SRC binding to the cytoskeleton, triggered by growth cone attachment to laminin, is protein tyrosine phosphatase-dependent

Steve Helmke1,2,*, Kathryn Lohse1,2,‡, Keith Mikule1,2, Malcolm R. Wood1,2,§ and Karl H. Pfenninger1,2,¶

1Department of Cellular and Structural Biology, University of Colorado School of Medicine, and 2University of Colorado Cancer Center, Denver, Colorado, 80262, USA
*Present address: Division of Cardiology, University of Colorado Health Sciences Center, 4200 E. Ninth Avenue, Box B-139, Denver, CO 80262, USA
‡Present address: Alpha Spectra Inc., 715 Arrowest Ct., Grand Junction, CO 81505, USA
§Present address: The Scripps Research Institute, SBR 9, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA
¶Author for correspondence

Accepted 9 June; published on WWW 30 July 1998

SUMMARY

The interaction of the non-receptor tyrosine kinase, Src, with the cytoskeleton of adhesion sites was studied in nerve growth cones isolated from fetal rat brain. Of particular interest was the role of protein tyrosine phosphatases in the regulation of Src-cytoskeleton binding.

Growth cones were found to contain a high level of protein tyrosine phosphatase activity, most of it membrane-associated and forming large, multimeric and wheat germ agglutinin-binding complexes. The receptor tyrosine phosphatase PTPα seems to be the most prevalent species among the membrane-associated enzymes. As seen by immunofluorescence, PTPα is present throughout the plasmalemma of the growth cone including filopodia, and it forms a punctate pattern consistent with that of integrin β1. For adhesion site analysis, isolated growth cones were either plated onto the neurite growth substratum, laminin, or kept in suspension. Plating growth cones on laminin triggered an 8-fold increase in Src binding to the adherent cytoskeleton. This effect was blocked completely with the protein tyrosine phosphatase inhibitor, vanadate. Growth cone plating also increased the association with adhesion sites of tyrosine phosphatase activity (14-fold) and of PTPα immunoreactivity (6-fold). Vanadate blocked the enzyme activity but not the recruitment of PTPα to the adhesion sites.

In conjunction with our previous results on growth cones, these data suggest that integrin binding to laminin triggers the recruitment of PTPα (and perhaps other protein tyrosine phosphatases) to adhesion sites, resulting in de-phosphorylation of Src’s tyr 527. As a result Src unfolds, becomes kinase-active, and its SH2 domain can bind to an adhesion site protein. This implies a critical role for protein tyrosine phosphatase activity in the earliest phases of adhesion site assembly.

Key words: Cellular adhesion, Protein tyrosine phosphatase, Src, Integrin, Cytoskeleton, Growth cone

INTRODUCTION

The nerve growth cone, the leading edge of the growing neurite, is a structure specialized for amoeboid locomotion and pathfinding (see e.g. Lankford et al., 1990). Therefore, the nerve growth cone must be able to respond to the presence or absence of specific cell adhesion molecules in its microenvironment, and it must be capable of making and breaking contacts with such molecules in a rapidly cycling manner. There is an increasing body of evidence that cell adhesion molecules, such as integrins, perform a ‘sensory’ function and signal to the cell interior the presence of the appropriate ligands in the environment. In addition, the cell is capable of regulating its attachment to a particular substratum, presumably by modulating the conformation of certain cell adhesion molecules (for review see, e.g. Clark and Brugge, 1995; Machesky and Hall, 1996; Parsons, 1996). Most of these phenomena appear to take place at adhesion sites, where cell adhesion molecules are clustered and linked to the substratum on the one hand and to the cytoskeleton, primarily the actin cytoskeleton, on the other hand (see, e.g. Burridge et al., 1988; Gumbiner, 1993; Craig and Johnson, 1996). These interactions enable the cell to exert force against the substratum via the adhesion sites and, thus, to crawl (see, e.g. Stossel, 1993; Huttenlocher et al., 1995; Zigmond, 1996).

Adhesion sites are known to contain a variety of proteins, many of which have been identified (e.g. Gumbiner, 1993). A family of molecules of particular interest, concentrated in these adhesion sites, are non-receptor protein tyrosine kinases, such as Src, Fyn and focal adhesion kinase (FAK; Clark and Brugge, 1995; Parsons, 1996). In numerous studies Src and FAK have been implicated in the signalling to the cell interior of ligand interactions of adhesion molecules.

