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Translocation of an SH2-containing protein tyrosine phosphatase (SH-PTP1) to the cytoskeleton of thrombin-activated platelets

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Abstract

A significant protein tyrosine phosphatase (PTP) activity was found to be associated with the cytoskeleton of thrombin-stimulated platelets. Translocation of the enzyme became maximal within 1–2 min of thrombin stimulation and was suppressed by cytochalasin D or upon inhibition of aggregation. Immunoblotting as well as immunoprecipitation revealed that a PTP with two SH2 domains (SH-PTP1) displayed the same behaviour, translocation to the cytoskeleton showing the same time course as that observed for pp60$^{c\text{-}src}$. We conclude that SH-PTP1 might represent a critical enzyme in the complex interplay between the various proteins regulating protein tyrosine phosphorylation in the cytoskeletal matrix.

Key words: Platelet; Thrombin; Protein tyrosine phosphatase; Cytoskeleton; SH2

1. Introduction

Besides regulating cell growth, differentiation and transformation [1,2], protein tyrosine phosphorylation is also important for the function of terminally differentiated cells such as platelets [3,4]. These anucleated cells contain a number of non-receptor PTK such as pp60$^{c\text{-}src}$ and related proteins [4,5], pp72^{syk} [6], or focal adhesion kinase (pp125^{fak}) [7]. Tyrosine phosphorylation results in the formation of protein complexes via specific recognition of well defined phosphotyrosyl residues by proteins containing SH2 domains [8,9]. Most of these proteins also contain SH3 regions, which are thought to direct their association with cytoskeleton [8–10]. In activated platelets, this kind of association was previously described for various phosphoinositide kinases (including PI 3-kinase), phospholipase C, diacylglycerol kinase, pp60$^{c\text{-}src}$ and pp125^{fak} [11–17]. This was also observed in nucleated cells [10,18–20], leading to the suggestion that recruitment of various signalling proteins to the cytoskeleton might enable multiple interactions and cascade events on the cytoskeletal matrix [19].

PTP are very critical in regulating the level of tyrosine phosphorylation. Two families of PTP have been defined and include transmembrane and intracellular enzymes (reviewed in [21,22]). Previous studies on the biochemistry of platelet PTP included the characterization of a 53-kDa PTP displaying a membrane localization [23] as well as the identification of PTP-1B as a substrate of calpain in thrombin-stimulated platelets [24]. In addition, two other PTP appear particularly interesting for platelets, specially when considering possible interactions with the cytoskeleton: PTPH1, also called MEG, is a 104–106-kDa protein identified by molecular cloning in HeLa cells and megacaryocytes, and displaying homology with the cytoskeletal proteins band 4.1, ezrin and talin [25,26]; SH-PTP1, also referred to as PTP1C, HCP, SHP or PTPN6, is predominantly expressed in hematopoietic cells and contains two SH2 domains [27–30].

In the present study, we have investigated the subcellular distribution of platelet PTP activity, with special interest for its possible association with cytoskeleton during the course of thrombin-stimulated aggregation. Owing to its potential participation in signalling complexes via its SH2 domains, SH-PTP1 was more specifically investigated.

2. Materials and methods

2.1. Materials

Poly(Glu,Tyr) (4:1) (molecular mass 20–50 kDa), synthetic peptide RGDS, cytochalasin D, human thrombin, anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate antibody, anti-rabbit IgG peroxidase conjugate and anti-mouse IgG peroxidase conjugate were purchased from Sigma. [γ-^{32}P]ATP and ECL (immunoblotting detection system) were from Amersham. An immunoaffinity-purified, rabbit polyclonal antibody specific for SH-PTP1 was obtained from UBI (Lake

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Abbreviations: PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; PI, phosphatidylinositol.

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Placid, NY, USA). Protein A-Sepharose CL-4B was purchased from Pharmacia-LKB, Uppsala, Sweden.

