Phosphotyrosine phosphatase activity associated with c-Src in large multimeric complexes isolated from adrenal medullary chromaffin cells

Monique L. VAN HOEK, Colleen S. ALLEN and Sarah J. PARSONS¹

Box 441, Health Sciences Center, Department of Microbiology, University of Virginia, Charlottesville, VA 22908, U.S.A.

Chromaffin cells, which secrete catecholamines in response to acetylcholine, express high levels of the Src-family tyrosine kinases. These kinases contain protein–protein interaction domains which bind signal transduction proteins that participate in a variety of cellular processes. To determine if signalling proteins bind c-Src in chromaffin cells, we examined c-Src immunocomplexes for co-precipitating proteins. We discovered a phosphotyrosine phosphatase (PTPase; EC 3.1.3.48) activity which associates with specific subcellular pools of c-Src *in vivo* and which preferentially binds the SH2 (Src homology 2) domain of c-Src *in vitro*. Known PTPases were not identified by blotting of c-Src immunocomplexes with a panel of anti-PTPase anti-

bodies, suggesting that the PTPase may be a novel family member. The c-Src–PTPase complex is enriched in the plasma membrane fraction and exists in several large complexes, as revealed by gel-filtration analysis. This PTPase activity is altered rapidly following stimulation by secretagogues, decreasing within 30 s and returning to basal levels by 60 s of stimulation. Both the subcellular localization and rapid activity changes suggest that the c-Src-associated PTPase may function in early signalling events emanating from the nicotinic acetylcholine receptor. In support of this is the co-precipitation of a PTPase activity with the nicotinic acetylcholine receptor and co-chromatography of this receptor with one of the c-Src–PTPase complexes.

INTRODUCTION

Chromaffin cells reside in the medulla of the adrenal gland and are responsible for the synthesis, storage and regulated exocytosis of catecholamines. *In vivo*, acetylcholine is the primary stimulus for catecholamine secretion, signalling through the acetylcholine receptor (AChR). *In vitro*, nicotine functionally replaces acetylcholine. Upon binding to the nicotinic AChR (nAChR), nicotine activates a variety of signal transduction pathways (including a rapid influx of calcium through voltage-gated calcium channels), which culminate in the movement of secretory vesicles to the plasma membrane, fusion and release [1–4].

Studies from our laboratory have indicated that tyrosine kinases are required for chromaffin cell exocytosis [5]. Since the nAChR does not exhibit intrinsic tyrosine kinase activity, cytoplasmic tyrosine kinases, including Src family members, have been implicated in this process. Of those expressed in chromaffin cells, our laboratory has focused on c-Src, because it is abundant [6], its activity is modulated following secretory stimulation [7], and it is appropriately localized to play an important role in exocytosis [6,8].

Members of the Src family of tyrosine kinases are highly related [9] and contain well-defined domains, including the N-terminal Unique region, the Src homology 3 (SH3) and SH2 regions, the kinase domain and the C-terminal regulatory domain. The N-terminal Unique region is the least conserved among family members and contains the myristoylation site, which is required for membrane localization. The SH3 and SH2 domains are protein–protein interaction domains that bind proline-rich and phosphotyrosine-containing moieties, respectively, in other proteins. These structural features of Src family members suggest that the c-Src molecule may have two separate functions: a catalytic function and an ‘adapter’ function.

Studies using vaccinia virus vectors to overexpress c-Src in chromaffin cells revealed a non-catalytic role for c-Src in se-

cretion; specifically, overexpressed c-Src reversed the inhibitory effect of the vaccinia virus vector on secretion [10]. In the nicotine-activated signalling pathway, this function of c-Src was localized to or immediately downstream of the nAChR and did not require c-Src tyrosine kinase activity. Based on these findings, it was postulated that the protein–protein interaction domains of c-Src might be mediating its ability to rescue secretory activity. Because the vaccinia virus expresses phosphotyrosine phosphatases (PTPases) [11], which could be functioning as inhibitors of secretion, it was postulated that a likely candidate for a protein that binds to and is sequestered by c-Src could be a PTPase. Several reports have indicated that PTPases associate with c-Src in other systems [12–14], providing a model for our studies. Thus immunoprecipitates of c-Src from uninfected chromaffin cells were examined for such an activity to determine if PTPases could function in one or more signalling pathway(s) emanating from the neuronal nAChR.