Studies from this and other laboratories have shown in the past that Src and other Src family tyrosine kinases are highly enriched in the nerve growth cone (Maness et al., 1988;
most recent, Helmke and Pfenninger (1995) demonstrated that Src family members in growth cones are reversibly bound to the cytoskeleton and that, at least in the case of Src, this interaction is regulated by phosphorylation and de-phosphorylation of a tyrosine residue near the C terminus (in Src, tyr 527) (see also Kaplan et al., 1994). Our data indicated that only Src de-phosphorylated on tyr 527 (which is not folded upon itself and has the SH2 region free to interact with other phosphotyrosines; Fukui et al., 1991; Amrein et al., 1993) can combine with the cytoskeleton. In contrast, Src phosphorylated on tyr 527 is not attached to the cytoskeleton. This is consistent with the interesting observation that cytoskeleton-bound Src is much more active catalytically (Helmke and Pfenninger, 1995). These results suggested that control of the phosphorylation state of Src’s tyr 527 by c-Src kinase (Csk; apparently constitutively active) and one or multiple protein tyrosine phosphatases (PTPases) would regulate the cytoskeletal binding of Src (see, e.g. Mustelin and Burn, 1993). As a consequence, these enzymes, especially (a) regulated PTPase(s), would control the linkage of the plasma membrane, in which Src is anchored, to the cytoskeleton.

These observations and conclusions prompted the formulation of the following hypotheses: (1) attachment of the growth cone to an appropriate substratum may trigger Src-cytoskeleton linkage; and (2) this interaction may be regulated by one or more PTPases whose activity at adhesion sites would be controlled by substrate attachment. Parts of these hypotheses are consistent with previous observations, such as the induction by platelet aggregon of Src binding to the cytoskeleton (Horvath et al., 1992) and of the association of PTPase with the cytoskeleton (Ezumi et al., 1995).

The present report describes the results from testing the proposed hypotheses. Src-cytoskeleton interactions were studied in a novel assay involving the plating of isolated growth cones, so-called growth cone particles (GCPs), on a physiological neurite growth substratum, laminin; PTPases in these GCPs were partially characterized; PTPase recruitment and/or activation in, adhesion sites were assessed; and the role of PTPases in the Src-cytoskeleton interaction was investigated.

MATERIALS AND METHODS

Materials

Aprotinin was obtained from CalbioChem; Soybean trypsin inhibitor, pepstatin A, leupeptin, benzamidine, phenanthroline, phenylmethylsulfonyl fluoride (PMSF) and aminoethylbenzoylsulfonyl fluoride (AEBSF) were from Sigma. Wheat germ agglutinin (WGA)-Sepharose and Sephacryl S-300 were from Pharmacia. N-acetylglucosamine, Nonidet-P40 (NP-40), Triton X-100 (TX100), CHAPS, saponin, β-escin, phosphoserine (P-ser), phosphothreonine (P-thr) and phosphotyrosine (P-tyr) were from Sigma. Nitrocellulose (Biotrace NT) was from Gelman Sciences. Sodium orthovanadate, Na3VO4 (vanadate) was from Fisher.

Brain subcellular fractions

Subcellular fractions were prepared from brains of fetal Sprague-Dawley rats (18 days gestation). Timed-pregnant rats were purchased from Harlan. All procedures were performed on ice or at 4°C, and aprotinin (10 µg/ml) was added to solutions as a protease inhibitor. In some cases, the following protease inhibitors were also included: PMSF (1 mM), pepstatin A (1 µM), leupeptin (1 µM), benzamidine (10 mM), and phenanthroline (5 mM).

Growth cone particles (GCPs)

These were prepared essentially as published by Pfenninger et al. (1983), but modified as described by Lohse et al. (1996). Fetal brains were homogenized in approximately 8 volumes of 0.32 M sucrose containing 1 mM MgCl2 and 2 mM TES buffer, pH 7.3. The homogenate was spun at 1,660 g for 15 minutes, and the resulting low-speed supernatant was loaded onto a discontinuous density gradient with steps of 0.83 M and 2.66 M sucrose in the same buffer. The gradients were spun to equilibrium at 242,000 g for 40 minutes in a vertical rotor (VTI50, Beckman). The A fraction at the 0.32/0.83 M sucrose interface containing the GCPs was collected. Unless indicated otherwise, this fraction was diluted 3- to 4-fold with 0.32 M sucrose, and GCPs were pelleted at 39,800 g for 30 minutes.

GCP membranes

Membranes were prepared from GCPs as previously described (Ellis et al., 1985), with slight modifications. The GCP pellet was resuspended in 6 mM Tris-HCl, pH 8.1, with 0.5 mM EDTA. Material was allowed to lyse with stirring for 30 minutes and then was spun at 200,000 g for 40 minutes. This resulted in a pellet of ‘crude GCP membranes’ or ‘lysed GCPs’ and a supernatant, the cytosolic GCP fraction. To prepare ‘salt-washed GCP membranes’ (GCM; Ellis et al., 1985), the crude GCP membrane fraction was treated with 0.3 M Na2SO4 plus 20 µg/ml saponin for 30 minutes with stirring. This suspension was spun at 200,000 g for 40 minutes to collect salt-washed GCP membranes. The salt-wash supernatant also was collected and used in some experiments.

