Low molecular weight phosphotyrosine protein phosphatase translocation during cell stimulation with platelet-derived growth factor

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Abstract Low M<sub>r</sub> phosphotyrosine protein phosphatase (PTP) is a cytosolic enzyme whose activity upon platelet-derived growth factor (PDGF) and insulin receptors has been demonstrated in vivo. In our study we demonstrate that this enzyme, both naturally expressed and overexpressed in NIH/3T3 fibroblasts, translocates from the cytosol to the Triton X-100 insoluble fraction following stimulation with PDGF. It emerges that the phosphorylation of a defined population of PDGF receptors, which is localized in this fraction and seems to be endowed with peculiar features and functions, is particularly affected by low M<sub>r</sub> PTP overexpression.

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Key words: Phosphotyrosine protein phosphatase; Signal transduction; Enzyme translocation; Platelet-derived growth factor receptor

1. Introduction

Tyrosine phosphorylation of specific cellular proteins is a crucial mechanism typical of those pathways involved in the transmission of signals from a large variety of stimuli. Phosphotyrosine protein phosphatases (PTPs) together with phosphotyrosine protein kinases regulate tyrosine phosphorylation.

Low M<sub>r</sub> PTP is a cytosolic enzyme [1] which exists in two isoenzymatic forms. While nothing is known regarding the physiological role of the first isoform (also named ‘fast’), more and more details are emerging concerning the second (‘slow’), which is the subject of this study. This isoenzyme seems to be rather ubiquitous since it was detected in liver, brain, fibroblasts, placenta, heart and erythrocytes from different species [2–7]. It possesses the characteristic PTP homology domain CXXXXYR in its active site and its phosphate binding loop shows three-dimensional identity with that contained in PTP1B and Yersinia PTP, as is apparent from the resolved crystal structure of the enzyme [8]. Nevertheless it does not share any sequence homology with the other members of the non-receptor cytosolic phosphotyrosine phosphatase subfamily. In particular, this enzyme does not show any of the structural features which usually mediate stable or temporary association of PTPs with the particulate fraction as SH2 domains, myristoylation sites or cytoskeletal protein-like domains [9]. However, its ability to act upon membrane substrates has already been demonstrated: first its activity upon epidermal growth factor receptor (EGFr) was evidenced in vitro [10], subsequently the overexpressed enzyme displayed a phosphatase activity upon platelet-derived growth factor receptor (PDGFr) [4] and insulin receptor [11] in NIH/3T3 cells.

Low M<sub>r</sub> PTP seems to be regulated by Src-kinase, in fact it is phosphorylated and activated by the oncogenic variant pp60<sup>v-src</sup> [12,13]. This evidence further supports its integration in a complex of molecular interactions which take place in the particulate fraction.

Several so-called cytosolic PTPs are known to translocate to the particulate fraction following growth factor stimulation or cell transformation: for example PTP1D interacts with activated EGFr and PDGFr via its SH2 domains [14,15]; PTP1 is localized to focal adhesions as well as to the endoplasmic reticulum of Src-transformed fibroblasts, through its carboxy-terminal hydrophobic tail [16]. In these cases such PTP redistribution could participate in the definition of their substrate specificity.

It is well known that PDGFr is localized in cell membrane (Triton X-100 soluble fraction). Recently Coats et al. demonstrated that a sub-population of PDGFr is distributed in a Triton X-100 insoluble fraction and it colocalizes with several cytoskeletal proteins [17]. Here we demonstrate that low M<sub>r</sub> PTP (both naturally expressed and overexpressed) exclusively undergoes a translocation from the cytosol to this Triton insoluble fraction following PDGF stimulation. We show a greater and more specific decrease in tyrosine phosphorylation of Triton insoluble than Triton soluble PDGFr when low M<sub>r</sub> PTP is overexpressed. These results allow us to hypothesize the involvement of such translocation in the determination of low M<sub>r</sub> PTP substrate choice.

