New perspectives in PDGF receptor downregulation: the main role of phosphotyrosine phosphatases

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Summary

Uncontrolled activation of receptor tyrosine kinases (RTKs) is implicated in the proliferation of cancerous cells, and deficiencies in RTKs results in pathological conditions such as developmental abnormalities and immunodeficiencies. Tight regulation of RTK cascades is therefore critical for eliciting an appropriate type and level of response to external stimuli. The aim of this work is to compare different RTK downregulation mechanisms, such as ligand-induced internalisation, ubiquitin-mediated proteolysis and dephosphorylation by protein phosphotyrosine phosphatase (PTPs). We choose platelet-derived growth factor receptor (PDGF-r) in NIH3T3 cells as a model of RTK. Our data suggest that PDGF-r internalisation could be mainly considered as a positive signaling system, as it is involved in MAPK activation rather than a downregulation of the mitotic signal. Inhibition of receptor ubiquitination does not result in regulation of PDGF-r tyrosine phosphorylation and does not lead to variation of intracellular signalling pathways. The overall PDGF-r protein degradation upon PDGF stimulation does not exceed 30-40% of the total receptor; thus the receptor remains functionally active for further stimulation. On the contrary, PTP-dependent dephosphorylation of the activated receptors appears to play a crucial role. In fact, inhibition of PTP upon PDGF stimulation results in upregulation of receptor phosphorylation level, of PI3K recruitment and activation and of cell cycle rate. On the contrary, PTP-dependent dephosphorylation does not affect the endosomic pool of activated receptor. Furthermore, we demonstrate that PDGF-r downregulation by means of PTP dephosphorylation is important for both short term (2 hours) and long-lasting (up to 8 hours) PDGF-r activation. Herein we propose a revisited model of PDGF-r downregulation in which PTPs dephosphorylation retains a major role, conferring on receptor internalisation a signal transduction function.

Key words: Phosphotyrosine phosphatases, Tyrosine kinase receptor downregulation, Endocytosis, Ubiquitination, Redox regulation

Introduction

PDGF is a potent mitogen for mesenchymal cells, smooth muscle cells and glial cells. Binding of PDGF to the extracellular domain of the receptor is thought to induce receptor dimerisation, allowing transphosphorylation of adjacent dimerised receptors on specific tyrosine residues within the intracellular region. The phosphorylated tyrosines serve as docking sites for recruitment of cytosolic signalling proteins with appropriate binding motifs. The cellular function of receptor-associated proteins is believed to be modified as a consequence of association with, and phosphorylation by, the receptor itself. Thus, ligand binding initiates intracellular signalling events involving the synthesis of second messenger molecules, activation of small G proteins, protein phosphorylation cascades and ultimately gene transcription. All these signalling pathways are thought to mediate biological responses to PDGF through cell cycle progression (Heldin et al., 1998). The mechanism used to fine-tune receptor-induced signalling and the full diversity of the signalling potential of the receptor remain unclear. It has been demonstrated that PDGF-r expression is downregulated during the second phase of the cell cycle and during cell differentiation. In particular, PDGF-r downregulation is most probably regulated at two levels: (1) at a short time level, which is thought to be controlled essentially by an immediate ligand-induced internalisation of ligand-receptor complexes, and (2) at a long lasting level, when downregulation response is caused by receptor degradation by ubiquitin-dependent proteolysis (Joazeiro et al., 1999) and reduction of PDGFβ-r mRNA expression (Oster et al., 2000). Internalisation of the PDGF-r upon stimulation with the agonist is one of the earliest responses among those elicited by PDGF. This internalisation is mediated by clathrin-coated pits in which the 100 kDa GTPase dynamin is responsible for the pinch-off step of the vesicle. Internalised receptors can recycle back to the cell surface or can be sorted to lysosomal degradation (Joly et al., 1995). Only this latter process results in a downregulation of cell surface receptors. Since mutations that impair the ability of some tyrosine kinase receptors to be internalised and degraded cause cell transformation, receptor downregulation by ligand-induced internalisation appears to be an important element in the control of cellular proliferation. It is not clear what the mechanism guiding the internalisation is, although it has been demonstrated to depend on tyrosine phosphorylation of the receptor itself. Both Src-tyrosine-kinase- and phosphatidylinositol-3-kinase-binding (PI3K) sites on PDGF-r

have been shown to be involved in receptor internalisation (Mori et al., 1994; Shpetner et al., 1996). Furthermore, it has been reported that inhibition of insulin receptor internalisation leads to a partial inhibition of Shc phosphorylation and MAPK inhibition (Ceresa et al., 1998; Faure et al., 1999; Chow et al., 1998). These findings indicate that the internalised insulin receptors have a positive signal transduction role, at least in Shc and MAPK roads.

c-Cbl, one of the numerous SH2-domain-containing proteins that bind to the activated PDGF-r has been recently identified as a proteasomal E3-like ubiquitin ligase involved in the downregulation of tyrosine kinase receptors (Levkowitz et al., 2000). C-Cbl ubiquitination is reported to target receptors for degradation by the proteasome (Joazeiro et al., 1999; Bao et al., 2000). By this, cells became less sensitive to further PDGF stimulation. Both ligand-induced internalisation and c-Cbl-mediated ubiquitination require tyrosine phosphorylation of the receptor and can be considered downregulation mechanisms that terminate receptor signalling through degradation of the molecule (Miyake et al., 1998; Levkowitz et al., 1999).