Protein chemistry

Wheat germ agglutinin (WGA)-binding subfraction of GCPs

Either crude GCP membranes or salt-washed GCP membranes were solubilized for stirring for at least 30 minutes in Tris-buffered saline (TBS) with 0.5% Nonidet-P40 (NP-40). Insoluble material was spun out at 200,000 g for 40 minutes. The detergent-solubilized fraction was loaded onto WGA-Sepharose either in batch or in a column. In batch, the WGA-Sepharose mixture was incubated with agitation for at least 2 hours or overnight; on a column, the detergent-soluble fraction was passed through at a low flow rate (<5 ml/hour). Following incubation with WGA-Sepharose, the supernatant or column flow-through was collected and the adsorbent washed with 1-2 column volumes of TBS/NP-40. Low-affinity glycoconjugates were eluted with at least 1-2 column volumes of TBS/NP-40 plus 20 mM N-acetylglucosamine (GlcNAc).

High-affinity WGA-binding glycoproteins were eluted subsequently with 300 mM GlcNAc in TBS/NP-40 using 2-3 column volumes. Eluate was recovered in bulk or with a fraction collector. For some experiments, the fractions containing the highest amounts of protein, as determined by Bradford assay (1976), were pooled (‘WGA-binding subfraction’).

Gel filtration

Crude GCP membranes were solubilized with 20 mM CHAPS in TBS with stirring for 30 minutes at 4°C. Insoluble material was spun out at 200,000 g. The supernatant was loaded onto Sephacryl S-300 gel filtration columns (load was less than 5% of column volume). Proteins were eluted with TBS plus 5 mM CHAPS at a flow rate of 10-15 ml/hour. Approximately two-thirds of the void volume was recovered in bulk before fractions of 1.5 to 3.0 ml were collected. Columns were calibrated in the same running buffer using colored molecular mass standards and Dextran Blue as a marker for the void volume.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Protein amounts were determined by the modified Lowry method...
et al., 1993), PTPs

ice for 15-30 minutes to allow protein to precipitate. After
by the addition of 3 volumes of 25% TCA (w/v). One volume of 10
incubated for 20 minutes at 37°C. Control samples devoid of either
amounts of GCP protein were used in the assays, and samples were
containing 0.1 M sodium acetate (pH 6.0), 0.2% TX100, and 1 mM
PTPase assay with phosphoamino acids
manufacturer's instructions (Amersham or NEN). Moist blots were

temperature. Following extensive washing with blocking solution

ammonium molybdate/ascorbic acid method (Chen et al., 1956). A
phosphate released into the supernatants was determined using the

MBP is known to be a suitable exogenous substrate for many PTPases,
greater specificity as well as sensitivity of the assays. Phosphorylated
substrate was on tyrosine (data not shown). A trace of phosphorylation

2 mM and ammonium molybdate to 100 mM. Finally, [γ-32P]ATP (200
cpm/pmol) was included at 4 mM. The reaction was started by the

MBP , obtained from Sigma, was resuspended in 25 mM imidazole,

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ph 7.2, and dialyzed against at least two changes of the same buffer.
MBP (about 2 mg/ml) was phosphorylated in the following solution:
40 mM imidazole-HCl, pH 7.2, 50 mM NaCl, 12 mM magnesium
acetate, 4 mM MnCl2, 0.1 mM Na3VO4, 0.2 mM EGTA, 0.05% (v/v)
TX100, and 0.02% (v/v) glycerol (Tonks et al., 1991a). Insulin and
epidermal growth factor were added at 300 and 200 nM, respectively.
The partially purified receptor kinases were added at 1/5 to 1/10 the
final volume of phosphorylation. Dithiothreitol (DTT) was added to
2 mM and ammonium molybdate to 100 mM. Finally, [γ-32P]ATP (200

The PTPase assay using 32P-tyr-MBP substrate was carried out as described by Tonks et al. (1991a). Samples containing PTPase with 2
mM DTT and 2 mM EDTA added, were preincubated at 30°C for 5
minutes. Reaction was initiated by the addition of 5 to 10 µl of 32P-

MBP (about 2 mg/ml) was phosphorylated in the following solution:

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were replaced by buffer alone were included to determine background
counts.

Plating assay
Nitrocellulose-coated 35-mm Petri dishes (Lagenaur and Lemmon, 1987) were incubated with laminin for 1 hour with shaking and then
blocked twice for 30 minutes with TBS-5% nonfat dry milk. 2 ml of
A fraction containing GCPs, without prior pelleting, were gently
mixed with 2 ml of a 2× intracellular buffer (40 mM Hepes pH 7.3,
100 mM KCl, 10 mM NaCl, 6 mM MgCl2) and permeabilized with
0.02% β-escin (Kobayashi et al., 1989). These GCP suspensions were
incubated in the presence or absence of 1 mM ATP for 5 minutes at
37°C. Some GCP mixtures were then treated with 1% TX100 (plus
10 µg/ml aprotinin, 0.1 mM leupeptin, 1 mM AEBSF) on ice for 10
minutes and the detergent-insoluble cytoskeletal fraction isolated by
centrifugation for 1 hour at 4°C, at 100,000 g. To measure phosphatase
activity in the cytoskeleton of these GCPs in suspension, the
cytoskeleton fractions isolated by centrifugation were resuspended in
0.1 M Na acetate, pH 6, plus 1 mM EDTA and incubated with 10 mM
P-tyr or P-ser at 37°C for 20 minutes. Following chloroform/methanol
extraction, the aqueous layer was assayed for inorganic phosphate
(Chen et al., 1956) and the protein fraction for total protein (Peterson,
1983).

