Low M_r phosphotyrosine protein phosphatase activity on fibroblast growth factor receptor is not associated with enzyme translocation

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Abstract Fibroblast growth factor receptor (class IV) shares a certain degree of similarity with class III members like platelet-derived growth factor and macrophage-colony-stimulating factor receptors, which, once activated, are substrates of low M_r phosphotyrosine protein phosphatase. Up until now no phosphotyrosine phosphatase has been shown to act on this receptor in vivo. Here we demonstrate that low M_r phosphotyrosine protein phosphatase is able to reduce receptor tyrosine phosphorylation and cell proliferation in response to basic fibroblast growth factor. Contrary to what was previously observed for platelet-derived growth factor, during cell stimulation with basic fibroblast growth factor, no enzyme redistribution among cellular compartments is observed.

Key words: Phosphotyrosine protein phosphatase; Signal transduction; Fibroblast growth factor receptor; Macrophage-colony-stimulating factor receptor

1. Introduction

Evidence has been provided to support the hypothesis that localization is the most important factor influencing phosphotyrosine protein phosphatase (PTP) target selection [1–3]. The analysis of the sequences outside the catalytic domain of PTPs revealed a great variety of structures which may serve to regulate their targeting to a subcellular location, thereby restricting the range of available substrates.

Low M_r PTP (18 kDa) was previously considered a cytosolic enzyme, but we recently demonstrated that, at least in NIH/3T3 fibroblasts, it also resides in the Triton X-100-insoluble fraction, usually identified as the cytoskeleton [4,5]. Up until now low M_r PTP has been shown to interact in vivo only with proteins which reside in the particulate fraction: low M_r PTP is phosphorylated by $pp60^{src}$ [6], Lck and Fyn [7] and it dephosphorylates platelet-derived growth factor receptor (PDGFr) and macrophage-colony-stimulating factor receptor (M-CSFr) (class III receptors) [5,8]. A corresponding reduction in the mitogenic response to PDGF and M-CSF was also observed. Low M_r PTP activity upon epidermal growth factor receptor (EGFr) (class I receptor) has been previously evidenced in vitro [9], but we showed that this has no correspondence in vivo [10].

We recently demonstrated that low M_r PTP undergoes a redistribution from the cytosol to the Triton X-100-insoluble fraction following cell stimulation with PDGF, and that in this fraction low M_r PTP exerts most of its activity on PDGFr [4]. This phenomenon, in contrast, was not observed with EGF and, correspondingly, no effect on EGFr activation was observed [10]. These findings, taken together with the fact that EGFr is a substrate for this PTP in vitro, i.e. in a condition where the necessity of enzyme compartmentalization is overcome, seemed to stress the importance of intracellular localization of low M_r PTP in the definition of enzyme specificity in vivo.

The high affinity fibroblast growth factor receptor (FGFr) family includes several members which bind more than one type of FGF. These receptors, though being allocated to a separate class (class IV), share several structure similarities with the members of class III, such that the first identified gene encoding FGFr was called *flg*, which stands for '*fms*-like gene', where *fms* is the gene encoding M-CSFr.

Here we demonstrate that these similarities extend also to the susceptibility of FGFr to low M_r PTP, as both receptor phosphorylation and mitogenic response elicited by the binding of basic FGF (bFGF) were affected by phosphatase overexpression. To our knowledge, this represents the first reported effect of a tyrosine phosphatase on FGFr phosphorylation. When we verified the connection between the activity of low M_r PTP and its translocation in response to M-CSF or bFGF, no redistribution of the phosphatase was observed.

2. Materials and methods

2.1. Cell culture

NIH/3T3 murine fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, supplemented with 10% (v/v) fetal calf serum (FCS) (complete medium). BAC-1.2F5 murine macrophages were cultured in DMEM supplemented with 10% FCS and 3000 U/ml human recombinant M-CSF. Low M_r PTP-overexpressing NIH/3T3 (NIH/3T3-PTP) and controls had been previously obtained through calcium phosphate precipitation transfection and characterized [11]. In order to maintain stable enzyme expression cells were periodically cultured in selective medium containing 400 μg/ml G418 (Sigma).

