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Low M_r Phosphotyrosine Protein Phosphatase Interacts with the PDGF Receptor Directly via Its Catalytic Site

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Many proteins bind to the activated platelet derived growth factor receptor (PDGF-R) either directly or by means of adapter molecules. Up to now all these proteins were shown to transmit and amplify the signal started with PDGF-R stimulation. In a recent study our group has demonstrated that low Mr phosphotyrosine protein phosphatase (LMW-PTP) specifically interacts with PDGF-R in NIH3T3 cells. In the present study we have attempted to clarify the modality of interaction, both *in vivo* and *in vitro,* of these two proteins, using a catalytically inactive LMW-PTP mutant. Our results indicate that LMW-PTP and PDGF-R interact directly, without the necessity of any adapter protein. This interaction leads to PDGF-R dephosphorylation and, presumably, interrupts one or more of the mitogenic pathways that originate from receptor activation.

Phosphotyrosine protein phosphatases can be classified into two subfamilies: one receptor-like family, whose members contain an extra cellular domain, a transmembrane region and, typically, two repeated phosphatase domains; and a second non-receptor like family, whose members feature a single catalytic phosphatase domain (1). LMW-PTP is a cytosolic enzyme that does not have extensive sequence homology to the other two classes of PTPases, but does contain a CXXXXXR motif which is the active site signature of all PTPase members (2). Both arginine and cysteine are essential for the catalytic activity of the LMW-PTP (3), as in the other PTPases, and it is almost certain that all PTPases share a common catalytic mechanism which includes a covalent cysteinyl phosphate intermediate. In our laboratories we have previously demonstrated that the mutation of Cys 12 to Ser, included in the signature motif, causes the complete loss of catalytic activity (4), yet, at the same time, the LMW-PTPC12S protein is still able to bind substrates (5). Overexpression of LMW-PTP in murine fibroblasts inhibits both normal and transformed cell growth during serum or PDGF-BB cell stimulation (6, 7, 8): we have recently demonstrated that transfection of the LMW-PTPC12S mutant into NIH3T3 cells causes a remarkable increase of both thymidine incorporation and cellular growth rate, thus confirming the *in vivo* dominant negative effect of the LMW-PTPC12S mutant (9). In addition, we have demonstrated that this phenotype was accompanied by an interaction between LMW-PTPC12S and PDGF-R. We have proposed that the phenotypic effect observed in NIH3T3-LMW-PTPC12S cells could be due to a slower PDGF receptor inactivation, resulting in an enhanced mitogenic signal.

In this paper we have attempted to identify the kind of interaction between LMW-PTP and PDGF-R. In particular, the data here reported demonstrate that LMW-PTP directly interacts with PDGF-R via its catalytic site, without any adapter molecule.

MATERIALS AND METHODS

Cell culture. Transfected NIH3T3 were cultured in 10% additioned fetal calf serum DMEM in 5% CO_2 modified

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Abbreviations: PTPase: phosphotyrosine protein phosphatase; LMW-PTP: low molecular weight phosphotyrosine protein phosphatase; PDGF-R: platelet derived growth factor receptor; PDGF-BB: platelet derived growth factor-BB; GST-LMW-PTP: glutathione S-transferase low molecular weight phosphotyrosine protein phosphatase fusion protein; GST: glutathione S-transferase.

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atmosphere. PDGF-BB (Peprotech) stimulation was performed treating 1×10^6 cultured cells for 5 minutes with 50 ng/ml of the growth factor, after 24 h serum starvation.

Enzyme preparation. GST-LMW-PTP fusion protein purification was carried out as previously indicated (10).

In vitro PDGF-R binding assay. After PDGF stimulation the NIH3T3-LMW-PTPC12S cells were lysed and the phosphorylated receptor was immunoprecipitated for 4 h at 4°C with 1 μg anti-PDGF receptor antibodies. Immune complexes were collected on protein G Sepharose (Pharmacia) according to (9). Agarose bounds receptors were washed several times with binding buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM $MgCl_2$, 100 mM NaCl and 0.1% Triton X-100) and then incubated in a 100 μl binding buffer assay with 1 μg of purified GST-LMW-PTP.