Other GCP suspensions (permeabilized and in intracellular buffer),
with or without 3 mM vanadate, were applied to the laminin-coated
dishes and centrifuged at 8,000 g (6,500 rpm) in a Beckman JS 7.5
rotor for 15 minutes at 25°C to bring the GCPs into contact with the
substratum. The dishes were then incubated for 10 minutes at 37°C
to allow adhesion complexes to form. Because vanadate gradually
becomes inactive at physiological pH (Gordon, 1991), it was added
immediately prior to the centrifugation so that it would be maximally
active during adhesion complex formation. The supernatant was
removed and the dishes were washed for 5 minutes at 25°C with a

(57×533)
The experimental system for these studies consisted of GCPs, sheared-off and re-sealed fragments (about 0.3-0.5 μm diameter) of nerve growth cones isolated from fetal rat brain. GCPs were characterized in the past, both morphologically and biochemically (Pfennninger et al., 1983; Ellis et al., 1985; Katz et al., 1985, Simkowitz et al., 1989). Although GCPs are derived from many different neuron types they are now known to be primarily of axonal origin (Lohse et al., 1996). When GCPs (permeabilized or non-permeabilized) were collected from the gradient and immediately analyzed for Src bound to the TX100-resistant cytoskeleton, a large percentage of Src (about 70%; Helmke and Pfenninger, 1995) was found in the cytoskeletal fraction (Fig. 1). It is known that GCPs are depleted of ATP under these conditions (Lockerie et al., 1991). However, if permeabilized GCPs were transferred to intracellular buffer containing ATP a much smaller fraction of Src was associated with the cytoskeleton. Such suspended (and not previously pelleted), permeabilized GCPs in ATP-containing intracellular buffer served as the controls for the plating experiments described here.

**Plating of GCPs on laminin triggers Src-cytoskeleton binding**

These experiments were designed to address the question of whether GCP attachment to laminin-coated Petri dishes triggered the association of Src with the cytoskeleton. As described in detail in Materials and Methods, the permeabilized GCPs were either kept in suspension or plated on laminin-coated dishes, always in the presence of exogenous ATP. Based on turbidity measurements of the GCP-containing fraction prior to plating versus the supernatant removed from the Petri dishes after plating, approximately 80-90% of GCPs bound to laminin in our experimental conditions. Binding was reduced to ~20% of GCPs applied if the nitrocellulose-coated Petri dishes were simply quenched with milk rather than coated with laminin (data not shown).

Cytoskeletal fractions were prepared by TX100 extraction, solubilized with SDS and analyzed by western blot using the Src monoclonal antibody 327. In a typical assay, approximately 40 μg TX100-insoluble protein was recovered from a single 35-mm dish. As shown in Fig. 1, plating on laminin increased cytoskeletal bound Src 8-fold in the presence of ATP, indicating an effect of integrin-laminin interaction on Src-cytoskeleton binding. Our previous studies predicted that this effect should be blocked with the PTPase inhibitor, vanadate.

**RESULTS**

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**Fig. 1.** Src recruitment to growth cone adhesion complexes formed upon plating on laminin. GCPs in suspension were permeabilized with 0.02% β-escin and incubated for 5 minutes in the absence (1) or presence (2) of 1 mM ATP. Thereafter the TX100-insoluble CS fractions were isolated. Permeabilized GCPs incubated with ATP also were plated onto laminin-coated dishes in the absence (3) and presence (4) of 3 mM vanadate. After plating the TX100-resistant, adherent cytoskeleton fractions were isolated. The level of Src was determined by western blot using 20 μg protein of each cytoskeleton fraction. A representative western blot is shown; the densitometric quantitation of Src immunoreactivity, normalized to permeabilized GCPs incubated with ATP, is presented below. SEM, standard error of the mean.
We incubated as described in Materials and Methods with 32P-tyr-inhibition of GCP PTPase activity by P-tyr or vanadate. GCPs Fig. 3. Note the preponderance of tyrosine phosphatase activity. The inorganic phosphate released from the substrates was quantitated. Note the preponderance of tyrosine phosphatase activity.