2. Materials and methods

2.1. Cell culture and transfection

NIH/3T3 murine fibroblasts (normal or v-src transformed) came from the Laboratory of Cellular and Molecular Biology of the NCI, National Institutes of Health, Bethesda, MD, USA and were cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose, supplemented with 10% fetal calf serum (FCS) (complete medium). All cells were cultured in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

Normal and v-src transformed NIH/3T3 overexpressing low M<sub>r</sub> PTP were previously obtained in our laboratory through cotransfection of pSVPTP and pKNeo plasmids [18,19].

In order to obtain c-src overexpressing cells, control (neomycin resistant) and PTP overexpressing NIH/3T3 were cotransfected with 8 µg of pSGT plasmid, containing the cDNA coding for pp60<sup>v-src</sup> (obtained from Sara Courtneidge, EMBL, Heidelberg, Germany [20]),

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and 1 µg of pBABE-puro plasmid, carrying the resistance to puromycin [21], in a 60-mm Petri dish, using the calcium phosphate precipitate technique. Control cells were obtained with transfection of pBABEpuro plasmid only. Stable puromycin resistant clones were isolated by supplementing medium with 2 µg/ml antibiotic.

In order to maintain stable expression all transfected cell lines were periodically cultured in selective medium.

2.2. Evaluation of pp60src overexpression

In order to individuate c-src overexpressing clones, cells were collected in Laemnii sample buffer, boiled for 10 min and centrifuged at 10 000×g for 10 min. Equal protein amounts from the lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot and immunore cognition with monoclonal 327 anti-pp60src antibody followed by incubation with peroxidase-conjugated anti-mouse antibody (Transduction Laboratories) and ECL detection (Amersham).

The activity of the enzyme was verified following its immunoprecipitation. Cells (both control and Src overexpressing) were lyed in hypotonic buffer (10 mM HEPES, pH 7.5, 10 mM NaCl, 1 mM K2HPO4, 5 mM NaHCO3, 1 mM CaCl2, 0.5 mM MgCl2, 5 mM EDTA, 1 mM sodium vanadate, 20 mM NaF, 10 mM sodium pyrophosphate, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 1 mM PMSF) supplemented with 1% Triton X-100. The lysate was centrifuged at 100 000×g for 30 min, then the kinase was immunoprecipitated by adding 15 µg of anti-PTP antibodies and 35 µl of protein G-Sepharose (Pharmacia). Following washes, the resin-bound kinase was assayed on Raytide peptide (Oncogene) in the presence of [γ-32P]ATP following the manufacturer’s instructions. The reaction products, adsorbed on phosphocellulose, were analyzed by liquid scintillation counting and revealed the presence of active overexpressed Src-kinase.

2.3. Stimulation of cells with mitogens

Cells were grown to sub-confluence, then they were serum starved for 24 h and stimulated with 20 ng/ml PDGF-BB (Peprotech) for the indicated time periods at 37°C. To stop growth factor stimulation, dishes were placed on ice and rapidly washed twice with ice-cold phosphate-buffered saline (PBS) (10 mM Na phosphate, pH 7.4) supplemented with 1% Triton X-100. The residual Triton X-100 resistant material was washed twice with hypotonic buffer and sonicated twice for 15 s in ice. Cell debris was removed from the lysate by centrifugation at 10 000×g for 3 min and then the lysate was centrifuged at 100 000×g for 30 min at 4°C; the resulting supernatant represented the cytosolic fraction.

The pellet was washed twice with hypotonic buffer, then it was extracted for 30 min in ice with 30 µl of the same buffer supplemented with 1% Triton X-100 and centrifuged at 100 000×g for 30 min. The Triton X-100 soluble supernatant was supplemented with Laemnii sample buffer and boiled.