2.2. Determination of the mitogenic response to bFGF in PTP-expressing NIH/3T3 and controls

1.5×10^4 cells/cm^2 were plated in complete medium on multi-well dishes and allowed to adhere for 3 h. Medium was then changed and cells were grown for the subsequent 72 h in DMEM containing 0.5% FCS and 100 ng/ml bFGF (Boehringer Mannheim). Every 24 h, cell number was determined using a Bürker chamber and the remaining

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Abbreviations: PTP, phosphotyrosine protein phosphatase; EGFr, epidermal growth factor receptor; PDGFr, platelet-derived growth factor receptor; M-CSFr, macrophage-colony-stimulating factor receptor; FGFr, fibroblast growth factor receptor; bFGF, basic FGF; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BCA, bicinchoninic acid; ECL, enhanced chemiluminescence

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wells were supplemented with fresh medium. Every experimental point was assessed in quadruplicate.

2.3. Stimulation of cells with mitogens

Fibroblasts were grown to sub-confluence, serum-starved for 24 h and then stimulated with bFGF for the indicated times. To up-regulate M-CSFr expression, macrophages were maintained in medium containing 10% FCS, deprived of M-CSF, for 18 h prior to growth factor addition. To stop growth factor stimulation, dishes were placed on ice and rapidly washed twice with PBS (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) supplemented with 100 μM sodium vanadate.

2.4. Analysis of the tyrosine phosphorylation pattern following cell stimulation with mitogens

The pattern of tyrosine phosphorylation in response to mitogen stimulation was obtained and evaluated as already described [10]. The immunorecognition of basic FGFr (bFGFr) was performed on the same membrane, following incubation for 30 min at 50°C in stripping buffer (62.5 mM Tris-HCl pH 6.7, 100 mM 2-mercaptoethanol, 2% (w/v) SDS) and extensive washing, using VBS-7 anti-bFGFr monoclonal antibody (Calbiochem) according to the procedure cited above. Note that the antibody used did not allow us to distinguish between different types of FGFr capable of interacting with basic FGF, but NIH/3T3 are known to express predominantly FGFr-1 [12]. A densitometric analysis of bands was performed.

2.5. Cell fractionation and analysis of fractions

The separation of cytosolic, Triton X-100-soluble and -insoluble fractions was performed as already described [4]. Nuclei were isolated according to the method described by Schreiber et al. [13]. They were then lysed through a 10 s sonication in 100 μl of a 50 mM HEPES pH 7.5 buffer, containing 1% (v/v) Triton X-100, 50 mM NaCl, 5 mM EDTA, 0.5 mM PMSF, 1 mM sodium vanadate, 10 μg/ml leupeptin, 0.1 mM benzamidine. Nuclear lysates were finally clarified at $10\,000 \times g$ for 10 min. The purity and integrity of the nuclear fraction were confirmed assaying 5′ nucleotidase activity as a marker of plasma membranes, and acid phosphatase activity as a marker of lysosomes, and evidencing the presence of the typical histonic pattern by SDS-PAGE and gel staining with Coomassie blue stain (Pierce).

Subcellular fractions from different samples were normalized for protein concentration before their subsequent analysis. In order to determine low M_r PTP distribution, samples were subjected to 12.5% SDS-PAGE and immunoblotting with a polyclonal anti-low M_r PTP antibody [14] followed by a peroxidase-conjugated anti-rabbit antibody (Calbiochem), according to the procedure cited above. A densitometric analysis of bands was performed.

3. Results

The mitogenic response of NIH/3T3 cells to bFGF was evaluated by counting at different times of incubation (Fig. 1). Growth of NIH/3T3-PTP cells was significantly reduced compared to that of controls (−65% at 72 h).