Using phosphoamino acids as substrates, we first measured total phosphatase activities in GCPs. Fig. 2 shows that under the conditions used the release of inorganic phosphate from phosphoamino acids is much higher for P-tyr than for either P-ser or P-thr. Subsequent measurements of PTPase activity were performed with an exogenous protein substrate, 32P-tyr-MBP. That these measurements were specific was demonstrated in competition experiments with the two phosphoamino acids, P-ser and P-tyr. 10 mM P-tyr reduced 32P release from the substrate by nearly one-half, whereas the same amount of P-ser left the activity unaffected. At 20 or 30 mM P-tyr, the enzyme activity was blocked completely (Fig. 3). Fig. 3 also shows that millimolar concentrations of vanadate reduced growth cone PTPase activity substantially, and at 3 mM, inhibited it by 90%.

In the next set of analyses we addressed the question of whether PTPase activity was enriched in GCPs relative to fetal brain homogenate, and whether it was membrane-associated or cytosolic. Equal amounts of protein were collected from the different fractions generated during the GCP preparation procedure: fetal brain homogenate, low-speed supernatant (the parent fraction of GCPs), and GCPs. Furthermore, we prepared lysed GCPs devoid of the bulk of their soluble contents, and GCP membranes, prepared by salt-wash of lysed GCPs. Fig. 4A shows the PTPase activity, measured with 32P-tyr-MBP as a substrate in the various fractions. We observed in GCPs an only modest enrichment (approx. 1.7-fold) of PTPase activity per unit protein. However relative to GCPs, membrane subfractions (GCMs) contained a much higher, and the soluble fractions a much lower, specific activity. This indicated that the bulk of PTPase activity was associated with membranes.

This observation raised the question of whether PTPase activity was associated with membrane glycoproteins. In order to examine this issue, we prepared a TX100 extract from lysed GCPs, spun out the cytoskeletal fraction and loaded the soluble membrane extract onto a WGA affinity column. After the column was washed, glycoproteins were eluted in two steps: low-affinity species at 20 mM GlcNAc and high-affinity species at 300 mM GlcNAc. PTPase activity was measured in the eluted fractions as shown in Fig. 4B. Most of the PTPase activity was retained on the WGA column. Some of the activity was eluted at 20 mM GlcNAc, but elution of the bulk of the activity necessitated the use of 300 mM hapten sugar. This result indicated that most of the growth cone's PTPase activity was associated with a membrane glycoprotein fraction.

A number of antibodies were used to screen western bots of GCPs for known PTPases (see e.g. Streuli, 1996). An antibody against the cytosolic T-cell PTPase (kindly provided by Dr N. Tonks; Cool et al., 1990) recognized multiple bands in western blots of soluble GCP proteins, with a particularly strong band at 65 kDa (data not shown). This could possibly represent the PTPase, Syp (Klinghoffer and Kazlauskas, 1995). Of the several receptor PTPase antibodies (to PTP-α, -β, -κ, -σ; all generously provided by Dr J. Sap) we used, only anti-PTP-α revealed prominent specific immunoreactivity (Fig. 4C; data for the other antibodies not shown). In Fig. 4C a very strongly reactive PTP-α band was observed at the expected 130 kDa in the TX100 membrane extract prepared from lysed GCPs. When loaded on the WGA affinity column, no PTP-α immunoreactivity was observed in the flow-through, but it was eluted at 20 mM and, especially, at 300 mM GlcNAc.

In order to characterize further the membrane-associated PTPase activity of GCPs, we fractionated a CHAPS extract of lysed GCPs by size exclusion chromatography and analyzed...
each fraction for PTPase activity and PTPα immunoreactivity. The results are shown in Fig. 5. The resolving range of the Sephacryl S-300 column used for these experiments is about 10^4 to 10^6 Da. Almost all of the PTPase activity in the fraction was eluted between approx. 250 and 450 kDa, with two main peaks around 400 kDa. PTPα (with an apparent molecular mass of 130 kDa) co-migrated with these major peaks as shown in Fig. 5B. In fact, immunoreactivity was particularly prominent at about 82.5 and 84 ml and then again at about 88.5 ml of eluted volume, precisely coinciding with the highest activity peaks. These results are compatible with the view that most (if not all) membrane-associated PTPase activity of GCPs is present in high-molecular mass complexes, and that PTPα accounts for a significant portion of membrane-associated PTPase activity of growth cones.

Indirect immunofluorescence was performed on primary
neuron cultures to localize PTPα in intact growth cones. Fluorescence was observed throughout the entire neuron. Examination of distal neurites and growth cones revealed punctate plasmalemmal PTPα staining extending into the finest filopodia (Fig. 6). The punctate pattern consisted of spots of various sizes and often was enhanced at the proximal base of the growth cone and in its most distal periphery.