2.4. Cell fractionation

Cell fractionation was performed according to Oude Weernink et al. [22] with minor modifications. Cells from a sub-confluent 10-mm dish were collected in 500 µl hypotonic buffer and sonicated twice for 15 s in ice. Cell debris was removed from the lysate by centrifugation at 100 000×g for 3 min and then the lysate was centrifuged at 100 000×g for 30 min at 4°C; the resulting supernatant represented the cytosolic fraction.

The pellet was washed twice with hypotonic buffer, then it was extracted for 30 min in ice with 30 µl of the same buffer supplemented with 1% Triton X-100 and centrifuged at 100 000×g for 30 min. The Triton X-100 soluble supernatant was supplemented with Laemnii sample buffer and boiled.

The residual Triton X-100 resistant material was washed twice with 1% Triton and further solubilized in 30 µl of RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium vanadate, 20 mM NaF, 10 mM sodium pyrophosphate, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 1 mM PMSF) for 30 min in ice, then 15 µl of boiling 3× Laemnii sample buffer was added (final SDS concentration: 2.1%). This fraction, referred to as Triton insoluble fraction, was clarified through centrifugation at 10 620×g for 10 min.

2.5. Low M, PTP immunoprecipitation

The detection of low M, PTP in the cytosol was rather difficult due to the large volume of this fraction (500 µl). For this reason, the enzyme was immunoprecipitated by incubating for 3 h in ice with 5 µg polyclonal anti-low M, PTP antibodies, which had been raised in rabbits and affinity purified as previously described [23]. We verified that the amount of antibody used was enough to completely clear a lysate from the enzyme. The immunoprecipitate was recovered upon addition of 5 µl protein A-Sepharose (SIGMA), during 1 h of incubation in a shaker at 4°C. The resin bound complex was washed three times with PBS and then it was eluted with boiling 2× Laemnii sample buffer. When we analyzed the PTP distribution during the time course of PDGF stimulation, samples from each time point containing equal protein amounts were subjected to immunoprecipitation.

2.6. Western blotting analysis

In order to determine the PTP distribution the immunoprecipitate from the cytosol as well as aliquots of 20 µg of proteins from Triton soluble and insoluble fractions were subjected to SDS-PAGE and immunoblotting with peroxidase-conjugated specific antibodies. The immunoreactive bands were visualized by ECL (Amersham) detection on a Biomax Light film (Kodak). In order to evidence the endogenous enzyme the detection was performed using the highly sensitive ECL plus.

The various fractions were sometimes analyzed also for the presence of pp60src (using 327 mAb antibodies as cited above) and PDGF receptor (using polyconal anti-PDGFr, Santa Cruz, and peroxidase-conjugated anti-rabbit, Calbiochem). Their tyrosine phosphorylation pattern was determined using RC20 peroxidase-conjugated anti-phosphotyrosine antibodies (Transduction Laboratories).

Successive immunore cognitions were performed on the same nitrocellulose membrane following the stripping procedure described by the manufacturer for the ECL detection method. A densitometric analysis of bands was performed.

3. Results

NIH/3T3 fibroblasts were stimulated with PDGF-BB for increasing time periods, cell lysates were then subjected to fractionation. Low M, PTP was immunoprecipitated from the cytosolic fraction while, in the case of Triton soluble and insoluble fractions, which had a much lower volume (45 µl), it was not necessary to recover the enzyme through immunoprecipitation.

The immunoblot analysis of fractions with anti-PTP antibodies, following the electrophoretic separation of bands, showed a gradual reduction in the amount of the cytosolic enzyme and a subsequent return to the basal level during growth factor stimulation (Fig. 1). This time course was accompanied by a complementary variation in the PTP level in the Triton insoluble fraction. From the densitometric analysis of bands it emerges that, at 10 min stimulation, about 50% of the enzyme moved from the cytosol to the Triton X-100 insoluble fraction. At these time points of stimulation no enzyme could be detected in the Triton soluble fraction.