In order to ascertain the possibility of a negative effect of low M_r PTP on FGFr phosphorylation, we analyzed the tyrosine phosphorylation pattern following cell stimulation with increasing bFGF doses. As shown in Fig. 2B, the phosphorylation of a 110 kDa band was significantly reduced in NIH/3T3-PTP (lanes 5–8) compared to control cells (lanes 1–4). This band was identified as FGFr by subsequent recognition with a specific antibody (Fig. 2A); the presence of an equal receptor amount in the two cell lines was verified through densitometric analysis of the bands (not shown). As in Fig. 2 the tyrosine phosphorylation level of several proteins was identical in NIH/3T3-PTP and in control cells, the possibility of a general non-specific activity of low M_r PTP due to its overexpression was excluded.

FGFr is known to migrate from the plasma membrane to the nucleus during prolonged stimulation with bFGF [12]. Following stimulation with bFGF for up to 40 min, cells

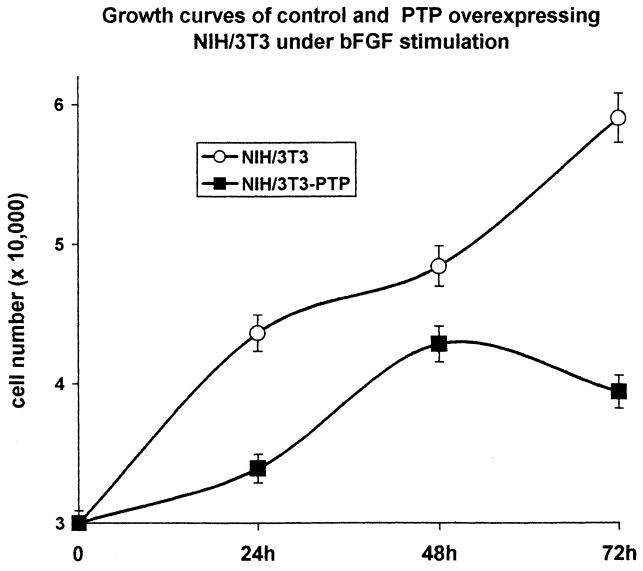

Fig. 1. Growth curves of NIH/3T3-PTP and controls following bFGF stimulation. Cells were plated in complete medium. After 3 h they were washed and grown in DMEM containing 0.5% FCS and 100 ng/ml bFGF for 3 days. Every 24 h cell number was determined in quadruplicate and the remaining wells were supplemented with fresh medium. Data from three independent experiments are reported as mean ± S.E.M.

were subjected to fractionation in order to obtain the nuclear and non-nuclear fractions, and the phosphatase distribution was determined through electrophoresis and immunoblotting. Fig. 3 shows that low M_r PTP was uniformly distributed in the non-nuclear fraction (lanes 1–4), while it was never retrieved from the nucleus (lanes 5–8). Under our conditions, FGFr did translocate to the nucleus, being already detectable 5 min after stimulation (data not shown), in keeping with Maher et al. [12].