Plating-induced recruitment of PTPase to cell adhesion sites

Our previous observations (Helmke and Pfenninger, 1995) and the data presented here suggested that growth cone plating increased PTPase activity associated with the cytoskeleton, either by increasing the specific activity of the enzyme(s) or by recruiting enzyme to adhesion sites. In order to test this hypothesis, we plated GCPs on laminin and assayed for PTPase in the TX100-resistant cytoskeletal fraction. Table 1 shows the results of cytoskeletally-associated PTPase activity in suspended GCPs (in the absence or presence of ATP) vs. plated GCPs in the absence or presence of vanadate. In these experiments we used the phosphoamino acid P-tyr (and, for comparison, P-ser) as a substrate in order to avoid the possibility that steric hindrance or other factors might distort the activity measurable with the exogenous protein substrate, 32P-tyr-MBP. As shown in Table 1, low levels of PTPase were associated with the cytoskeleton in suspended GCPs, in the presence or absence of ATP. When GCPs were plated on laminin, however, we observed a nearly 14-fold increase of cytoskeletally-associated PTPase activity. This increase was not observed if P-ser was used as a substrate. These experiments also showed that vanadate blocked nearly 90% of the cytoskeletally-associated PTPase activity in plated GCPs. It is important to note that, in these experiments, vanadate was added after the formation and extraction of the TX100-resistant cytoskeletal fraction so that we were measuring the inhibition of PTPase activity in the adhesion complexes already formed under control conditions. This is in contrast to the experiments shown in Figs 1 and 6 where vanadate was added prior to plating to test its effect on the recruitment of proteins to newly formed adhesion complexes.

In view of our result that PTPα was a significant contributor of PTPase activity in growth cones, we addressed the question of whether PTPα was recruited to the cytoskeleton, i.e. to adhesion sites, during plating. This was done by western blot with the PTPα antibody. Fig. 7 shows the results. In fact, the figure shows the exact same blot as Fig. 1, first reacted for Src and subsequently re-probed with the PTPα antibody. PTPα can be seen as a sharp band at 130 kDa as before. The weaker and somewhat lower band observed may be a breakdown product in these experiments. For quantitation, only the 130 kDa band was used. As observed for Src, a significant amount of PTPα was cytoskeletally associated in suspended GCPs, in the absence of ATP. When ATP was added, however, there was a substantial decrease in cytoskeletal association of PTPα. When these ATP-containing GCPs were plated on laminin, there was

Table 1. Specific activity of phosphatase associated with the cytoskeleton in suspended vs plated GCPs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>GCPs in suspension</th>
<th>GCPs plated on laminin</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>−ATP</td>
<td>+ATP</td>
</tr>
<tr>
<td>P-tyr</td>
<td>42±11</td>
<td>36±6</td>
</tr>
<tr>
<td>P-ser</td>
<td>36±23</td>
<td>37±5</td>
</tr>
</tbody>
</table>

*Data are means of three experiments ± s.d., in nmoles/minute per mg protein. n.d., not done.
PTPα activity was enriched in membrane fractions prepared from GCPs. In contrast, soluble supernatants recovered from these preparations contained relatively low levels of PTPase activity. Thus, PTPase activity was most concentrated in membranes, and this membrane association was resistant to high ionic strength. On size-exclusion chromatography, the bulk of CHAPS-solubilized, membrane-associated PTPase activity eluted at about 400 kDa, well above the apparent molecular masses of known PTPases. This suggests that most growth cone PTPase activity is present in large, multimeric complexes that are likely to include other membrane proteins. The majority of growth cone PTPase activity (and all of PTPα) was retained on WGA, by direct binding of glycosylated receptor PTPases and/or indirectly, via association (resistant to mild detergent) with membrane glycoproteins, as suggested by gel filtration. Such interaction between PTPases and transmembrane glycoproteins would be consistent with a possible role of PTPases in GCP transmembrane signalling.

Screening GCPs for known PTPases revealed PTPα as the only prominently represented species. PTPα is a receptor PTPase of approximately 130 kDa, with a very small but glycosylated external domain (Sap et al., 1990; Kaplan et al., 1990; Matthews et al., 1990; Daum et al., 1994; Streuli, 1996). It was confined, as expected, to membranes prepared from GCPs (data not shown) and retained on the WGA affinity column. Whether binding to WGA was due to PTPα’s own glycosylation or caused by its association with other glycoproteins is not known. However, PTPα eluted at about 400 kDa from Sephacryl S-300, indicating its presence in large multimeric complexes. Particularly striking was the close correspondence between the two maximum peaks of eluted PTPase activity on the one hand and the bimodal elution of anti-PTPα immunoreactivity on the other hand. This observation suggests that PTPα may account for the bulk of these two peaks of PTPase activity. As a corollary, PTPα may well be the most prominent membrane-associated PTPase in growth cones.