2.2. Incubation of platelet suspensions and isolation of platelet cytoskeleton

Platelet suspensions (5×10^8 cells/ml) in Tyrode's albumin buffer containing 1 mM $CaCl_2$ were prepared from human blood as previously described [12]. After equilibration for 5 min at 37°C, platelets were incubated in the presence of thrombin (1 NIH unit/ml) for appropriate times. In some experiments, the 5-min preincubation was performed in the presence of RGDS (200 μg/ml), EDTA (5 mM) or cytochalasin D (20 μM). Incubations were terminated by addition of an equal volume of 'CSK buffer' [12] containing Triton X-100 and cytoskeletons were isolated as in [12].

2.3. Determination of PTP activity

[^{32}P]Phosphotyrosyl poly(Glu,Tyr) was used as a substrate of PTP as in [31], except in experiments dealing with immunoprecipitated SH-PTP1 (see Fig. 3), where it was replaced by *p*-nitrophenyl phosphate [31].

2.4. Protein determination, gel electrophoresis and immunoblotting

Protein content was determined as in [32]. Proteins were separated by electrophoresis in a 10% polyacrylamide gel under reducing conditions [33] and blotted onto nitrocellulose as in [34]. Immunodetection was performed with a polyclonal antibody specific for SH-PTP1 (0.2 μg/ml) or the monoclonal antibody Ab-1 specific for pp60$^{c\text{-}src}$ (1 μg/ml). Final detection was achieved using anti-rabbit or anti-mouse immunoglobulins conjugated either to horseradish peroxidase (ECL system) or to alkaline phosphatase as secondary antibodies. 5-Bromo-4-chloro-3- indolyl phosphate was used as substrate of alkaline phosphatase.

2.5. Immunoprecipitation of SH-PTP1

SH-PTP1 was immunoprecipitated from cytoskeletons solubilized in 'RIPA buffer' [35,36], using 2 μg/ml of rabbit polyclonal antibody specific for SH-PTP1.

3. Results

Using [^{32}P]phosphotyrosyl poly(Glu-Tyr) as a substrate, platelets displayed a significant PTP activity (325 ± 8 pmol $\times$ min^{-1} per 10^9 cells, mean ± S.E.M.), which was distributed between cytosol (60%) and particulate fraction (40%, data not shown). PTP activity remained very low in cytoskeletons from resting platelets but increased significantly upon thrombin stimulation, attaining 8-fold the basal level within 2 min (Fig. 1A). By comparison (Fig. 1B), the increase of total cytoskeletal protein, which mainly reflects actin polymerization, occurred within the first seconds and attained 3- to 4-fold the basal level within 10–30 s. This suggests that association of PTP activity with platelet cytoskeleton was not a passive event resulting from non specific attachment of the enzyme(s) to the newly generated actin filaments.

By immunoblotting of subcellular fractions from resting platelets, a specific polyclonal antibody recognized a protein displaying the expected size of SH-PTP1 (67–68 kDa). In agreement with previous observations [37], a stronger signal was detected in the membrane fraction compared to cytosol (not shown). As shown in Fig. 2, SH-PTP1 was barely detected in the cytoskeleton of resting platelets, but following thrombin addition it displayed a stronger immunological signal, which became maximal after 1 min of thrombin stimulation. Finally, SH-PTP1 could also be detected by immunoprecipitation followed by immunoblotting. Here again, the 68-kDa protein was found to increase in the cytoskeleton of thrombin-stimulated cells with a time course of translocation identical to that previously observed (Fig. 3A). Interestingly, the immunoprecipitate was determined for hydrolytic activity towards *p*-nitrophenyl phosphate, which is currently used as a convenient substrate of various PTP [31,38]. As illustrated in Fig. 3B, immunoprecipitated phosphatase activity was found to associate to the cytoskeleton of thrombin-stimulated cells with a time course identical to that previously observed for SH-PTP1 and pp60$^{c\text{-}src}$.