Until now all observations of low M, PTP activity in vivo had been performed on overexpressed enzyme, particularly we were able to evidence its effect upon PDGF signaling. From the immunoprecipitation comparing control and PTP overexpressing cells [4]. We then decided to verify if also the overexpressed enzyme was sub-

Fig. 1. Low M, PTP translocation in NIH/3T3 fibroblasts. Fractions obtained from cells stimulated with PDGF-BB were subjected to 12% SDS-PAGE and immunoblotting with anti-PTP antibodies conjugated to peroxidase, followed by ECL-plus detection. A: Immunoprecipitates obtained with anti-PTP antibodies from the cytosolic fraction. B: Triton X-100 insoluble fraction. Lane 1: unstimulated; lane 2: 5 min stimulation; lane 3: 10 min stimulation; lane 4: 20 min stimulation; lane 5: 30 min stimulation.
Fig. 2. PDGFr tyrosine phosphorylation in control and PTP overexpressing NIH/3T3. Cells were stimulated with PDGF-BB for 10 min. Fractions were subjected to SDS-PAGE, Western blotting and ECL detection. A: Immunorecognition of PDGFr band, performed with anti-PDGFr antibodies and peroxidase-conjugated anti-rabbit, showing PDGFr content in lanes of panel B. B: Tyrosine phosphorylation pattern, obtained with peroxidase-conjugated anti-phosphotyrosine antibodies. Lanes 1–4: Triton X-100 soluble fractions; lanes 5–8: Triton X-100 insoluble fractions; lanes 1, 2, 5–6: control NIH/3T3; lanes 3, 4, 7, 8: PTP overexpressing NIH/3T3. Arrow: PDGF receptor. C: Low M, PTP distribution in PTP overexpressing cells, obtained with peroxidase-conjugated specific antibodies. Lanes 1, 2: immunoprecipitates obtained with anti-PTP antibodies from the cytosol; lanes 3, 4: Triton insoluble fraction.

jected to such a translocation following PDGF stimulation. The analysis of low M, PTP overexpressing NIH/3T3 revealed a time course of migration from the cytosol to the Triton insoluble fraction practically identical to that typical of the naturally expressed enzyme.

The translocation of the PTP to the Triton X-100 insoluble fraction was very interesting. In fact, given the abundant distribution of PDGFrs in the Triton soluble fraction (which can be identified with the membrane) one could expect to observe a movement of low M, PTP in this compartment. Recently Coats et al. [17] demonstrated that a well defined pool of PDGFr resides in a Triton insoluble fraction which codistributes with cytoskeleton. Taking into account this observation we decided to verify the phosphorylation state of PDGFr in Triton X-100 soluble and insoluble fractions from control and PTP overexpressing cells. Cells were stimulated for 10 min with PDGF, fractions were then obtained and analyzed through 10% SDS-PAGE, Western blot and immunorecognition with anti-phosphotyrosine antibodies. Fig. 2B shows a decrease in the staining of phosphorylated PDGFr band in NIH/3T3-PTP compared to control cells. It is noteworthy that, as regards the Triton soluble fraction, besides a 54.3% decrease (from the densitometric analysis of bands) in PDGFr staining there is a general decrease in the tyrosine phosphorylation level of various proteins in NIH/3T3-PTP compared to control cells. This does not happen in the Triton insoluble fraction where the reduction in the phosphorylation level pertains essentially to PDGFr band (−72%). An equivalent amount of receptor was present in the compared lanes (Fig. 2A). In Fig. 2C we verify that, at this time point of stimulation (10 min) overexpressed low M, PTP effectively translocated from the cytosol to the Triton insoluble fraction.