**DISCUSSION**

**Partial characterization of growth cone PTPases**

PTPase activity in GCPs was very compared to that of phosphatases hydrolyzing P-ser or P-thr under the experimental conditions used in our assays. That release of 32P from the MBP substrate was not due to the presence of phosphatases other than PTPase was shown by inhibition of the activity with P-tyr but not P-ser. About 85% of PTPase activity cleaving the 32P-tyr-MBP was inhibitable with vanadate (at 3 mM) as expected (Gordon, 1991). When different subfractions of fetal brain were compared, the rate of 32P-tyr-MBP hydrolysis in GCPs was nearly twice that observed in the homogenate, indicating moderate enrichment of the enzyme activity. This is not surprising considering the known enrichment of protein tyrosine kinases of the non-receptor type (Src and other Src-family members) as well as receptor tyrosine kinases in growth cones (e.g. receptors for IGF-1, trks and others; Maness et al., 1988; Cheng and Sahyoun, 1990; Helmke and Pfennigner, 1995; Quiroga et al., 1995). However, it is far from clear whether PTPases detected in GCPs serve a role primarily as negative regulators, i.e. to turn off the responses to protein tyrosine kinase activation, or whether some PTPases may be involved in positive signalling.

**PTPase responses to substrate attachment of GCPs**

Our novel, cell-free GCP plating assay was designed to investigate early molecular changes in the growth cone upon interaction with an appropriate substratum, laminin. Freshly isolated GCPs have very low ATP levels but are capable of generating ATP, maintaining physiological intracellular calcium levels, phosphorylating proteins, and vesicle fusion with the plasmalemma (Lockerie et al., 1991; K. Mikule and K. H. Pfenninger, unpublished observations). However, they are not known to synthesize polypeptides. Assay conditions were established in which GCPs could be plated onto laminin either intact or after permeabilization with β-escin to allow for the introduction into cytosol of various agents, such as ATP and vanadate. The results for Src and PTPase distribution were the same for sealed GCPs suspended in Krebs buffer (data not shown) as for permeabilized GCPs in intracellular buffer containing ATP. Upon mild solubilization of plated GCPs with TX100, most GCP proteins were removed, but cytoskeletal elements remained attached to laminin (see also Gordon-Weeks, 1987; Cheng and Sahyoun, 1988; Sobue and Kanda, 1989; Meiri and Gordon-Weeks, 1990) and could be recovered with SDS for further analysis. These TX100-resistant proteins
conducted the adhesion complexes, presumably representing early and/or transient phases of adhesion site formation.

In intact growth cones in culture PTPα immunoreactivity was observed throughout the plasma membrane in a punctate pattern strikingly similar to that observed for talin and paxillin (in primary neurons; K. Mikule, unpublished observations) and for integrins (especially β1) and talin (in growth cones of PC12 cells and primary neurons; Arregui et al., 1994; Grabham and Goldberg, 1997). Because growth cones do not form the focal adhesions typical of cultured fibroblasts this punctate pattern is consistent with PTPα clustering at adhesion sites or ‘point contacts’ (Arregui et al., 1994). In isolated GCPs, non-attached and suspended in buffer, in the presence or absence of exogenous ATP, only very low levels of PTPase activity were associated with the TX100-resistant cytoskeleton. However, plating onto laminin enhanced cytoskeleton-associated PTPase activity (but not serine phosphatase activity) nearly 14-fold. Simultaneously the bulk of PTPα immunoreactivity became associated with the TX100-insoluble adhesion complexes. Vanadate inhibited most of the cytoskeletonally-associated PTPase activity in plated GCPs but did not affect the redistribution of PTPα immunoreactivity. Therefore, the recruitment of PTPα (and, perhaps, other PTPases) to adhesion complexes was not dependent on PTPase activity or ‘upstream’ of the increase of PTPase activity in adhesion complexes.

The effect of GCP plating on Src distribution

In freshly isolated, suspended GCPs (essentially free of ATP; Lockerbie et al., 1991), a significant portion of Src immunoreactivity was associated with the cytoskeletal subfraction (Helmke and Pfenninger, 1995). However, in the presence of ATP, over 90% of the Src previously associated with the cytoskeleton became TX100-soluble. Presumably, ATP-depleted GCPs are in a quasi ‘rigor’ state, in which regulatory functions, such as protein phosphorylation, are blocked. The addition of ATP restores physiological conditions, and adhesion complexes that have no ligands externally can be dismantled. This view is consistent with the findings of Crowley and Horwitz (1995) who reported that ATP addition to permeabilized fibroblasts promotes focal adhesion breakdown by stimulating tyrosine phosphorylation as well as the generation of tension in the actin-myosin system.

Plating GCPs on laminin in the presence of ATP resulted in an almost 8-fold increase in cytoskeletal association of Src. However, this effect could be blocked by adding the PTPase inhibitor, vanadate, to the GCPs prior to plating. This result indicated that Src-cytoskeleton interaction, triggered by membrane binding to laminin (presumably via integrins), was dependent upon PTPase activity. In other words, the binding of Src to the cytoskeleton must be downstream of an increase in PTPase activity in adhesion complexes.

