Synaptophysin: a substrate for the protein tyrosine kinase pp60$^{c\text{-}src}$ in intact synaptic vesicles

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Expression of pp60$^{c\text{-}src}$, the first well defined proto-oncogene product, is developmentally regulated and tissue-specific, with neuronal tissues displaying high amounts of the *c-src* encoded pp60$^{c\text{-}src}$ kinase activity. In the central nervous system pp60$^{c\text{-}src}$ is preferentially expressed in regions characterized by a high content of grey matter and elevated density of nerve terminals. In this study we show for the first time a direct interaction between pp60$^{c\text{-}src}$ and synaptophysin as a physiological target protein in neurons by demonstrating that endogenous pp60$^{c\text{-}src}$ is able to phosphorylate synaptophysin (p38). p38 is a major constituent of the synaptic vesicle membrane protein and is thought to play a key role in the exocytosis of small synaptic vesicles and possibly small clear vesicles in neuroendocrine cells.

Introduction

The cellular homologue of the viral *src* oncogene (*v-src*) of Rous sarcoma virus, the proto-oncogene, *c-src* is structurally highly conserved during evolution and has been demonstrated to be present and expressed for the first time in phylogenesis in the most primitive multicellular animals, the sponges (Barnekow & Schartl, 1984). A similarly well conserved expression pattern, with highest levels of pp60$^{c\text{-}src}$ kinase activity found in neural cells in all animals tested e.g. *Hydra*, insects, amphioxus, lampreys, sharks, bony fish, amphibians, birds and mammals, points to a very basic physiological function for this enzyme within this cell type (Schartl *et al.*, 1989; Schartl & Barnekow, 1984). During ontogenesis it has been demonstrated in various species that pp60$^{c\text{-}src}$ kinase activity is found preferentially in post-mitotic neurons of the central nervous system (CNS). The regional occurrence of the *c-src* gene product has been studied in detail (Maness, 1986; Sudol *et al.*, 1988; Ross *et al.*, 1988). A marked correlation of changes in *c-src* expression and the terminal differentiation of neural cells has been demonstrated in a variety of experimental systems (for review see Sudol, 1988, Barnekow 1989). In the rat CNS, pp60$^{c\text{-}src}$ is present in all regions, being enriched in areas characterized by high content of grey matter containing a high density of nerve terminals (Walaas *et al.*, 1988). A comparative localization of *c-src* mRNA and of the gene product indicated that the protein is translocated to the processes of the nerve terminals after translation (Maness *et al.*, 1988; Raulf *et al.*, 1989). A slower migrating and more active form of

pp60$^{c\text{-}src}$ containing an insertion of six amino acids within its amino terminal domain (Levy *et al.*, 1987; LeBeau *et al.*, 1987; Martinez *et al.*, 1987) is preferentially detected in membranes of neural growth cones and processes, leading to the suggestion of its possible role in growth cone-mediated neurite extension (Maness *et al.*, 1988). The physiological function of pp60$^{c\text{-}src}$ is, however, still obscure. Since it is widely accepted that it exerts its biological effects via tyrosine phosphorylation of specific cellular proteins, these target proteins need to be identified in order to obtain a better understanding of the molecular mechanism of pp60$^{c\text{-}src}$ function.

Recently, four major tyrosine-containing phosphoproteins with apparent molecular masses of 105, 94, 38, and 30 kD were described in synaptic vesicles, the exocytotic organelles of nerve terminals that store and release neurotransmitters upon electrical stimulation (Pang *et al.*, 1988a, b). The 38 kD protein was identified as synaptophysin (p38), a well characterized integral membrane protein of small synaptic vesicles (Jahn *et al.*, 1985). Furthermore, it was shown by subcellular fractionation of rat neocortex that crude synaptic vesicle fractions were highly enriched for pp60$^{c\text{-}src}$, whereas other fractions, in particular the cytosol and mitochondrial fractions contained only low levels (Hirano *et al.*, 1988). However, no experimental data exist whether the vesicular pp60$^{c\text{-}src}$ is the tyrosine kinase responsible for synaptophysin phosphorylation or whether other, yet unidentified kinases are involved. Using highly purified synaptic vesicles from rat brain, we present here evidence that synaptophysin is a substrate for the endogenous protein tyrosine kinase pp60$^{c\text{-}src}$ present in intact synaptic vesicles.