EXPERIMENTAL

Culture of chromaffin cells and secretion assay

Primary cultures of chromaffin cells were prepared from adrenal glands as described by Cox et al. [5]. For secretory stimulation, 20 μ M nicotine (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used, as described in Allen et al. [15]. [³H]Noradrenaline release assays (secretion assays) were performed as outlined previously [5,15].

Immunoprecipitation and immunoblotting

These protocols were performed as described in [7,10,15,16] with the following antibodies: c-Src-specific monoclonal antibodies

Abbreviations used: AChR, acetylcholine receptor; nAChR, nicotinic AChR; PTPase, phosphotyrosine phosphatase; SH3 domain, Src homology 3 domain; MAb, monoclonal antibody; RCML, reduced, carboxymethylated lysozyme; GST, glutathione S-transferase; SHPTP2, Src homology 2-containing phosphotyrosine phosphatase 2.

¹ To whom correspondence and reprint requests should be addressed.

(MAbs) GD11 and EB8 [17,18]; MAb 2-17 (Quality Biotech, Camden, NJ, U.S.A.); c-Src-family rabbit polyclonal antibody Q0 [15,19]; c-Src-specific rabbit polyclonal antibodies Q8 and Q9 [7]; Syp/SHPTP2 (Src homology 2-containing phosphotyrosine phosphatase 2)-specific antibody (UBI, Lake Placid, NY, U.S.A.); AChR α - and AChR β -specific MAbs (Transduction Laboratories, Lexington, KY, U.S.A.), AChR-specific MAb 35 [20]; and the negative antibody controls p120 RasGAP-specific MAb 6F2 [21], anti-phosphotyrosine MAb 4G10 (UBI) and mouse γ -globulin (Jackson ImmunoResearch Laboratory, West Grove, PA, U.S.A.). For immunoprecipitation, antigen-antibody complexes were captured on Protein A-Sepharose (Pharmacia). Unless otherwise stated, all immunoprecipitations were performed with 5 μ g of antibody and 500 μ g of chromaffin cell lysate, prepared in PTPase Lysis Buffer (see below).

For Western immunoblotting, Q0 antiserum was diluted 1:1000, and 125 I-labelled Protein A (Amersham, Arlington Heights, IL, U.S.A.) was used for detection. The relative amounts of c-Src in immunocomplexes were determined by densitometric analysis of autoradiograms using ImageQuant software.

32 P-labelling of reduced, carboxymethylated lysozyme (RCML) on tyrosine residues

RCML was prepared using a modification of the Tonks procedure [22] and was phosphorylated on tyrosine by v-Src obtained from IV5 cells [23]. v-Src was immunoprecipitated from 20 mg of IV5 cell lysate using 12 μ g of anti-Src MAb GD11 and 500 μ l of Protein A-Sepharose beads. This immunocomplex was washed with a high-salt buffer (1 M NaCl, 50 mM Tris, pH 7.2, 0.1 % Nonidet P40), a low-salt buffer (50 mM Tris/HCl, pH 7.2, 1 % Nonidet P40, 0.5 % deoxycholate, 1 mM EDTA, 150 mM NaCl), and Hepes-buffered saline (pH 7.2). The phosphorylation reaction was prepared in 1 ml [24] of 0.5 M Hepes, 5 mM Na₃VO₄, 15 M NaCl, 10 mM MgCl₂, 2 mM MnCl₂, 0.2 mM ATP, 2 M RCML (Sigma), the v-Src immunocomplex and 0.5 mCi of [γ - 32 P]ATP (DuPont NEN, Boston, MA, U.S.A.). After vortexing, the reaction was incubated at 30 °C for 48 h with constant mixing.

To terminate the reaction, Protein A-Sepharose beads were removed by centrifugation at 16000 *g* for 5 min at 4 °C, and the supernatant was brought to a final concentration of 15 % (w/v) trichloroacetic acid to precipitate the labelled RCML. The mixture was centrifuged at 4 °C for 10 min at 16000 *g*, and the pellet was washed three times with cold 10 % trichloroacetic acid and twice with acetone to remove the trichloroacetic acid. The pellet was dissolved in 100 μ l of 0.1 M NaOH and brought to 1 ml with 500 μ M Hepes, pH 7.0. Incorporation of radioactivity was measured in a Beckman LS5801 scintillation counter. Substrate was prepared both with and without the addition of 0.2 mmol of unlabelled ATP during the phosphorylation reaction. Incorporation in the absence of unlabelled ATP averaged 0.4 mmol of phosphotyrosine/mol of RCML; that in the presence of unlabelled ATP was 80 mmol/mol. However, all assays were performed under conditions in which 10–50 % of the available counts were released by the active samples, thus remaining in the linear range of the reaction [24]. Similar results (% phosphotyrosine released) were obtained regardless of which substrate was used.