We recently demonstrated [4] that, following NIH/3T3 stim-

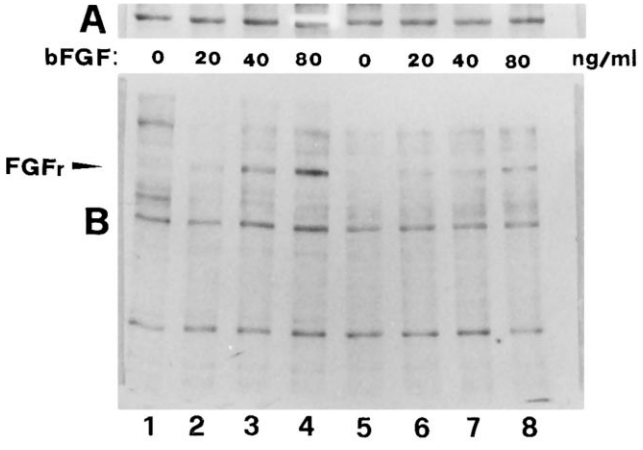

Fig. 2. Tyrosine phosphorylation pattern in NIH/3T3-PTP and controls following bFGF stimulation. Cells were serum-starved for 24 h and then stimulated for 5 min with 20, 40 or 80 ng/ml bFGF. Total cell lysates were analyzed through SDS-PAGE and Western blotting followed by ECL detection. A: Immunoblot with anti-FGFr antibodies. B: Immunoblot with anti-phosphotyrosine antibodies. Lanes 1–4: controls; lanes 5–8: NIH/3T3-PTP. Arrow: FGFr. Densitometric analysis of phosphorylated FGFr bands: lane 7, −56% compared to lane 3; lane 8, −65% compared to lane 4 (data obtained using a Quanti Scan (Biosoft) program).

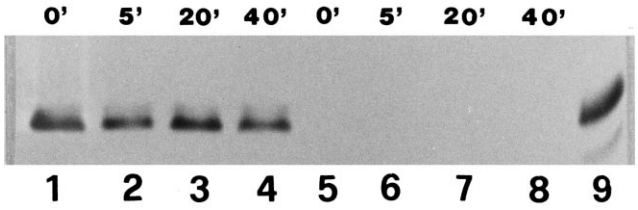

Fig. 3. Low M_r PTP distribution in the nuclear and non-nuclear fractions, in PTP-overexpressing NIH/3T3. Cells were stimulated for the indicated time periods and then subjected to fractionation. Nuclear and non-nuclear fractions were analyzed by SDS-PAGE and immunoblotting with anti-low M_r PTP antibodies. Lanes 1–4: non-nuclear fraction; lanes 5–8: nuclear fraction; lane 9: control low M_r PTP.

ulation with PDGF, low M_r PTP translocates from the cytosol to a Triton X-100-insoluble fraction. Cytosol and Triton X-100-soluble and -insoluble fractions from bFGF-stimulated cells were analyzed for low M_r PTP distribution. Fig. 4A shows that an invariant level of low M_r PTP was observed in both the cytosol and the Triton-insoluble fraction. No enzyme was ever detected in the Triton-soluble fraction (that is in membranes) at these time points of stimulation (not shown). In a previous study, we demonstrated a reduction in both the mitogenic response elicited by M-CSF and the tyrosine phosphorylation of its receptor in N3FP cells, NIH/3T3 cells artificially induced to express M-CSFr and overexpressing low M_r PTP [5]. In this cell line (not shown) and in BAC-1.2F5 murine macrophages which do not overexpress the phosphatase, the enzyme distribution among cell fractions was then analyzed (Fig. 4B). In both cases, following M-CSF stimulation, an invariant level of low M_r PTP was observed in the cytosol (1) and in the Triton-insoluble fraction (2), while no enzyme was detected in the Triton-soluble fraction (not shown). It is worth noting that the naturally expressed enzyme in BAC-1.2F5 cells appears as a doublet. The meaning of this pattern is currently under investigation. On stripping and reprobing membranes with anti-receptor antibodies, bFGFr and M-CSFr were clearly displayed in the Triton-insoluble fraction (data not shown), a result never reported before.

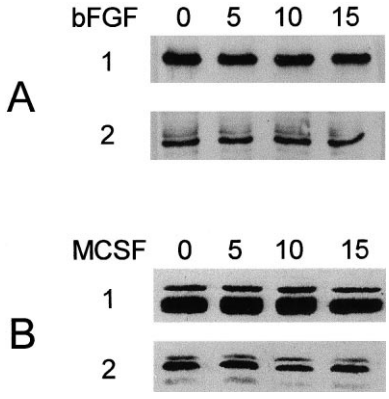

Fig. 4. Low M_r PTP distribution following stimulation with bFGF or M-CSF. Serum-starved fibroblasts (A) or macrophages deprived of M-CSF for 18 h (B) were stimulated with 40 ng/ml bFGF or 5000 U/ml M-CSF, respectively, for 0, 5, 10 or 15 min. Cell fractions were analyzed by SDS-PAGE and immunoblotting with anti-low M_r PTP antibodies, followed by ECL detection. 1: cytosol; 2: Triton X-100-insoluble fraction.