Results

Synaptic vesicles display pp60$^{c\text{-}src}$ specific tyrosine kinase activity

To test whether pp60$^{c\text{-}src}$ is specifically associated with synaptic vesicles, its distribution was monitored in subfractions obtained following vesicle isolation according to the procedure described by Hell *et al.* employing as the last purification step a chromatography on controlled-pore glass beads (Hell *et al.*, 1988). This procedure yields a vesicle fraction, which is free from contamination by other cellular membranes. Aliquots of fractions were used for immunoprecipitation, using three different antibodies known to react with rat pp60$^{c\text{-}src}$ and subsequently the kinase assay was performed essentially as described (Schartl & Barnekow, 1982). In parallel, the preparations of synaptic vesicles were routinely analysed for synaptophysin as described in Hell *et al.* (1988). Highest pp60$^{c\text{-}src}$ kinase activity was

found in the vesicle fraction also containing the highest amounts of synaptophysin (Hell et al., 1988). Performance of 2-dimensional phosphoamino acid analysis revealed that the heavy chain of the pp60^{c-src} antibody was phosphorylated exclusively in tyrosine residues (data not shown). These data are consistent with the results of Hirano et al. who demonstrated a good correlation between the level of tyrosine phosphorylation and the enrichment of synaptic vesicles (Hirano et al., 1988).

Synaptophysin is phosphorylated on tyrosine by immunoprecipitated pp60^{c-src} in vitro

In the following experiments, we have investigated whether solubilized synaptophysin is phosphorylated by exogenous pp60^{c-src} in vitro. For this purpose, a detergent extract of synaptic vesicles was incubated with pp60^{c-src} purified by immunoprecipitation from c-src transfected NIH3T3 cells (Shalloway et al., 1984; Johnson et al., 1985) using the monoclonal antibodies Mab 327 (Lipsich et al., 1983) and Mab GD11 (Parsons et al., 1984). Aliquots of synaptic vesicle extract were added to the purified pp60^{c-src}, followed by addition of gamma-[^{32}P]ATP to initiate phosphorylation. In Figure 1a the data obtained with Mab 327 are shown. Identical results were obtained using Mab GD 11. As can be seen from Figure 1a, lane 1, phosphorylation of both synaptophysin and of the pp60^{c-src} molecule itself occurred under these conditions. The phosphorylated band of 52 kD represents a degradation product of the c-src gene product, which is often seen under those experimental conditions (Erikson et al., 1980). Under the same experimental conditions but without addition of the pp60^{c-src} reactive monoclonal antibody to the c-src transfected NIH3T3 cell extract, phosphorylation of synaptophysin could not be detected (Figure 1a, lane 2), indicating that the phosphorylation of p38 depends on the presence of pp60^{c-src}. The failure to detect phosphorylated p38 under such experimental conditions shows that the amount of endogenous pp60^{c-src} present in 5 µg of vesicle extract is not sufficient to yield a detectable phosphorylation of synaptophysin on autoradiographs which had been exposed for 1 to 3 h. Therefore the results indicate that phosphorylation of p38 as shown in Figure 1a, lane 1 is due to the addition of significantly higher amounts of exogenous pp60^{c-src} compared to the low amounts of endogenous pp60^{c-src}. A comparative and quantitative Western Blot analysis of exogenously added and vesicular-bound endogenous pp60^{c-src} is shown in Figure 2.

Interestingly, when synaptophysin was immunoprecipitated from the vesicle extracts with Mab C7.2, a monoclonal antibody against synaptophysin (Navone et al., 1986) and the immunocomplex added to immunoprecipitated pp60^{c-src}, only the autophosphorylation of pp60^{c-src} but no phosphorylation of synaptophysin was observed. This result suggests that Mab C7.2 blocks the potential pp60^{c-src} phosphorylation site(s) in p38 (Figure 1a, lane 3).