PTPase assay

The protocol used to measure PTPase activity was adapted from that of Shriner and Brautigan [25]. Cells (or medullae) were disrupted in PTPase Lysis Buffer containing 25 mM Hepes, pH 7.0, 5 mM EDTA, 5 mM EGTA, 1 % Triton X-100, 45 mM

2-mercaptoethanol, 1.0 mM PEFABLOC (Boehringer Mannheim), 50 μ g/ml leupeptin and 0.5 % aprotinin (Sigma). Extracts were clarified by centrifugation at 16000 *g* for 5 min. Immunoprecipitates were washed three times in Diluent Buffer (25 mM Hepes, pH 7.2, 5 mM EDTA, 2 mM EGTA, 0.1 mg/ml BSA and 30 mM 2-mercaptoethanol) and assayed for PTPase activity by incubation with $\sim$ 100000 c.p.m. of [32 P]RCML in 40 μ l of Diluent Buffer for 45 min at 30 °C. Samples were precipitated with trichloroacetic acid and subjected to centrifugation. The amount of [32 P]P_i released into the supernatant was determined by scintillation counting. Data are expressed as percentage release, determined by subtracting the counts released from beads alone from the sample counts and dividing by the total counts. In some cases, to facilitate statistical analysis of different experiments, relative activities were used; however, between experiments, the percentage phosphotyrosine released was generally comparable. Zinc inhibition was achieved by adding 2.1 mM ZnCl₂ to Diluent Buffer made with 2 mM EGTA but without EDTA for a final effective concentration of 100 μ M ZnCl₂. The zinc-containing buffer was used for the washes and in the PTPase reaction to inhibit activity. Na₃VO₄ was used similarly at 1–5 mM. Total PTPase activity (not c-Src associated) was assayed by measuring the release of P_i from a tyrosine-phosphorylated peptide substrate [END(pY)INASL] using a molybdate-dependent detection reaction for free phosphate (Promega Corp., Madison, WI, U.S.A.).

Fusion proteins

All fusion proteins were derived from chicken c-Src and were expressed from the GST-2T vector (Pharmacia Biotech, Piscataway, NJ, U.S.A.), except the Unique-domain protein, which was derived from murine c-Src sequences and was expressed from the GST-4T3 vector. The glutathione S-transferase (GST)-Unique-domain-(1–82) fusion construct was kindly donated by Dr. J. Park and Dr. C. Cartwright (Stanford University, Stanford, CA, U.S.A.). The GST-SH2-(amino acid residues 149–262), GST-SH3-(residues 79–144) and GST-SH3/2-(residues 79–262) tandem domains were provided by Dr. T.-H. Leu and Dr. J. T. Parsons (University of Virginia, Charlottesville, VA, U.S.A.). Fusion proteins were expressed in XL1-Blue bacteria (Stratagene) and used at a concentration of 1 μ g of fusion protein/100 μ g of cellular protein.

Hypotonic lysis and cellular fractionation

Hypotonic lysates were prepared from cultured chromaffin cells or medullae as described in Reynolds et al. [26], but in the absence of PTPase inhibitors. The supernatant (cytosol) was brought to 45 mM 2-mercaptoethanol, 5 mM EDTA, 5 mM EGTA and 1 % Triton X-100. The pellet was resuspended in a volume of PTPase Lysis Buffer equal to that of the supernatant, in order to generate fractions that represented equivalent portions of cells. The resuspended pellet was again subjected to Dounce homogenization and centrifugation at 2000 *g* to prepare supernatants for immunoprecipitation. Fractions enriched in cellular membranes, secretory vesicles or secretory vesicle membranes were prepared by sucrose-gradient fractionation and resuspended in equal volumes of PTPase Lysis Buffer to generate cell-equivalents, as described by Allen et al. [15].

