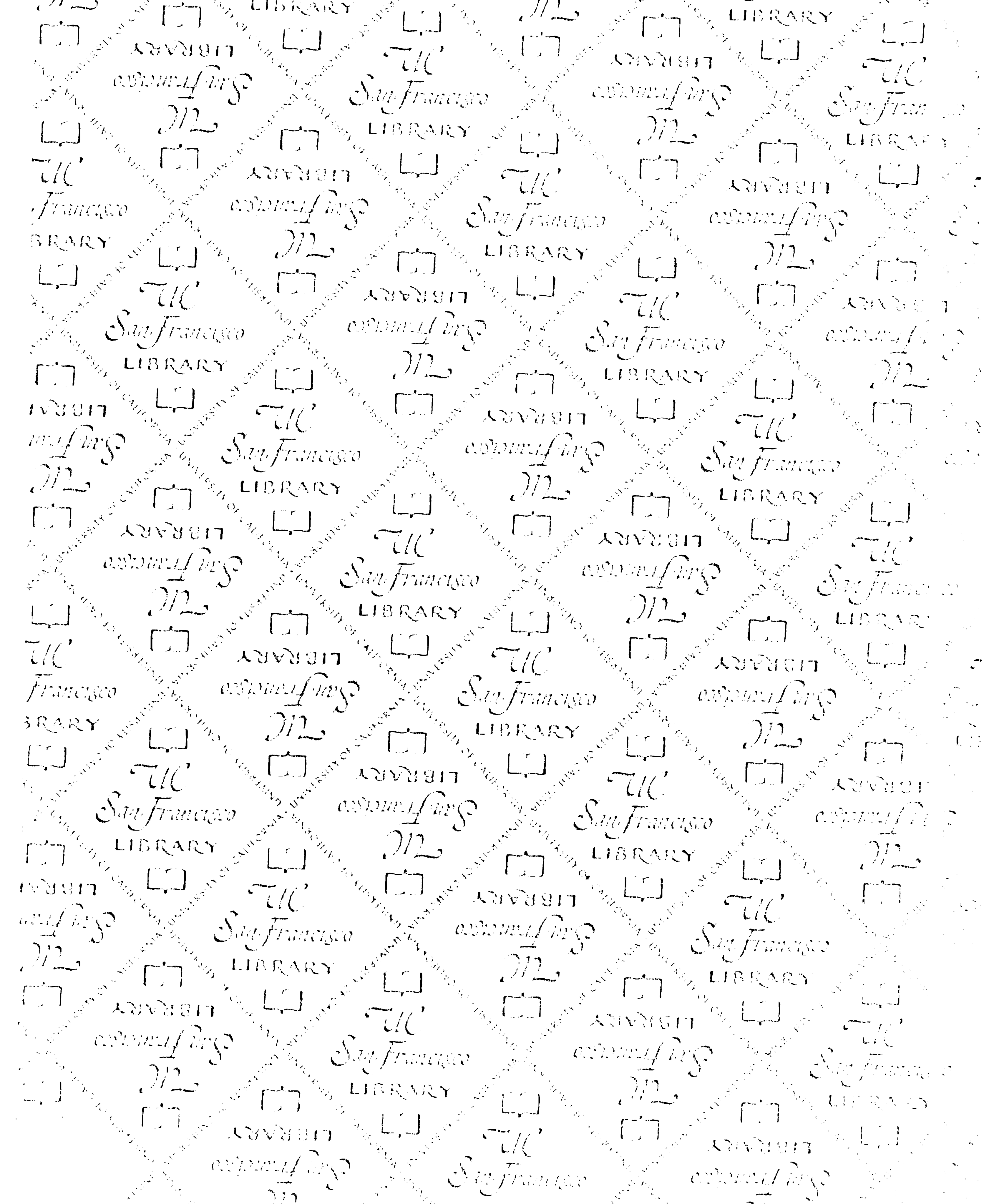

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