Using synaptophysin, purified according to the procedure described by Navone et al. (1986), as a substrate for immunoprecipitated pp60^{c-src} a distinct phosphorylation of synaptophysin can be detected (Figure 3, lane 1). In the presence of Mab C7.2, the degree of phosphorylation of synaptophysin decreases drastically,
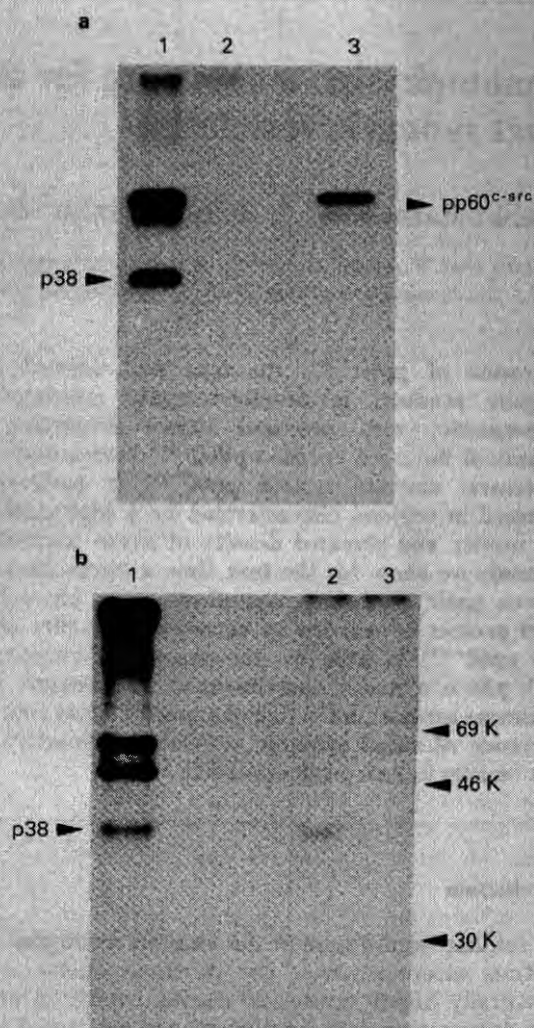

Figure 1 Phosphorylation of synaptophysin in vitro (a) and in intact synaptic vesicles (b). (a) Lane 1 = 5 µg synaptic vesicle protein extract added to immunoprecipitated pp60^{c-src} before the phosphorylation was started; lane 2 = same preparation in the absence of the pp60^{c-src} specific monoclonal antibody; lane 3 = immunoprecipitated pp60^{c-src} in kinase buffer added to synaptophysin, immunoprecipitated from 50 µg synaptic vesicle extract using the monoclonal antibody C7.2, immediately before the in vitro phosphorylation reaction was started. Exposure time of X-ray film 1 h using an intensifying screen. For further details see Material and methods. (b) 15 µl intact vesicles (= 45 µg vesicle protein) were incubated with 5 µCi gamma-[^{32}P]ATP in 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]-propane, 50 mM ZnCl$_2$ for 10 min at 25°C. The sample was divided into 3 aliquots. The reaction was stopped by addition of sample buffer (sample 1) and 100 µl RIPA buffer (150 mM NaCl, 10 mM Tris, 50 mM NaF, 2 mM EDTA, 0.1% DOC, 0.01% SDS, 1 mM PMSF, 5% Trasylol, 1% Triton X-100) (samples 2 and 3). For further details see (a) and Material and methods. Lane 1 = phosphorylated intact synaptic vesicles; lane 2 = phosphorylated synaptophysin from intact synaptic vesicles immunoprecipitated after lysis using 2 µl monoclonal antibody C7.2 and 2 µl rabbit-anti-mouse antibody; lane 3 = sample treated as described in lane 2, but in the absence of Mab C7.2. Only the rabbit-anti-mouse antibody and protein A containing bacteria were added to the labeled incubation mixture. The samples were run on the same gel, but due to the loss of radioactivity during washing procedures of the immunoprecipitated samples 2 and 3, this part of the gel had to be exposed to X-ray film for a longer period

whereas the degree of autophosphorylation of pp60^{c-src} increases proportionately (Figure 3, lane 2). These results strongly support the suggestion of a blocking of potential phosphorylation sites in the synaptophysin molecule by Mab C7.2. Additional evidence for the
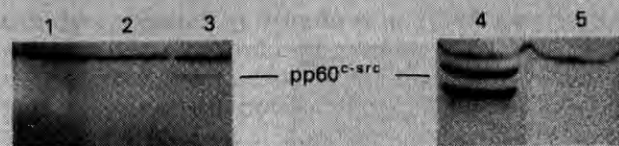
Figure 2 Quantitative Western Blot analysis of exogenously added and vesicular-bound endogenous pp60$^{c\text{-}src}$. Lane 1: 1.25 μg vesicle extract (for details see Figure 1a and Material and methods); lane 2: 3.75 μg vesicle extract; lane 3: 10.00 μg vesicle extract; lane 4 and lane 5: 50 μg extract of c-src transfected 3T3 cells, prepared in extraction buffer (see Material and methods). Samples 1–4 were incubated with Mab 327 (diluted 1:2000 with PBS-0.25% Tween20 and sample 5 was incubated with PBS-0.25% Tween20 in the absence of antibodies for at least 20 h at 4°C followed by incubation with biotinylated anti-mouse antibody, diluted 1:1000 in PBS-0.25% Tween20 for 60 min at 25°C. For further details see Material and methods