Gel filtration

Chromatography through a 90 ml Sepharose 4B-200 resin (Sigma) gel-filtration column was carried out at 4 °C in a 0.1 M

buffer (25 mM Tris, 0.1 M NaCl, pH 7.2), at 5 ml/h per cm². Medullary tissue (40–100 mg of protein), lysed in PTPase Lysis Buffer and cleared by centrifugation (16000 g, 10 min, 4 °C), was loaded in 1–2 % of the column volume. The detergent concentration of the load was adjusted with respect to the protein concentration for appropriate solubilization [27]. Fractions, each of which represented approx. 2 % of the column volume, were assayed by immunoprecipitation of c-Src from 500 μ l samples with MAb 2-17 and performing the PTPase assay as described above.

RESULTS

c-Src-associated PTPase activity: assay conditions for optimal detection

As part of our overall effort to identify chromaffin cell proteins that bind to c-Src, we examined c-Src immunocomplexes for associated PTPase activity. Figure 1(A) shows that PTPase activity was specifically co-immunoprecipitated with c-Src when compared with the activity detected in immunocomplexes of the negative antibody and positive Syp controls. Syp (also known as SHP-2, SHPTP2 or PTP1D) is an intracellular (non-receptor-type) PTPase, containing two SH2 domains, which is present in many cell types [28], including chromaffin cells. The Western immunoblot in Figure 1(B) shows that Syp and c-Src were immunoprecipitated by the appropriate antibodies and that no Syp was detectable in the c-Src immunoprecipitate. This analysis also revealed that the anti-Syp antibody was able to precipitate less than 10 % of the total Syp present in whole-cell extracts (compare lanes 6 and 7 of Figure 1B), helping to explain the

Table 1 Inhibition of c-Src-associated PTPase activity by zinc and vanadate

Samples were prepared by immunoprecipitation from 500 μ g portions of chromaffin cell lysate with the indicated antibodies and determination of the PTPase activity of each sample, as described in the Experimental section ($n = 3$ –27). The results are expressed as the percentage phosphotyrosine release from RCML, and are means $\pm$ S.E.M.

Antibody	PTPase activity (% release)		
	Control	+ 5 mM Vanadate	+ 100 μ M ZnCl ₂
Anti-Src	28.1 $\pm$ 2.4	2.6 $\pm$ 1.6	9.3 $\pm$ 2.3
Anti-Syp	36.3 $\pm$ 4.5	7.1 $\pm$ 1.5	5.1 $\pm$ 1.2
Negative antibody	10.8 $\pm$ 1.8	3.5 $\pm$ 1.8	4.8 $\pm$ 3.1

relatively low level of PTPase activity detected in the Syp immunocomplex. For the c-Src immunocomplex, maximum PTPase activity was found when 5 μ g of MAb 2-17 was used to precipitate c-Src from 500 μ g of lysate and the immunocomplex was incubated in a 45 min reaction (results not shown). The PTPase activity associated with c-Src was inhibited by zinc and vanadate [29] (Table 1), verifying the activity as a PTPase.

Mapping the region on c-Src to which PTPase binds

To determine if the detection of the c-Src-associated PTPase was restricted to immunoprecipitates obtained with MAb 2-17, and to gain insights into the region of c-Src involved in the binding of the PTPase activity, a panel of anti-c-Src antibodies whose epitopes map to different regions of the c-Src molecule was used. The two C-terminally directed anti-c-Src antibodies (Q8 and Q9) exhibited associated PTPase activity that was comparable with or slightly greater than the activity associated with the N-terminally directed MAb 2-17. In contrast, antibodies that bind the SH3 domain of c-Src (GD11 and EB8) precipitated significantly less activity. When the PTPase activity was normalized to the amount of c-Src in the immunocomplexes (Table 2), the 2-17, Q8 and Q9 antibodies were found to be associated with intermediate to high specific PTPase activities, whereas GD11 and EB8 precipitates exhibited the lowest specific activities.

The low level of PTPase activity co-precipitated by the SH3-directed MABs (GD11 and EB8) suggested that the SH3 or neighbouring SH2 domain of c-Src might mediate binding of c-Src to the PTPase(s). To investigate this possibility, GST fusion proteins of c-Src protein-interaction domains (SH2, SH3 and Unique) were used to affinity-precipitate proteins from chromaffin cell lysates, and the resulting complexes were assayed for PTPase activity. Table 3 shows that PTPase activity was associated with the various domains in the following order: SH2 > SH3/SH2 > SH3 > Unique = GST control beads.