v-src transformed NIH/3T3 grow in the absence of growth factors and if they are stimulated with PDGF-BB neither phosphorylation of the specific receptor nor an increase in the mitogenic rate can be observed [12]. In fact Zhang et al. demonstrated a reduction in PDGFr mRNA in v-src transformed NRK fibroblasts [24]. The analysis of overexpressed low M, PTP distribution in v-src NIH/3T3 showed that it is abundantly represented in the Triton insoluble fraction (Fig. 3A), besides being present in the cytosol. Considering that only 1/9 of the Triton insoluble fraction was subjected to electrophoresis, while the immunoprecipitate from the cytosol was entirely applied to the gel, we evaluate that about 68% was present in the former fraction and 32% in the latter (densitometric analysis). Traces of the enzyme can be detected in the Triton soluble fraction too, but we think that they should be considered a contamination of the preparation, given the large amount of overexpressed PTP in this cell line. Following stripping of this nitrocellulose filter and incubation with anti-phosphotyrosine antibodies we noticed that low M, PTP was tyrosine-phosphorylated both when present in the cytosol and in the Triton insoluble fraction (Fig. 3B), though to different extents: 44% in the former and 113% in the latter fraction, as revealed by the densitometric analysis.

pp60
src
is known to migrate from membrane to cytoskeleton (and precisely to focal adhesions) during PDGF stimulation [23], while pp60
src
is permanently localized in focal adhesions [25]. We then decided to verify if there could be a cofractionation of low M, PTP and pp60
src
and a temporal coincidence in their translocation. Cells overexpressing both the PTP and the kinase were stimulated with PDGF for 10 min, lysates were obtained in hypotonic buffer and then subjected to fractionation. The various fractions were examined through electrophoresis and Western blotting with both anti-PTP and anti-Src antibodies. Fig. 4B reveals the expected migration of a part of the cytosolic PTP to the Triton insoluble fraction during growth factor stimulation. In lane 7 we analyze a control lysate which was completely cleared of PTP using 5 µl of the specific antibody. In Fig. 4A, which was obtained through stripping and re-probing of the same nitrocellulose filter, we can appreciate the concomitant translocation of a certain amount of c-src from the Triton soluble to the insoluble fraction. The two bands which stain positive in Fig. 4A, lanes 1 and 2 (corresponding to the cytosol, where Src-kinase is not localized), represent a cross-reaction of the

Fig. 3. Low M, PTP distribution and tyrosine phosphorylation in v-src transformed NIH/3T3. Cell lysates were subjected to fractionation followed by 12% SDS-PAGE and Western blot. A: Immunorecognition with anti-PTP antibodies. B: Immunorecognition with anti-phosphotyrosine antibodies. Lane 1: immunoprecipitate with anti-PTP antibodies from the cytosol; lane 2: control immunoprecipitate with non-specific immunoglobulins; lane 3: Triton X-100 soluble fraction; lane 4: Triton X-100 insoluble fraction; lane 5: standard low M, PTP.
rabbit immunoglobulins used to immunoprecipitate low \( M_r \) PTP with the anti-mouse used as secondary conjugated antibody in this blot.

It can be seen that the PTP band is double, which may indicate that the enzyme is subjected to some modification in certain conditions (i.e. during Src overexpression). By reprobing this filter with anti-phosphotyrosine antibodies we verified that the modification responsible for this variation in electrophoretic mobility does not consist in tyrosine phosphorylation. Further investigation is needed to clarify this evidence.

4. Discussion

Low \( M_r \) PTP structurally belongs to the cytosolic PTP subfamily, nevertheless so far it has been shown to interact only with proteins which are localized in the particulate fraction: it dephosphorylates PDGFr [4] and insulin receptor [16] and it is phosphorylated and activated by pp60\(^{src}\) [12,13].

We decided to verify if this enzyme could undergo a translocation from the cytosol to the particulate fraction where it performs its enzymatic activity. The analysis of the PTP distribution revealed that it moves, during the course of PDGF stimulation, from the cytosol to the Triton X-100 insoluble fraction and then again to the cytosol as we prolonged growth factor stimulation. It is noteworthy that the enzyme is present, though at a low level, in the Triton insoluble fraction of unstimulated cells too.