These observations are consistent with and complementary to our earlier findings (Helmke and Pfenninger, 1995). Our previous study demonstrated that the binding of Src to the cytoskeleton, known to occur via its SH2 domain (Fukui et al., 1991), is inhibited by phosphorylation of tyr 527. In other words, when tyr 527 is phosphorylated, Src is folded upon itself, and its SH2 domain is blocked by P-tyr 527 (Amrein et al., 1993; Liu et al., 1993; Kaplan et al., 1994). Therefore, dephosphorylation of tyr 527, by increase of an appropriate PTPase activity at adhesion sites, is expected to trigger the binding of Src to the cytoskeleton. Our results show indeed that Src binding to the cytoskeleton is enhanced by GCP attachment to laminin, presumably by the interaction of an integrin with its ligand. GCP attachment to laminin also increased PTPase activity in adhesion complexes, and this phenomenon was vanadate-insensitive, whereas the binding of Src to the cytoskeleton was highly vanadate-sensitive. These data provide evidence that one or more PTPases are mediators between the event of integrin binding to laminin and Src attachment to the cytoskeleton. In other words, such PTPase(s) seem to be positive regulators of Src function.

Studies by others (Zheng et al., 1992; den Hertog et al., 1993) have identified PTPα as one of the enzymes capable of hydrolyzing phosphorylated tyr 527 of Src. Therefore, it is of particular interest that PTPα immunoreactivity is increased in adhesion complexes upon plating on laminin. Based on the approximately sixfold increase of PTPα immunoreactivity, compared to a nearly 14-fold increase in enzyme activity, recruitment of PTPase (PTPα and, perhaps, others) to the adhesion complexes seems to account for most of the increased enzyme activity, but an additional increase in specific PTPase activity is possible.

Our observations are consistent with the concept that certain PTPases, such as PTPα, form complexes with adhesion molecules (see e.g. the interactions between PTPα and E-cadherin; the localization of LAR to focal adhesions; the increase, by (ωβ)3 integrin-dependent platelet aggregation, of PTP1β binding to the cytoskeleton; for review, see Streuli, 1996; Ezumi et al., 1995). Of particular interest is the lymphocyte-specific transmembrane PTPase CD45 (see, e.g. Mustelin and Burn, 1993), which activates the two Src-family non-receptor tyrosine kinases Lck and Fyn by dephosphorylating them on tyrosines homologous to Src’s tyr 527 (Sieh et al., 1993). This situation appears to be analogous to the proposed positive regulation of Src by integrin via PTPα.

Early stages of adhesion complex formation

As summarized in several excellent reviews (e.g. Clark and Brugge, 1995; Craig and Johnson, 1996; Parsons, 1996), an early step in the formation of adhesion sites is activation of two protein tyrosine kinases, focal adhesion kinase (FAK, which binds to integrin β subunits) and an Src family tyrosine kinase. Integrin binding to a ligand is known to result in the phosphorylation of tyr 397 of FAK, thus generating a high-affinity binding site for the SH2 domain of Src and other members of the Src family (Cobb et al., 1994; Schaller et al., 1994; Xing et al., 1994). FAK phosphorylation on tyr 397 may occur autocatalytically (Schaller et al., 1994), but upon platelet aggregation, other polypeptides are tyr-phosphorylated before FAK (Huang et al., 1993), and catalytically inactive Src does not associate with the cytoskeleton (Hamaguchi and Hanafusa, 1987; Helmke and Pfenninger, 1995). Therefore, the earliest tyr-phosphorylation events upon cell attachment may involve the activation of Src, and not FAK.

Certain integrins and PTPα may form complexes that result in greatly increased local PTPase activity if they are clustered by ligand binding. Src family members in the vicinity, not catalytically active and not linked to cytoskeletal elements, would become de-phosphorylated on tyr 527 or its homolog. The unfolded Src family member would then be free to interact with tyr 397 of FAK, which it may phosphorylate (see
Calalb et al., 1995). FAK and Src may then become the primary anchor(s) for the many cytoskeletal proteins to be assembled at the cellular attachment site. Both FAK and PTPα could form the link, via GRB2 binding, to further intracellular signalling (Schlaepfer et al., 1994; Su et al., 1994). The process of adhesion site assembly would be reversed by c-Src kinase (Csk), whose overexpression has been shown to perturb focal adhesions (Bergman et al., 1995). Thus, our data are consistent with the hypothesis that PTPα, and perhaps other PTPases, are positive regulators of Src family members and of the earliest stages of integrin signalling and adhesion complex formation.

We thank Drs Nicholas Tonks and Jan Sap for helpful discussions and for making their antibodies available to us. Assistance by Ms Gray Grether and Carmel McGuire with the completion of the manuscript also is acknowledged gratefully. This work was supported by NIH grant NS24672 and, in part, by NSF grant BNS 9109775 and funds from the University of Colorado Cancer Center.

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