Western blotting analysis. Cells were lysed in 100 μl of binding buffer and sonicated twice for 10 sec. After four 1 ml washes with the same buffer, the immunoprecipitates were resuspended in electrophoresis sample buffer, and they were run on SDS PAGE (10% gel). Proteins were then electrophoretically blotted onto nitro-cellulose membrane (Sartorius). The blot was blocked with 3% bovine serum albumin in phosphate buffered saline solution containing 0.1% Tween 20 for 1 h at 25°C. Anti-LMW-PTP polyclonal antibodies or anti-phosphotyrosine antibodies (Affiniti), both linked to horse radish peroxidase, were diluted 1:2000 and 1:2500 respectively in blocking solution and incubated for 2 additional hours in gentle agitation. After 3 washes in phosphate buffered saline solution with 0.5% Tween 20, the blot was developed by enhanced chemioluminescence (Amersham).

RESULTS AND DISCUSSION

LMW-PTP binds weakly to activated PDGF-R. NIH3T3 cells were transfected with genes coding for both wild-type and the mutated (C12S) inactive LMW-PTP, using the previously described procedure (9). PDGF-R was isolated from NIH3T3-LMW-PTPC12S cells by immunoprecipitation with anti-PDGF-R antibodies. 4×10^6 cells were PDGF-BB stimulated and the obtained immunoprecipitated receptor was used for LMW-PTP *in vitro* binding assay (11). Lane 2 of fig. 1A shows that the anti-PDGF-R antibodies cause the precipitation of the target antigen linked to LMW-PTP. We have also demonstrated that washing this immunoprecipitate three times with the binding buffer completely removes the LMW-PTP from the immunoprecipitates (see lane 3 of fig. 1A). This suggests that PDGF-R and LMW-PTP interact weakly, since so few washes are sufficient to remove LMW-PTP from the immunoprecipitates. Tyrosine kinase receptors bind many molecules in response to the stimulation of the agonists; these bonds are frequently mediated by interactions between phosphorylated tyrosines and protein domains like SH2 domains (12) and a novel class of binding regions called PTB domains (13). The binding of LMW-PTP to PDGF-R is

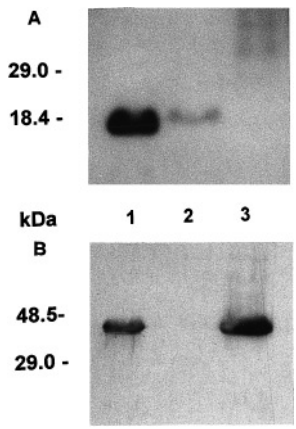

FIG. 1. Panel A: lysates from 1×10^6 cells were immunoprecipitated with 1 μg of anti-PDGF receptor antibodies. Immunoprecipitates were run on SDS PAGE, transferred onto nitrocellulose membrane and probed with anti-LMW-PTP polyclonal antibodies; lanes 1:200 ng of purified LMW-PTP as control; lane 2: anti-PDGF-R antibodies immunoprecipitated from NIH3T3-LMW-PTPC12S cells; lane 3: anti-PDGF-R antibodies immunoprecipitated from NIH3T3-LMW-PTPC12S cells, washed several times with binding buffer. Panel B: anti-LMW-PTP antibodies western blot of GST-LMW-PTPC12S *in vitro* binding assay. Lane 1: 200 ng of purified GST-LMW-PTPC12S as control; lane 2: PDGF receptor from unstimulated cells incubated with 1 μg of GST-LMW-PTPC12S; lane 3: PDGF receptor from PDGF-BB stimulated cells incubated with 1 μg of GST-LMW-PTPC12S.