Several studies previously showed that activation-dependent association to platelet cytoskeleton of a number of proteins actually involved integrin α_{IIb}/β_3 and actin polymerization. This is the case for instance for pp60$^{c\text{-}src}$ or PI 3-kinase [13–17]. From data presented in Fig. 4A, the same behaviour was observed for platelet PTP activity, since the tetrapeptide RGDS as well as EDTA, which inhibit fibrinogen binding to integrin α_{IIb}/β_3 by different mechanisms, both abolished the translocation of PTP activity to platelet cytoskeleton. Moreover, Fig. 4B shows that RGDS abrogated the association of SH-PTP1 with platelet cytoskeleton promoted by thrombin

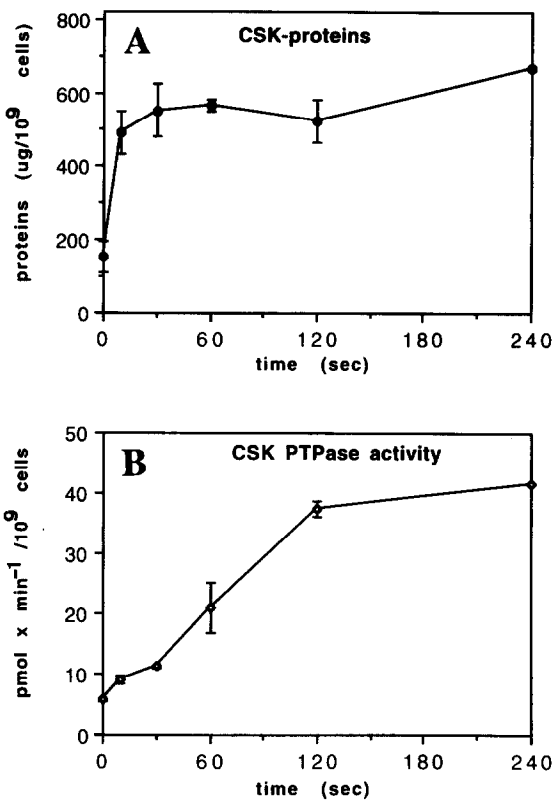

Fig. 1. Translocation of proteins and PTP activity to the cytoskeleton of thrombin-stimulated platelets. Platelets were incubated for the indicated times in the presence of thrombin (1 NIH unit/ml), cytoskeletons were isolated and determined for PTP activity (A) and for protein content (B). Data are means ± S.E.M. of 3 different experiments.

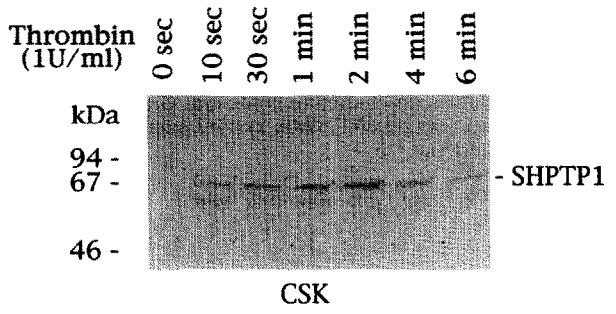

Fig. 2. Translocation of SH-PTP1 to the cytoskeleton of thrombin-stimulated platelets. Platelets were incubated as described in Fig. 1 and cytoskeletons were analysed by immunoblotting for the presence of SH-PTP1, detection being achieved by alkaline phosphatase. Data are representative of at least 5 experiments with identical results.

addition. In the presence of cytochalasin D, the thrombin-stimulated increase of PTP activity sedimenting with Triton-insoluble fraction was also suppressed (not shown).