Figure 4 Phosphoamino acid analysis of synaptophysin. (a) Synaptophysin phosphorylated by pp60$^{c\text{-}src}$ in vitro (for details see Figure 1a and Material and methods). (b) Synaptophysin phosphorylated in intact vesicles (for details see Figure 1b, lane 1) was treated as described in Material and methods. (c) Synaptophysin phosphorylated in synaptic vesicle extracts after previous elimination of pp60$^{c\text{-}src}$ was treated as described in Material and methods

blocking of potential phosphorylation sites within the synaptophysin molecule comes from experiments using intact synaptic vesicles which will be described in detail below. Lanes 3 and 4 show the results of control experiments whereby sample 3 was incubated in the absence of pp60$^{c\text{-}src}$ (Figure 3, lane 3). Thus unspecific phosphorylation due to contaminating proteins from the cell extract being present in the incubation mixture can be excluded. Addition of gamma-[^{32}P]ATP to purified synaptophysin demonstrates that purified p38 itself does not exhibit a phosphorylating activity (Figure 3, lane 4). In order to define our assay conditions, we examined further properties of the in vitro phosphorylation reaction. Comparing the influence of divalent cations on the phosphorylation reaction, a preference for Zn^{2+} versus Mn^{2+} and Mg^{2+} was observed, with 50 mM $ZnCl_2$
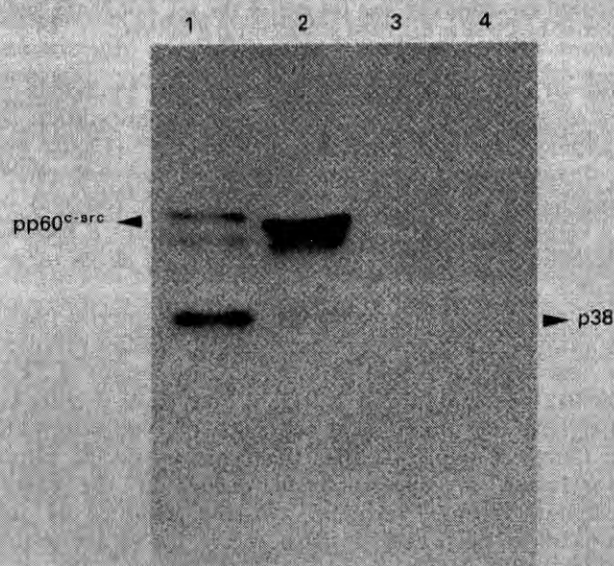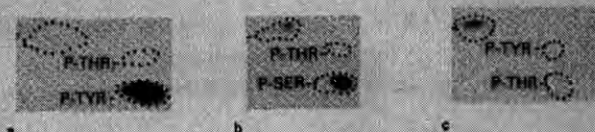
yielding the highest degree of [^{32}P] incorporation into p38 (data not shown). This is in line with a variety of experiments using artificial substrates for the in vitro kinase reaction (Barnekow & Bauer, 1984; Findik & Presek, 1988). Analysis of the time-dependent incorporation of gamma-[^{32}P]ATP into p38 by immunoprecipitated pp60$^{c\text{-}src}$ revealed that maximum incorporation occurred after 5 min (data not shown). These results are consistent with the data reported by Pang et al. who described a 50% of maximal level of tyrosine phosphorylation of synaptophysin within three minutes (Pang et al., 1988).

Identification of phosphoamino acids in synaptophysin from synaptic vesicle extracts phosphorylated by the pp60$^{c\text{-}src}$ immunocomplex is shown in Figure 4a. The data indicate that synaptophysin is exclusively phosphorylated in tyrosine residues in vitro.