Attempts to identify the c-Src-associated PTPase

We have been unable to identify the c-Src-associated PTPase activity by Western immunoblotting of c-Src immunocomplexes with a panel of anti-PTPase antibodies, even though several of the PTPases we have examined can be detected in extracts of chromaffin cells, e.g. Syp (SHPTP2) (Figure 1B), R-PTP α [30], R-PTP β [31], PTP1B [32], ERP (extracellular-regulated PTPase) and MKP-1 (mitogen-activated protein kinase phosphatase) [33] (results not shown). Our inability to cross-blot the c-Src-associated PTPase could be due to its low abundance in the complex or to the possibility that it is a novel member of the family of PTPases.

Figure 1 Parameters of the PTPase assay

(A) Time of incubation. Anti-c-Src (MAb 2-17), anti-Syp or negative control (Neg Ab) immunocomplexes, prepared from unstimulated chromaffin cells, were washed and incubated for the indicated times in a PTPase reaction mixture that contained 290 pmol of phosphotyrosine-containing RCML (44300 c.p.m. of ³²P). The percentage phosphotyrosine (pTyr) release as a function of incubation time is plotted. Similar results were obtained in three repetitions of this experiment. (B) The Western immunoblot depicts MAb 2-17 (anti-Src), anti-Syp or negative control (Neg) immunoprecipitates (IP Ab) blotted for either Src or Syp (Blot Ab; see arrows). The antibody heavy chain is indicated. Additionally, samples comprising one-tenth of the post-immunoprecipitation supernatants (P_{Src}, P_{Syp} and P_{Neg} respectively) were blotted to show the efficiency of the immunoprecipitations.

Table 2 Relative specific activity of c-Src-associated PTPase using a panel of c-Src-specific antibodies

Specific PTPase activities were calculated based on the amount of c-Src protein and the PTPase activity in each immunoprecipitate. The mean specific activity of the MAb 2-17 immunocomplexes was set as 1.0, and the specific activities of the other antibody complexes are listed relative to this. The mean phosphotyrosine release from the MAb 2-17 immunocomplex was 28%. Results are means $\pm$ S.E.M. of the numbers of experiments shown. Significant differences compared with MAb 2-17 value: * P = 0.05; ** P < 0.001 (Student's t test).

Antibody	Relative specific activity	<i>n</i>
2-17	1.00 $\pm$ 0.03	23
GD11	0.03 $\pm$ 0.19**	8
EB8	0.35 $\pm$ 0.09**	13
Q8	1.91 $\pm$ 0.53*	12
Q9	1.30 $\pm$ 0.36	12

Table 3 Preferential binding of PTPase activity to the c-Src SH2 domain

Affinity precipitates were prepared with 5 μ g of each fusion protein from 500 μ g of lysate prepared from cultured chromaffin cells, adrenal medullae or rat or bovine brain, and then subjected to a PTPase assay in the presence (n = 4) or absence (n = 17) of 5 mM vanadate. Affinity precipitates from the different tissue sources yielded qualitatively and quantitatively similar results, and were all included in this analysis. The PTPase activity associated with GST alone was set to 1.0, and the activities associated with the domains of c-Src were determined relative to this. n.d., not determined. The mean phosphotyrosine release from the GST affinity precipitates was 12%. Results are means $\pm$ S.E.M. Significant differences compared with control value: * P < 0.05; ** P < 0.001 (Student's t test).

c-Src domain	Relative PTPase activity — Vanadate	+ Vanadate
None (GST alone)	1.00 $\pm$ 0.03	0.49 $\pm$ 0.2**
Unique	0.91 $\pm$ 0.13	n.d.
SH3	1.30 $\pm$ 0.12*	0.36 $\pm$ 0.23**
SH2	2.55 $\pm$ 0.31**	0.58 $\pm$ 0.22**
SH3/2	1.78 $\pm$ 0.31*	0.46 $\pm$ 0.21**

Modulation of c-Src-associated PTPase activity

Since several PTPases appear to be regulated by receptor-linked signal-transduction pathways [34,35], we examined the levels of PTPase activity in c-Src immunocomplexes from chromaffin cells that had been stimulated for various times with the secretagogue nicotine, to determine if the c-Src-associated PTPase activity was altered by signalling through the nAChR. Figure 2(A) depicts the results of one experiment in which a decrease in the c-Src-associated activity was seen after only 30–45 s of stimulation, with a return to non-stimulated levels by 60 s. In repetitions of this experiment (n = 18), decreases were consistently observed, but the time at which the maximum decrease was seen varied between 15 and 45 s following stimulation. Table 4 lists the cumulative results, comparing the lowest PTPase activity in each experiment with the non-stimulated control value at the same time. It demonstrates that, on average, a 38% decrease (range 26–50%) in the level of c-Src-associated PTPase activity occurred at approx. 30 s after stimulation when compared with non-stimulated controls (P = 0.0003; Student's t test). No differences in PTPase activity between stimulated and unstimulated cells could be seen at times greater than 1 min, although catecholamine release continued to occur, as shown by the secretion assay in Figure 2(B).