4. Discussion

PTP can thus be added to the number of signalling proteins able to redistribute to cytoskeleton of activated platelets. The observation is of importance owing to the fact that platelets undergo dramatic increase in tyrosine phosphorylation under appropriate stimulation [3,4]. The time course of the association of both PTP activity and SH-PTP1 is very similar to that described for phospholipase C, diacylglycerol kinase, various phosphoinositide kinases (including PI 3-kinase and its regulatory subunit p85) as well as pp60$^{c\text{-}src}$ and pp125^{fak} [7,11–17]. In addition, like for pp60$^{c\text{-}src}$ and PI 3-kinase, translocation of PTP to the cytoskeleton requires both aggregation and actin polymerization, as deduced from the inhibitory effects of the peptide RGDS, of EDTA and of cytochalasin D. There is thus some circumstantial correlation between the appearance of PTP activity and of SH-PTP1 in platelet cytoskeleton, the third wave of tyrosine phosphorylation described in aggregating platelets [3,4] and the thrombin-stimulated increase of PI 3,4-*bis*phosphate synthesis [39]. However, the possible molecular mechanisms linking these events still remain unclear. A recent study of Pumiglia and Feinstein [17] showed that translocation of integrin α_{IIb}/β_3 and of pp60$^{c\text{-}src}$ does not occur in moderate-sized aggregates but requires the latter extensive formation of large aggregates. We can thus suggest that association of SH-PTP1 to the cytoskeletal matrix follows the same rule and probably involves the same mechanism. A common structural feature between pp60$^{c\text{-}src}$ and p85 is the presence of SH2 and SH3 domains. The mechanisms involving SH3 are probably not exclusive, but one can suggest that various signalling proteins might associate to the cytoskeleton through their SH3 domains, whereas others could bind via their SH2 domains to phosphotyrosyl proteins present in cytoskeletal complexes. This is probably the case for SH-PTP1.

As previously suggested [19], it is tempting to speculate that the cytoskeleton forms a matrix onto which attached proteins become able to interact with each other. A further step in understanding the role of SH-PTP1 will be to define its natural substrate, which might belong to a number of other phosphotyrosyl proteins also found in the cytoskeleton of activated platelets. A first candidate could be pp60$^{c\text{-}src}$, however dephosphorylation of Y^{527}, which activates the kinase activity of pp60$^{c\text{-}src}$, actually precedes its translocation to cytoskeleton [16]. Two proteins were found to undergo integrin-dependent dephosphorylation in response to thrombin or to a thromboxane-A_2 analogue [40]. It would thus be interesting to investigate whether these two phosphotyrosyl proteins also undergo a translocation to cytoskeleton.

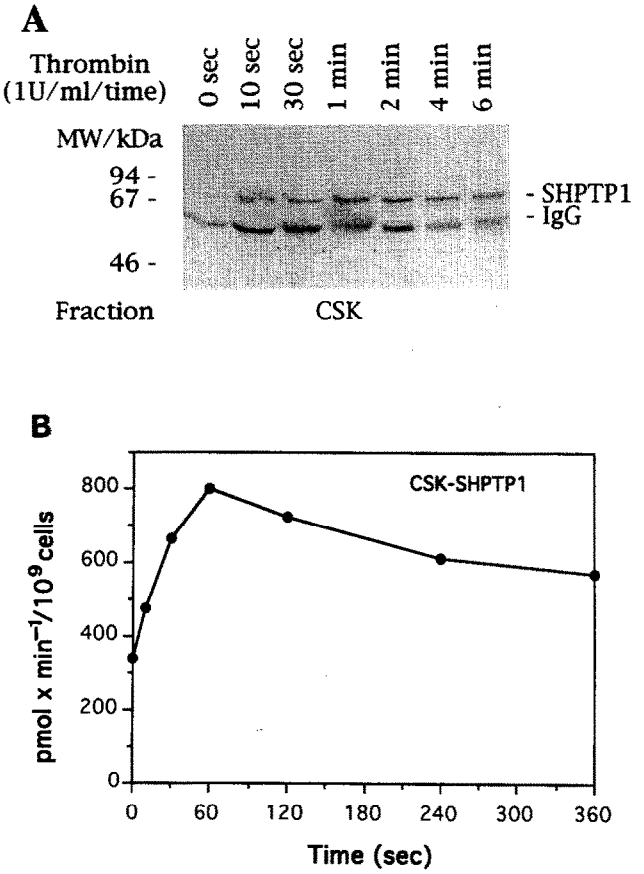

Fig. 3. Immunoprecipitation of SH-PTP1 and phosphatase activity from cytoskeletons isolated from thrombin-stimulated platelets. Platelet were incubated as described in Fig. 1, cytoskeletons (CSK) were isolated and SH-PTP1 was immunoprecipitated as described in section 2. Panel A shows the detection of SH-PTP1 by immunoblotting using alkaline phosphatase. Note the presence of immunoglobulin-G heavy chain (IgG) in the immunoprecipitated material below SH-PTP1. Panel B represents the phosphatase activity of the immunoprecipitate as determined with *p*-nitrophenyl phosphate as substrate. Data are representative of two experiments with identical results.