Synaptophysin is phosphorylated by pp60$^{c\text{-}src}$ tyrosine kinase activity in intact synaptic vesicles

To find out whether synaptophysin can also be phosphorylated by endogenous pp60$^{c\text{-}src}$ in intact synaptic vesicles, we incubated intact vesicles with gamma-[^{32}P] ATP. Under these conditions, only extravesicular domains of synaptophysin such as the tyrosine rich carboxy-terminal tail (Südhof et al., 1987) of the molecule should get phosphorylated by a protein kinase located in the same subcellular compartment as synaptophysin. As can be seen from Figure 1b, lane 1, p38 clearly shows incorporation of [^{32}P]. Two-dimensional phosphoamino acid analysis revealed a major phosphorylation of tyrosine residues and a minor phosphorylation of serine residues possibly due to a serine kinase of unknown origin being present in the vesicles (Figure 4b). Two additional protein bands of about 50–60 K are also labeled which were phosphorylated in serine and, to a lesser extent, in threonine residues as revealed by phosphoamino acid analysis (data not shown). A 29 kD protein, which was also seen on the autoradiograph after longer exposure, was phosphorylated in serine and to a lesser extent in tyrosine residues.

After lysis of the phosphorylated vesicles and subsequent immunoprecipitation of the extract using Mab C7.2, the radioactive labeled synaptophysin was seen running in the same molecular weight range as the unprecipitated 38 kD protein (Figure 1B, lane 2). In Figure 1b, lane 3 a control experiment is shown. An aliquot of sample 2 was processed without addition of monoclonal antibody against p38, to exclude unspecific reactions of the second antibody or the Protein A containing bacteria.

Figure 3 Phosphorylation of purified synaptophysin (p38) by immunoprecipitated pp60$^{c\text{-}src}$. pp60$^{c\text{-}src}$ was immunoprecipitated by Mab 327 as described in Figure 1. 0.1 μg purified synaptophysin was added immediately before the in vitro phosphorylation reaction was started as described in Material and methods. The reaction was stopped by addition of sample buffer and the samples treated as described in Figure 1. The result is shown in lane 1. In a parallel experiment 2 μl C7.2 were added to the incubation mixture immediately before the phosphorylation reaction was started (lane 2). In lane 3 a duplicate of sample 1 was incubated in the absence of Mab 327, but otherwise treated as described for lane 1. In lane 4 purified synaptophysin (0.1 μg) was incubated with 5 μCi gamma-[^{32}P]ATP in the absence of immuno-precipitated pp60$^{c\text{-}src}$ or pp60$^{c\text{-}src}$ containing cell extract.
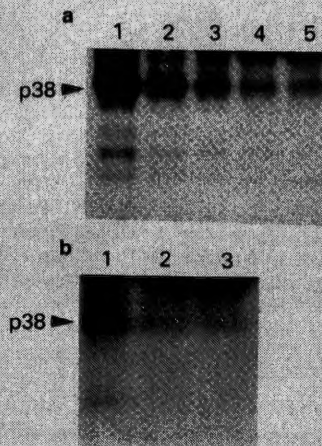
Figure 5 Phosphorylation of synaptophysin in intact vesicles in the presence of increasing amounts of Mab C7.2 (a) and in synaptic vesicle extracts after previous elimination of pp60^{c-src} (b). (a) 7 μl intact synaptic vesicles were phosphorylated in the absence or presence of Mab C7.2 as described in Figure 1b. except that 20 mM 1,3-bis-[tris(hydroxymethyl)methyl-amino]-propane was replaced by 50 mM Tris-(hydroxymethyl)-aminomethane, pH 7.8. 1 = in the absence of Mab C7.2, 2 = 0.5 μg, 3 = 5 μg, 4 = 25 μg, 5 = 50 μg Mab C7.2 were added. For further details see Figure 1b. (b) lane 1: 12 μg synaptic vesicle extract was incubated with 5 μCi gamma-[^{32}P]ATP in 50 mM Tris-(hydroxymethyl)-aminomethane, pH 7.8, 50 mM ZnCl$_2$ as described in Figure 1a. lanes 2 + 3: pp60^{c-src} was immunoprecipitated from synaptic vesicle extracts (12 μg each) using 1 μl Mab GD11 (2) or 1 μl Mab 327 (3) and a rabbit-anti-mouse antibody. The immunocomplexes were pelleted as described in Figure 1a and the supernatants incubated with 5 μCi gamma-[^{32}P]ATP for 10 min at 25°C. The reaction was stopped by addition of sample buffer and the probes processed as described in Figure 1a and Materials and methods. Exposure time of X-ray film 8 h using an intensifying screen