Thus the c-Src-associated PTPase may function at a very early point in the signalling cascade induced by nicotine and the

Figure 2 Comparison of the time courses of nicotine-induced secretion and changes in PTPase activity

(A) Change in c-Src-associated PTPase activity upon stimulation to secrete. c-Src immunoprecipitates were prepared at 0, 15, 30, 45 and 60 s after stimulation of intact chromaffin cells (●). Non-stimulated controls were subjected to mock stimulation before immunoprecipitation (■). The PTPase activity associated with the negative antibody control is indicated by ▲. PTyr, phosphotyrosine. (B) Secretion at early time points. The percentage of [3 H]noradrenaline ([3 H]NA) released at the indicated times was measured as described in the Experimental section. The inset depicts the same data, expanding the first 1 min. Data are means $\pm$ S.E.M. of triplicate samples.

nAChR. That these early time points could be significant and relevant for secretion is suggested by the inset of Figure 2(B), which shows that a significant amount of the total secreted [3 H]noradrenaline had been released within 60 s of stimulation.

Subcellular localization of the c-Src-PTPase complex

To assess the intracellular localization of the c-Src-associated PTPase activity, cells were fractionated following hypotonic lysis into soluble and particulate fractions. c-Src immunoprecipitates were prepared from each fraction and assayed for PTPase activity (Table 5a). The majority of the activity was localized in the particulate fraction, consistent with the localization of c-Src. Further fractionation of the particulate matter on sucrose gradients revealed that the majority of the PTPase activity was found in the crude plasma membrane fraction, while neither the secretory vesicle contents nor their membranes contained a significant amount of activity above controls (Table 5b). Since c-Src partitions between the plasma membrane and the secretory vesicle membrane fractions in an approximate 3:2 ratio [6,8], the differential distribution of the PTPase relative to c-Src suggests the existence of a population of c-Src at the plasma membrane

Table 4 Decrease in c-Src-associated PTPase activity following secretory stimulation

Samples were prepared by immunoprecipitation with MAb 2-17 of c-Src from chromaffin cells that had been stimulated with 20 μ M nicotine for 15, 30, 45 or 60 s, as described in the Experimental section. Within each experiment, PTPase activity at each time point of stimulation was determined relative to that at time zero, which was set to 1.0. Non-stimulated controls were subjected to mock stimulation at each time point. Results are means $\pm$ S.E.M. ($n = 18$). The mean of the lowest PTPase activity in stimulated cells was compared with the mean of that in the appropriate non-stimulated controls by Student's *t* test and found to be significantly different ($P < 0.0003$). The mean time at which the maximum decrease was observed was 31 ± 3 s post-stimulation. The mean decrease in PTPase activity from cells stimulated for 30 s was also significant compared with that at time zero ($P < 0.0001$).

Time (s)	Relative PTPase activity	
	Stimulated	Non-stimulated
0	1.0	1.0
31 ± 3	$0.75 \pm .03$	1.13 ± 0.09
60	0.93 ± 0.05	1.00 ± 0.06

Table 5 Subcellular localization of c-Src-associated PTPase activity

(a) Bovine medullary tissue was subjected to hypotonic lysis and fractionation. An equivalent amount of protein (500 μ g) from each fraction was immunoprecipitated with MAb 2-17 (anti-c-Src) or a negative control antibody, and the immunocomplex-associated PTPase activity was assayed. Results are expressed as percentage phosphotyrosine release from RCML, and are means $\pm$ S.E.M. ($n = 3$). The activity in all samples was inhibited by 5 mM vanadate. (b) Sucrose fractionation preserves the integrity of the secretory vesicles during initial purification. Cell equivalent volumes (corresponding to 200 μ g of the secretory vesicle membrane fraction, and an equal volume of each of the other fractions) were used to prepare immunoprecipitates.