not mediated by this kind of protein-protein interaction since the phosphatase completely lacks the above-mentioned domains. In order to identify the nature of the binding of the two molecules, we performed a PDGF-R purification by immunoprecipitation from 4×10^6 NIH3T3-LMW-PTPC12S cells either PDGF-BB stimulated or not. After several washes in binding buffer, we incubated the phosphorylated and native receptors with 1 μg of GST-LMW-PTPC12S fusion protein at 4°C for 30 minutes. In this *in vitro* experiment we have utilized the fusion protein to discriminate between the endogenous LMW-PTP and the exogenous one. Results are shown in fig. 1B: the non-phosphorylated PDGF-R does not bind to GST-LMW-PTPC12S (lane 2), while the phosphorylated receptor is able to bind *in vitro* to the GST-LMW-PTPC12S (lane 3). We underline that in this blot the LMW-PTP is not present since it has been removed by the washing with binding buffer. These *in vitro* results fit very well with our previous findings on *in vivo* LMW-PTP receptor binding: in fact, in both cases, the phosphatase is able to bind the receptor only in its phosphorylated, and thus activated, form.

LMW-PTP dephosphorylates the activated PDGF-R in vitro. In order to confirm the hypotheses of a direct LMW-PTP enzymatic action on Tyr-phosphorylated PDGF-R, we performed an *in vitro* assay between immunoprecipitated PDGF-R and LMW-PTP. For this purpose we purified GST-LMW-PTP from bacterial cultures by affinity chromatography and we tested its enzymatic activity on the activated PDGF-R. The anti-phosphotyrosine antibodies probed western blot (presented in fig. 2A) shows that the enzyme is able to dephosphorylate the phosphorylated receptor *in vitro.* This result is concordant with previous findings of Ramponi et al. (14) and Berti et al. (unpublished results) concerning the capacity of natural LMW-PTP to dephosphorylate (*in vitro*) activated EGF-R and PDGF-R respectively.

Competitive inhibitors prevent LMW-PTP association with the PDGF-R. We have previously demonstrated that the PDGF-R interacts *in vivo* with LMW-PTP forming a complex that can be immunoprecipitated either with the anti-PDGF-R or anti-LMW-PTP antibodies (9). Fig. 3 shows two hypotheses to explain the formation of the complex. The first (A) refers to the formation of the Michaelis enzyme-substrate complex between LMW-PTP and the target phosphotyrosine present on the activated PDGF-R. The second hypothesis concerns the existence of an adaptor that mediates the interaction between the PDGF-R and LMW-PTP. This adaptor protein could recruit the phosphatase to the plasma-membrane, facilitating its catalytic action on the receptor target phos-

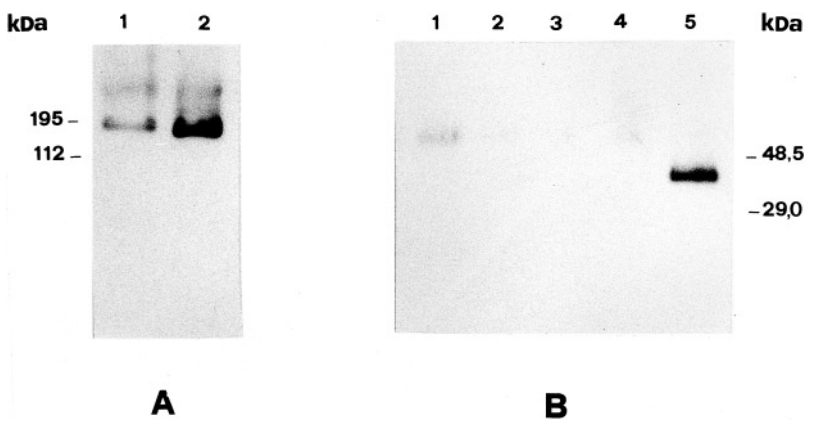

FIG. 2. Panel A: in vitro hydrolytic activity of GST-LMW-PTP on phosphorylated PDGF-R. The reaction mixture was run on SDS-PAGE, transferred onto nitrocellulose membrane and probed with anti-LMW-PTP polyclonal antibodies. Lane 1: PDGF-R with GST-LMW-PTP in the reaction mixture. Lane 2: PDGF-R as control. Panel B: in vitro binding experiment between the GST-LMW-PTPC12S and the phosphorylated PDGF-R. The western blot was probed with anti-LMW-PTP polyclonal antibodies. Lane 1 and lane 2: in presence, respectively, of 50 and 200 mM orthophosphate. Lanes 3 and 4: in presence, respectively, of 1 and 3 mM orthovanadate. Lane 5: 200 ng of purified GST-LMW-PTPC12S as control.