Incubation of intact synaptic vesicles with gamma-[^{32}P]ATP in the presence of increasing amounts of the monoclonal antibody against synaptophysin (Mab C7.2) results in a concentration-dependent decrease of synaptophysin phosphorylation (Figure 5a). This result supports the idea that the monoclonal antibodies partially block potential phosphorylation sites within the synaptophysin molecules. Due to the fact that Mab C7.2 is directed against a single epitope, a 100% blocking of all potential tyrosine phosphorylation sites would not be expected. On the other hand, the observation that the phosphorylation of synaptophysin was not completely abolished as observed in reactions containing immunoprecipitated synaptophysin from vesicle extracts (Figure 1b) points to a residual phosphorylation by serine kinases.

Finally, to prove the direct interaction of pp60^{c-src} and synaptophysin, aliquots of highly concentrated synaptic vesicle extracts were incubated with either 1 μl Mab 327 or 1 μl Mab GD 11 to eliminate the vesicular bound pp60^{c-src}. After separation of the pp60^{c-src} immunocomplexes from the incubation mixture, gamma-[^{32}P]ATP was added. As can be seen in Figure 5b, lanes 2 and 3, a significant reduction (about 45 to 50%) of synaptophysin phosphorylation can be observed compared to the sample shown in Figure 5b, lane 1, from which pp60^{c-src} has not been removed by previous immunoprecipitation. Immunoprecipitation using only the rabbit-anti-mouse antibody did not result in a significant reduction of synaptophysin phosphorylation (data not shown). Two-dimensional phosphoamino acid analyses of phosphorylated synaptophysin in the pre-
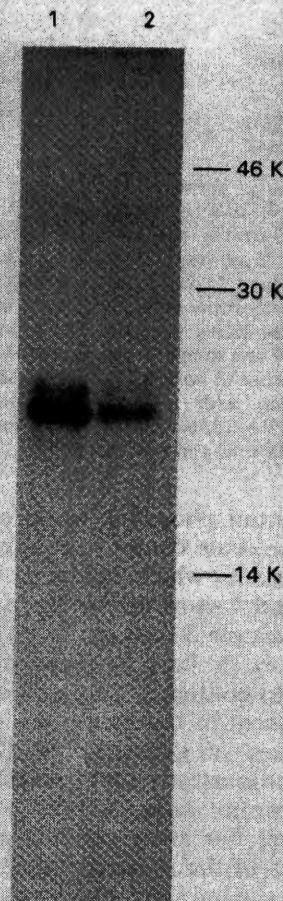

Figure 6 CNBr cleavage of synaptophysin phosphorylated *in vitro* (1) or in intact synaptic vesicles (2). Phosphorylation of synaptophysin was performed as described in Figure 1a and b. [^{32}P]-labelled synaptophysin was cut out of the gel, and eluted and cleaved by CNBr as described in Material and methods

sence or absence of pp60^{c-src} revealed only the serine — but no significant tyrosine-specific phosphorylation of synaptophysin after elimination of pp60^{c-src} (Figure 4b, c), demonstrating that no endogenous tyrosine phosphorylation of synaptophysin occurs after removal of the vesicular pp60^{c-src}, which clearly indicates a specific interaction of pp60^{c-src} and synaptophysin as its target protein.

Comparative CNBr cleavage of synaptophysin phosphorylated *in vitro* or in intact synaptic vesicles revealed an identical cleavage pattern of one major [^{32}P]-labelled fragment (about 20 kD) in both cases (Figure 6). Analysing the amino acid sequence of synaptophysin, we therefore conclude that the tyrosine-specific phosphorylation of synaptophysin occurs at the extra-vesicular carboxy terminal tyrosine residues of the molecule in both cases *in vitro* and in the intact vesicle (Südhof *et al.*, 1987).