Fraction	PTPase activity (% release)	
	MAb 2-17	Negative control
(a) Hypotonic fractionation		
Crude plasma membranes	23.67 ± 1.9	10.6 ± 1.2
Cytosol	14.7 ± 2.3	8.3 ± 3.7
(b) Sucrose fractionation		
Crude plasma membranes	57.9 ± 1.8	31.1 ± 1.4
Vesicle membranes	20.0 ± 1.5	15.4 ± 3.7
Vesicle contents	3.9 ± 0.2	2.4 ± 0.4

that has associated PTPase activity, and a population of c-Src at the secretory vesicle membrane that has less or none. The localization of the c-Src-PTPase(s) complex in the crude plasma membrane fraction positions it for participation in early signalling events associated with secretory stimulation of the nAChR. Indeed, PTPase activity could also be found associated with nAChR immunocomplexes (Table 6). However, it is not known if the PTPase activity associated with the nAChR is the same as the PTPase activity associated with c-Src.

Complexity of native c-Src-PTPase complexes

To determine the size and number of native complexes in which c-Src could be found in association with a PTPase activity, gel-filtration chromatography was performed on medullary tissue homogenates. Column fractions were assessed for the amount of c-Src and associated PTPase activity, as well as for the migration of the nAChR (Figure 3). The greatest c-Src-associated PTPase activity was consistently found in a high-molecular-mass peak (fractions 17–20; > 669 kDa) (Figure 3A). This peak contained readily detectable amounts of c-Src, as determined by immunoblotting of c-Src immunoprecipitates (Figure 3B). Other peaks

Table 6 Association of PTPase activity with the nAChR

Immunoprecipitates were prepared from adrenal medullary extracts (500 μ g), and the associated PTPase activity was determined as the percentage phosphotyrosine release from RCML (mean $\pm$ S.E.M.; $n = 6$). All activities were inhibitable by 1 mM vanadate.

Antibody	PTPase activity (% release)
Anti-Src MAb 2-17	33.3 ± 5.4
Negative control	13.4 ± 2.7
Anti-Syp	42.2 ± 4.9
Anti-nAChR	49.2 ± 4.6

were also observed, including a low-molecular-mass peak (fractions 34–44) which reproducibly migrated with a molecular mass corresponding to 450–500 kDa and contained the majority of c-Src by Western immunoblotting (note that every other fraction was blotted over this part of the column). The majority of the nAChR was also found in this low-molecular-mass peak (Figure 3C), further suggesting that these molecules may be in a native complex. Thus c-Src and an active associated PTPase were reproducibly found in at least two large-sized complexes, one (> 669 kDa) with a high specific activity (relative to c-Src protein) and the other (~ 475 kDa) with a low specific activity.

DISCUSSION

Subcellular localization and regulation of complex-formation

Enrichment of the c-Src-PTPase complex in the crude plasma membrane fraction positions the complex to interact with the nAChR and other signalling molecules involved in very early events following receptor stimulation. This intracellular positioning is consistent with the predictions derived from our earlier vaccinia virus studies, namely that c-Src may function immediately downstream of the receptor and upstream of the voltage-gated calcium channels [10], and suggests that c-Src and its associated PTPase could influence signalling through one or both of these molecules or, in turn, be influenced by them. The changes in c-Src-associated PTPase activity following nicotine stimulation is an example of the latter.

The different molecular masses of the c-Src-PTPase complexes found in chromaffin cells raise the question of whether complex-formation may be regulated. Regulation is also suggested by other results: (1) antibodies that co-precipitate the highest levels of PTPase activity also precipitate the lowest amount of c-Src (Table 2 and results not shown); and (2) the c-Src-associated PTPase(s) is found predominantly in the subcellular compartment enriched in plasma membranes, whereas c-Src itself can be found in both plasma membrane and granule membrane fractions. What determines the composition of the complexes remains unclear, but may reflect a physiological mechanism for localizing the c-Src-specific PTPase to the plasma membrane. Whether the same PTPases are found in both complexes is also not known. The identification of the c-Src-specific PTPase(s) is a goal of our current studies.