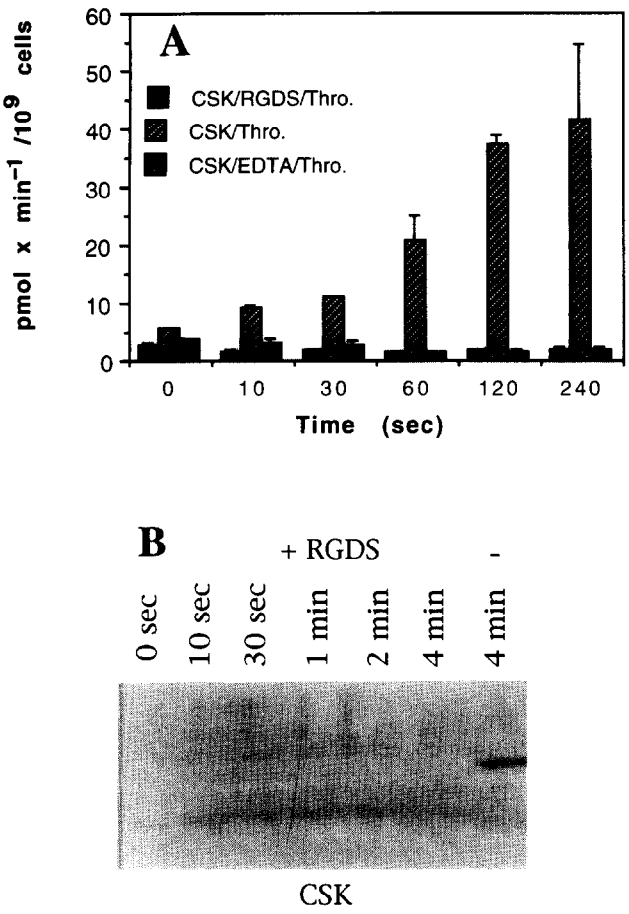

Fig. 4. Inhibition by RGDS and EDTA of the translocation to cytoskeleton of PTP activity and SH-PTP1. Platelets were stimulated with thrombin as described in Fig. 1 after a 5 min preincubation without any addition (CSK/Thro, hatched bars), or in the presence of 200 μg/ml RGDS (CSK/RGDS/Thro, filled bars), or in the presence of 5 mM EDTA (CSK/EDTA/Thro, dashed bars). In panel A, PTP activity was determined on cytoskeletons. Data are means ± S.E.M. of three different experiments. Panel B shows detection by immunoblotting of SH-PTP1 in the cytoskeletons of platelets incubated with RGDS and thrombin. The right lane (4 min −) corresponds to platelets incubated with thrombin in the absence of RGDS. Detection was achieved using ECL system.

Finally, our observation might offer some clue to understand the regulation of SH-PTP1. In vitro studies suggested three possible mechanisms, including proteolytic cleavage [38], tyrosine phosphorylation [41], as well as interaction with anionic phospholipids [37]. In contrast to pp60$^{c\text{-}src}$ [42] and PTP-1B [24], we did not detect any proteolytic cleavage of SH-PTP1. The second possibility would fit with the time course of pp60$^{c\text{-}src}$ translocation, although other protein tyrosine kinases are potential candidates to phosphorylate SH-PTP1. Finally, previous studies reported the association of various anionic phospholipids with cytoskeleton or some cytoskeletal proteins [44–46]. These might include phosphatidylserine [45], phosphatidic acid [47], or polyphosphoinositides [39,48].

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