Discussion

We have demonstrated that pp60^{c-src} phosphorylates specifically the synaptic vesicle constituent synaptophysin (p38) on tyrosine residues *in vitro*. Several findings indicate that synaptophysin is also a physiological substrate in the intact neuron. When intact synaptic vesicles were subjected to endogenous protein phosphorylation, synaptophysin was identified as the major tyrosine containing substrate. This is consistent

with data reported by Hirano et al. (1988) and Pang et al. (1988). The strongest evidence for synaptophysin as a physiological substrate for pp60^{c-src} comes from the fact that elimination of pp60^{c-src} from vesicle extracts by immunoprecipitation abolishes tyrosine phosphorylation of synaptophysin, while leaving its phosphorylation on serine residues unchanged. This indicates that the tyrosine phosphorylation of synaptophysin in intact synaptic vesicles is due to the activity of pp60^{c-src} kinase present in the same organelle. Recently, Grandori and Hanafusa described a protein of 38 kD which is stably associated with pp60^{c-src}, and found that this complex is highly enriched in chromaffin granule membranes of adrenal medulla (Grandori & Hanafusa, 1988). A similar complex was seen in rat and chicken brain. This p38, however, they identified to be different from calpactin I, II and synaptophysin, a finding which leads to the suggestion of additional potential target proteins in neuronal tissues.

The functional role of tyrosine phosphorylation of synaptophysin remains to be elucidated. Analogous to the regulatory role of serine/threonine phosphorylation in nerve cell function and tyrosine phosphorylation in neuronal signal transduction, tyrosine phosphorylation of synaptophysin may regulate specific processes, e.g. neurotransmitter release in nerve terminals (Nairn et al., 1985; Hopfeld et al., 1988). Synaptophysin has been shown to bind Ca^{2+} ions and to form ion channels when incorporated into planar lipid membranes, leading to the suggestion that it may be part of the fusion event during exocytosis, possibly by forming a fusion pore (Rehm et al., 1986). It will be of interest to determine whether the phosphorylation of synaptophysin affects its Ca^{2+} binding activity or whether the pp60^{c-src} kinase activity is regulated by Ca^{2+} in vivo. Interestingly, one of the Ca^{2+}-binding proteins associated with chromaffin granules, which also contain high amounts of pp60^{c-src} kinase activity, namely p36 (Calpactin), is a phosphorylation substrate for the viral pp60^{src} (Parsons et al., 1986).

The tyrosine phosphorylation may be involved in the modulation of the processes discussed above, resulting in a regulation of neurotransmitter release from the presynaptic nerve ending. Alternatively, synaptophysin phosphorylation may be related to the membrane traffic of small synaptic vesicles. For several vesicle membrane components, it has been shown that after synaptic vesicles undergo exocytotic release of their neurotransmitters, their membrane components are returned to the inside of the nerve terminals in coated vesicles (Heuser & Reese, 1981). pp60^{c-src} present at the cytoplasmic membrane of mature nerve cells could represent such routing molecules. Thus, tyrosine phosphorylation may regulate the biogenesis or the recycling of small synaptic vesicles or their direction to the nerve terminal. The aim of this paper was to demonstrate for the first time a direct interaction between pp60^{c-src} and synaptophysin as a physiological target protein in neurons. More work is clearly needed, however, to unravel the precise role of synaptophysin and its phosphorylation by the c-src proto-oncogene in the membrane dynamics of small synaptic vesicles. The availability of Mab C7.2 as a blocking antibody which interferes with synaptophysin tyrosine phosphorylation will help in the design of experiments aimed to elucidate the functional significance of this process.

Materials and methods

Reagents

All chemicals used were the purest grade available.

Antibodies

Antisera from RSV-tumor-bearing rabbits (TBR-sera) were prepared by simultaneous injection of SR-RSV-D and PR-RSV-C strain into newborn rabbits in a modification (Ziemiecki & Friis, 1980) of the procedure described by Brugge & Erikson (1977). The monoclonal antibody 327 to pp60^{src} (Lipsich et al., 1983) was obtained from J. Brugge (University of Pennsylvania, Philadelphia, USA), the monoclonal antibody GD11 (Parsons et al., 1984) from S. Parsons (University of Virginia, Charlottsville, USA). The monoclonal antibody C7.2 was generated as described previously (Navone et al., 1986). The rabbit-anti-mouse antibody was from Cooper Biochemicals Inc. (Malvern, USA). The biotinylated anti-mouse antibody was from Pansystems (Aidenbach, FRG).

Isolation of synaptic vesicles and purification of synaptophysin

Synaptic vesicles from rat brain were isolated according to the procedure described by Hell et al., 1988. Synaptophysin was purified by affinity chromatography as described previously (Navone et al., 1986).

Cells

The c-src transfected 3T3 cells were a gift from D. Shalloway (Pennsylvania State University, Pennsylvania, USA) and grown in Dulbecco-Vogt modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal calf serum.