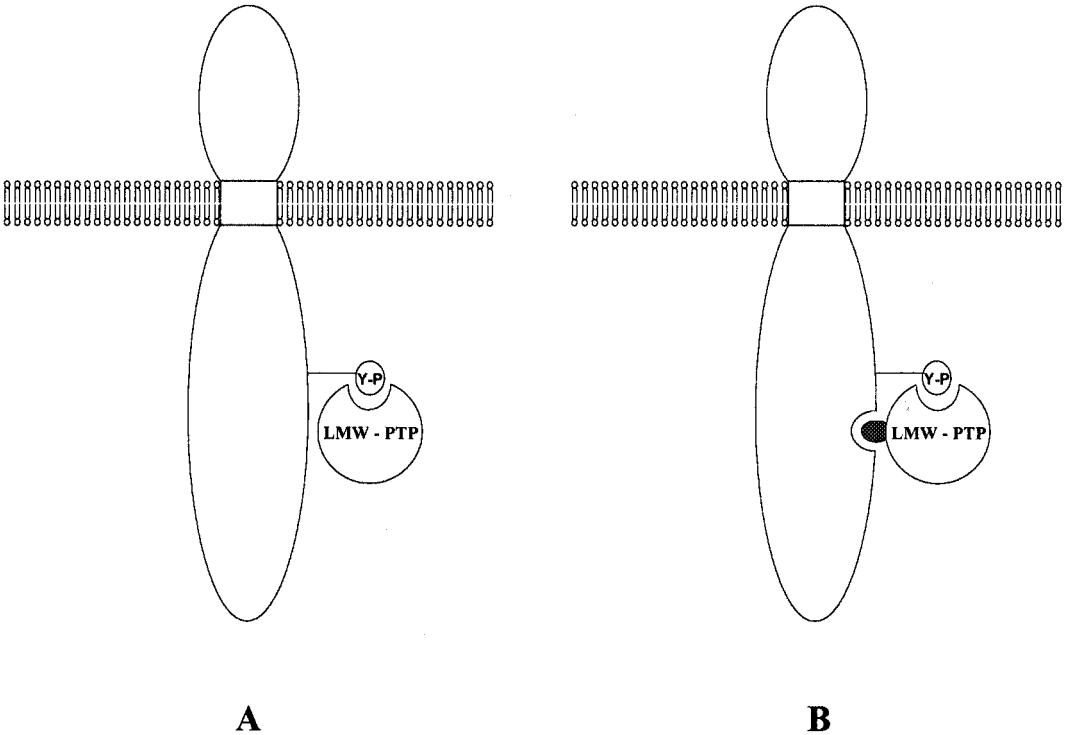

FIG. 3. Hypothetical association between the PDGF-R and LMW-PTP.

photyrosine. Inorganic phosphate and orthovanadate have been shown to competitively inhibit LMW-PTP with a Ki of respectively 2 mM and 0.03 mM (15). To test the action of these two inhibitors on the LMW-PTP binding with the phosphorylated receptor, we perform a series of *in vitro* binding assays with immunoprecipitated receptors and the GST-LMW-PTPC12S fusion protein in presence of Pi (50 and 200 mM) or orthovanadate (1 and 3 mM). Western blot of the obtained samples was probed with anti-LMW-PTP antibodies and is shown in fig. 2B.

Results clearly demonstrate that when either of the competitive inhibitors is present (lane 1 to 4), the association between LMW-PTP and phosphorylated PDGF-R is completely abolished. This is a further indication of a direct interaction between the receptor and LMW-PTP, performed via the phosphatase catalytic site (fig. 3 hypothesis A).

Hence, our results strongly suggest that the interaction between LMW-PTP and the PDGF-R does not involve any adapter protein, but instead performs directly via the LMW-PTP catalytic site. This agrees very well with our previous *in vivo* findings (9). The result of this interaction is the dephosphorylation of activated PDGF-R phosphotyrosine/s and, consequently, a negative regulation of the mitogenic signal starting with the activation of the PDGF-R.

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