Interestingly, we were able to evidence this translocation also in not overexpressing cells: this is the first evidence of the enzyme which is normally expressed in NIH/3T3 cells and it sustains all our previous lines of evidence which had been obtained through its overexpression, corroborating the thesis of a physiological (not pharmacological) role of this enzyme on PDGF signaling in vivo.

The analysis of the tyrosine phosphorylation pattern following growth factor addition shows that PDGF\(r\)r is more specific too, in fact there is a general phosphorylation decrease in the Triton soluble fraction of PTP overexpressing cells compared to controls, while only the phosphorylation of the PDGF\(r\) band is affected in the Triton insoluble fraction by PTP overexpression, at this time point of stimulation. Recent research indicates that several growth factor receptors can concentrate in specific regions where the plasma membrane interacts with the cytoskeleton to give specialized structures: for example, it has been reported that activated PDGFr moves in membrane ruffles [26] or in caveolae [27]. Finally it has become evident that a distinct subset of cellular PDGFr is present in a Triton X-100 insoluble fraction. Coats et al. [19] demonstrated that this sub-population of receptors is characterized by peculiar properties and functions such as higher basal tyrosine phosphorylation state, resistance to downregulation, cofractionation with cytoskeletal proteins and increased abundance with increasing cell density. They hypothesized that this receptor pool could be specifically involved in long-term responses to growth factor stimulation in those cellular modifications which involve a cytoskeletal rearrangement (such as membrane ruffling and chemotaxis). Our results raise the possibility of a regulatory role of low \( M_r \) PTP on this distinct pool of PDGFr.

We could distinguish between a PTP activity upon PDGF\(r\)r which is linked to its translocation in the same fraction (the Triton insoluble one) where this pool of receptors resides, and an activity on Triton soluble PDGFr\(s\), which is not accompanied by PTP redistribution. We may argue that the former activity is more specific, while the latter could be a pharmacological effect of low \( M_r \) PTP overexpression. The general reduction in tyrosine phosphorylation which we observed in the Triton soluble fraction could also reflect the decrease in PDGFr kinase activity once it is dephosphorylated by the PTP.

Some preliminary lines of evidence point to a possible role of pp60\(^{src}\) in low \( M_r \) PTP movement: in v-src transformed NIH/3T3 the overexpressed PTP is abundantly distributed in the Triton insoluble fraction, while the oncogenic kinase is known to be prevalently localized too. It is important to note that, given the unresponsiveness of this cell line to PDGF, we can exclude the involvement of those proteins which are recruited independently of Src by PDGFr in mediating the localization of the PTP.

The observation of the coincidence (at least for the time point which is considered in this experiment, Fig. 4) in the translocation of pp60\(^{src}\) and low \( M_r \) PTP to the Triton insoluble fraction during PDGF stimulation gives a basis for a hypothesis regarding a possible connection between the movements of the two enzymes. This colocalization is also an indirect confirmation of the goodness of our fractionation technique, since c-src redistribution during PDGF stimulation is a well known event.

As regards any hypothesis regarding the translocation mechanism and the role of pp60\(^{src}\) in it, for the moment we can only say that there does not seem to be a strict correlation between PTP localization and its tyrosine phosphorylation. In fact, as can be appreciated from Fig. 3B, in v-src transformed cells the enzyme is phosphorylated both in the cytosol and in the Triton insoluble fraction, while in normal and c-src overexpressing fibroblasts it is not phosphorylated in either of the two fractions (data not shown). We cannot exclude that, in this latter case, we failed to detect low \( M_r \) PTP phosphorylation due to its ability to auto-dephosphorylate and to the difficulty of completely inhibiting its activity. At any case, the possible loss of phosphorylation did not abrogate the enzyme redistribution.
Our current research is oriented to the identification of the precise site of low Mr PTP translocation, in particular we want to verify if this coincides with focal adhesions where Src kinase migrates following growth factor stimulation [28,29], also considering the reported corefractionation of the Triton insoluble pool of PDGFr with vimentin and focal adhesion kinase [17]. Structural features underlying this redistribution are under investigation.

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