Preparation of cell and vesicle extracts, immunoprecipitation and phosphorylation

Cells were washed, lysed and the extract clarified as described previously (Barnekow & Bauer, 1984). pp60^{c-src} was immunoprecipitated from solubilized extracts (200 µg) of c-src transfected 3T3 cells using either 1 µl Mab 327 or 1 µl Mab GD11 and a rabbit-anti-mouse antibody. After addition of a 10% suspension of Protein A containing Staphylococcus aureus bacteria (50 µl), the bacterial-bound immunocomplexes were washed. 5 µg vesicle extract, prepared by mixing equal volumes of purified vesicles in 0.32 M sucrose, 10 mM Hepes-KOH, pH 7.4 (Hell et al., 1988) and extraction buffer (10 mM sodium phosphate, 10 mM EDTA, 40 mM NaF, 1% Triton X-100, 5% Trasylol, pH 7.0) and centrifugation for 60 min at 4°C and 13 000g, was added immediately before the addition of 5 µCi gamma-[^{32}P]ATP in kinase buffer (20 mM 1,3-bis[tris-(hydroxymethyl)methylamino]propane), 0.5 to 50 mM ZnCl$_2$, followed by incubation for 10 min at 25°C. The reaction was stopped by addition of sample buffer containing 1.2% NaHCO$_3$ (Barnekow & Bauer, 1984). The mixture was boiled for 2 min, centrifuged for 2 min at 10 000g and the supernatant was loaded on a 12.5% polyacrylamide gel (Barnekow & Bauer, 1984). The labeled proteins were detected by autoradiography. The molecular weights of the proteins were determined using [^{14}C]-labeled protein markers.

Western Blot analysis

The samples were separated on a 10% polyacrylamide gel and transferred electrophoretically (60 mA, 45 min) to immobilon (Millipore, FRG) transfer membrane (prewashed with 0.3 M Tris/20% methanol, 25 mM Tris/20% methanol, 25 mM Tris/20% methanol/40 mM 6-amino-N-hexanoic acid). Then the

transfer membrane was blocked in PBS-0.5% Tween 20 for 20 min at 40°C and incubated with Mab 327 (diluted 1:2000 with PBS-0.25% Tween20 or with PBS-0.25% Tween20 in the absence of antibodies for at least 20 h at 4°C followed by incubation with biotinylated anti-mouse antibody, diluted 1:1000 in PBS-0.25% Tween20 for 60 min at 25°C. Alkaline phosphatase-streptavidin diluted 1:1000 in buffer 1 (0.1 M Tris, 0.1 M NaCl, 2.5 mM $MgCl_2$, 2% NaN_3, pH 7.4) was added. After incubation (20 min, 25°C) the membranes were washed in buffer 1 and subsequently in buffer 2 (1 M Tris, 0.1 M NaCl, 2.5 mM $MgCl_2$, pH 9.4) and the blots developed by addition of 1:30 0.5% 5-bromo-4-chloro-3-indolyphosphate and 0.03% 4-nitro-blue-tetrazolium-chloride (Serva, Heidelberg, FRG) for 3–4 min at 40°C.

Phosphoamino acid analysis

[^{32}P]-labelled IgG or synaptophysin phosphorylated by pp60^{c-src} *in vitro* were cut out of the gel and eluted over night at 37°C in 0.05 M NH_4HCO_3 containing 0.1% SDS. The sample was hydrolyzed in 6 N HCl for 140 min at 110°C and prepared for thin-layer electrophoresis according to the methods described by Cooper *et al.* (1983).

Protein determination

Determination of protein concentration in the supernatant of the clarified cell lysates was carried out on trichloracetic acid-precipitated aliquots according to the method of Lowry *et al.* (1951).

CNBr cleavage

[^{32}P]-labelled synaptophysin was cut out the gel and eluted as described above. 1/4 volume of 100% trichloracetic acid (TCA) was added (final 20% TCA) and the mixture incubated for 8–10 h at 4°C. After centrifugation, 2 × 0.5 ml ethanol-ether (1:1, −20°C) were added to the pellet, the supernatant discharged and the pellet dried by speed vacuum.

30 μl CNBr in 70% formic acid (50 mg ml^{-1}) was added, the pellet resuspended and incubated for 60 min at room temperature. After addition of 1 ml H_2O the mixture was lyophilized. The reaction products were separated on a 18% polyacrylamide gel and the [^{32}P]-labelled bands were detected by autoradiography. [^{14}C]-labelled peptides were used as molecular weight markers.

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