Mapping of the PTPase binding site on c-Src

The finding that the isolated SH2 domain of c-Src bound chromaffin-cell PTPases in preference to the isolated Unique or SH3 domains suggests that the association between the full-

Figure 3 High- and low-molecular-mass complexes contain c-Src and c-Src-associated PTPase activity

(A) Clarified medullary tissue homogenate (97 mg) in PTPase buffer was chromatographed on a Sepharose 4B gel-filtration column, and aliquots (500 μ l) of each column fraction (1.6 ml) were immunoprecipitated with Mab 2-17 and assayed for c-Src-associated PTPase activity (bars). The total PTPase activity in each fraction was also determined (●), using a tyrosine-phosphorylated peptide substrate [END(pY)INASL] and a molybdate-dependent detection reaction for free phosphate. The absorbance at 280 nm (OD280; broken line) is plotted to show the major protein peaks. Similar chromatographic patterns of PTPase activity were seen in four repetitions of this experiment. PTyr, phosphotyrosine. (B) The indicated fractions were immunoprecipitated with Mab 2-17 and blotted for c-Src with antibody Q0. (C) A 200 μ l aliquot from each fraction was blotted directly for nAChR with a combination of AChR α - and AChR β -specific MAbs.

length proteins may occur via a phosphotyrosine-SH2 interaction. Alternatively, recent reports have indicated that there may be phosphotyrosine-independent binding of proteins to SH2 domains [36,37]. Verification of whether the SH2-binding PTPase is identical with the PTPase that associates with intact c-Src awaits identification of the PTPase(s) involved. Curiously, MAbs directed to the SH3 domain of c-Src (GD11 and EB8) precipitated little PTPase activity. This could indicate that binding of the antibodies to the SH3 domain precludes the association of a PTPase, or, alternatively, that these antibodies only immunoprecipitate pools of c-Src that are free of proteins (including the PTPase) that bind to the SH3 domain. Since the epitope for these antibodies lies at the margin between the SH3 and SH2 domains [17], and the studies of Panchamoorthy et al. [38] suggest that occupation of one of these two domains can affect the ability of the other domain to bind to other proteins, association of an antibody with the SH3 domain could affect binding of the PTPase to the SH2 domain of c-Src.

PTPase activity changes on stimulation

An important aspect of this work is the demonstration of a change in c-Src-associated PTPase activity upon secretory stimulation. The decrease in c-Src-associated PTPase activity after 30 s of stimulation may reflect either a change in the activity of the associated PTPase due to some modification, or a change in the ability of the PTPase to associate with the c-Src molecule. This on-off mechanism might free the PTPase to act on other substrates to propagate the signal emanating from the nAChR. Additionally, these modulations of PTPase activity may represent

a larger physiological difference that may be difficult to capture and maintain throughout the extraction procedure.

Mutual regulation of c-Src and its associated PTPase

A number of different PTPases have been reported to associate with c-Src, either physically or functionally. These include Syp [13], PTPD1 [12], PTP1C [14] and R-PTP α [30,39,40]. All of these studies indicate that, in each system, c-Src and its associated PTPase appear to affect each other's activities *in vivo*, in some cases by phosphorylation/dephosphorylation mechanisms.

Results of preliminary studies that addressed the question of mutual regulation by c-Src and its associated PTPase in chromaffin cells suggest that mutual regulation may not be operative in this system. On examining c-Src immunocomplexes from stimulated chromaffin cells, we have been unable to detect any significant changes in c-Src kinase activity or c-Src phosphotyrosine content within the first 60 s of stimulation that could be attributed to the brief inactivation of the PTPase (results not shown). Thus our present investigations suggest that c-Src is not a substrate for its associated PTPase, and imply that the target of this PTPase is another molecule, perhaps the nAChR (see below). In contrast, our studies do not eliminate the possibility that c-Src modulates the activity of the PTPase.

A potential target of c-Src and its associated PTPase

Huganir and colleagues [41–43] have shown that the neuromuscular AChR in *Torpedo* is acted upon by both a tyrosine phosphatase and c-Src-related tyrosine kinases. Tyrosine

phosphorylation of the neuromuscular AChR [44] increases the rate of desensitization of the receptor [45], thereby decreasing the duration of the signal that the receptor can transmit. We have shown in the present study that PTPase activity associates with the neuronal nAChR in chromaffin cells (different from the neuromuscular form [46]), and that the nAChR co-chromatographs with the low-molecular-mass c-Src-PTPase complex on gel filtration, leading to a potentially interesting model of a tripartite interaction between tyrosine kinases, PTPases and the nAChR. As direct reagents become available, we will be able to test this model in the neuronal system and gain new insights into the regulation of exocytosis and signal transduction in chromaffin cells.

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