4. Discussion

The data presented in this paper demonstrate, for the first time, the existence of a phosphotyrosine phosphatase, low M_r PTP, whose overexpression in cells causes a reduction in the tyrosine phosphorylation level of activated bFGFr. This dephosphorylation produces an attenuation of the mitogenic signal departing from FGFr, which results in a lower proliferative response to bFGF, compared to control cells.

Our recent studies led us to hypothesize a correlation between the redistribution of low M_r PTP from the cytosol to the particulate fraction and PTP activity on a given receptor [4,10]. The results reported in this paper led us to reconsider the above hypothesis. As already observed for PDGFr, FGFr tyrosine phosphorylation was decreased by low M_r PTP overexpression, but no PTP redistribution was observed upon bFGF addition. An invariant level of enzyme was detectable in both the cytosol and the Triton X-100-insoluble fraction, while it was never retrieved from the Triton-soluble fraction. We verified that no PTP translocation in the Triton X-100-soluble fraction occurred during the very early phase (15 s) of cell stimulation with bFGF (data not shown). We also observed a similar 'unresponsiveness' to M-CSF (M-CSFr is a substrate of low M_r PTP in vivo [5]), on PTP redistribution in N3FP fibroblasts (data not shown) induced to express M-CSFr and overexpressing low M_r PTP [5] and BAC-1.2F5 murine macrophages, where no overexpression had been induced.

Many data suggest for bFGF a nuclear signalling function [12,15,16], which would be necessary, together with membrane receptor phosphorylation, to cause cell proliferation [17,18]. Nuclear translocation of bFGF occurs together with its plasma membrane receptors. The analysis of the nuclear fraction revealed that no low M_r PTP could ever be detected in it, even if we prolonged bFGF stimulation for time periods sufficient to induce receptor translocation (data not shown) [12]. This made us exclude the possibility that the PTP-dependent dephosphorylation of FGFr shown in Fig. 2 may be carried out in the nucleus.

We then concluded that bFGFr and M-CSFr dephosphorylation are not accompanied by low M_r PTP redistribution in a specific compartment. However, it is worth remembering here that the dephosphorylation of PDGFr was evident in the Triton-insoluble fraction (where low M_r PTP is redistributed following cell stimulation with PDGF), but also detectable in the Triton-soluble fraction (where PTP was never found) [4]. These results are understandable considering the contiguity of cytosol and membrane, which renders membrane-associated proteins accessible substrates for soluble enzymes. Thus, low M_r PTP displays a certain degree of in vivo substrate specificity that is achieved through a mechanism which does not necessarily involve a translocation of the enzyme. The latter event appears, at this point, to be a peculiar consequence of PDGF stimulation, whose meaning is currently under investigation.

Other considerations can be made concerning the ability of low M_r PTP to select its substrates. Jia et al. [19] showed the importance of the residues lining the deep crevice containing the active site in the definition of substrate specificity of several PTPs. In low M_r PTP these residues are mainly hydrophobic, and this seems to determine a clear preference of this enzyme for substrates containing two hydrophobic residues at

the phosphotyrosine *N*-side. A recent study demonstrates that, among nine Tyr-phosphorylated peptides containing the sequences surrounding the main phosphorylation sites of β-PDGFr, only one (corresponding to the requisite underlined above) was a substrate for which low M_r PTP displayed an in vitro affinity comparable to that typical of a number of other PTPs [20]. These data, while not excluding the involvement of an 'intermediate' (an adapter protein) allowing the interaction of low M_r PTP with its substrates, stress the importance of a sequence-specific recognition which could explain the observed in vivo preferences of this enzyme.

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