The long-term downregulation response for PDGF-r signalling is essentially mediated by messenger RNA expression reduction. It has been recently demonstrated that the repression of *pdgf-r* mRNA level is mediated by the c-Myc transcription early response gene. In particular, Myc-repression of PDGF-r expression is responsible for the downregulation of the receptor during the S and G2/M phase of the cell cycle (Oster et al., 2000).

Downregulation of tyrosine kinase receptors by phosphotyrosine phosphatase (PTPs) could be considered a simpler and faster way with respect to the removal of the activable receptors from the membrane by clathrin-mediated endocytosis and to the decrease of PDGF-r molecules by ubiquitin-mediated or lysosomal proteolysis, or mRNA repression (Östmann and Böhmer, 2001). Very little is known about the role of PTPs in PDGF-r dephosphorylation, although many PTPs interact with this receptor. In particular, the PTPs SHP-1 (Yu et al., 1998), SHP-2 (Qi et al., 1999), DEP-1 (Kovalenko et al., 2000), LMW-PTP (Chiarugi et al., 1995) and CD45 (Way and Mooney, 1993) have been described to dephosphorylate the activated PDGF-r, although the real meaning of this dephosphorylation is largely unclear. In fact, PTP action on tyrosine kinase receptors could lead to a general attenuation of receptor signalling by targeting all receptor phosphotyrosines or only the regulatory ones or, instead, by modulating signalling through site-selective dephosphorylation. In this context, we would like to elucidate the mechanisms of downregulation of tyrosine kinase receptors that follows their agonist-induced activation. In particular, we would like to compare the rate and the potency of each of the above mentioned mechanisms.

Materials and Methods

Materials

Unless specified, all reagents were obtained from Sigma. NIH-3T3 cells were purchased from ATCC; human recombinant platelet-derived growth factor BB (PDGF-BB) was from Peprotech; Enhanced Chemi-Luminescence Kit was from Amersham; all antibodies were from Santa Cruz, except those against P-Tyr-716, p85PI3K, which were from Upstate Biotechnology Inc. and those against P-Tyr751 and P-Tyr857, which were a generous gift of A. Kazlauskas (Schepens Eye Research Institute, Harvard Medical School, Boston, MA). BCA protein assay reagent is from Pierce. DCF-DA was obtained from Molecular Probes. GST-PLCγ1 fusion protein was a generous gift of A. Kazlauskas.

Cell culture and transfections

NIH-3T3 cells were routinely cultured in DMEM with the addition of 10% fetal calf serum in 5% CO_2 humidified atmosphere. All cell treatments were performed at 37°C. 10 μg of pRcCMV-C12A-LMW-PTP was transfected into NIH-3T3 cells using the calcium phosphate method (Chiarugi et al., 1995). Stable transfected clonal cell lines were isolated by selection with G418 (400 μg/ml). Mock-transfected cell lines were obtained by transfecting 2 μg of pRcCMVneo alone. The clonal lines were screened for expression of the transfected genes by (a) northern blot analysis and (b) ELISA using polyclonal anti-LMW-PTP rabbit antibodies.

Immunoprecipitation and western blot analysis

1×10^6 cells were seeded in 10 cm plates in DMEM supplemented with 10% FCS. Cells were serum starved for 24 hours before receiving 30 ng/ml PDGF-BB. Cells were then lysed for 20 minutes on ice in 500 μl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% Na-Deoxicholate, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenyl-methanesulphonyl-fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin). Lysates were clarified by centrifugation and immunoprecipitated for 4 hours at 4°C with 1-2 μg of the specific antibodies. Immune complexes were collected on protein A Sepharose (Pharmacia), separated by SDS/PAGE and transferred onto nitrocellulose (Sartorius). Immunoblots were incubated in 3% bovine serum albumin, 10 mM Tris/HCl pH 7.5, 1 mM EDTA and 0.1% Tween-20 for 1 hour at room temperature, probed first with specific antibodies and then with secondary antibodies conjugated with horseradish peroxidase, washed and developed with the Enhanced Chemi-Luminescence kit.

Cell adhesion on fibronectin

1×10^6 cells were seeded in 10 cm plates in DMEM supplemented with 10% FCS. Cells were serum starved for 24 hours before detachment with 0.25% trypsin for 1 minute. Trypsin was blocked with 0.2 mg/ml soybean trypsin inhibitor, centrifuged at 900 ***g*** for 10 minutes and then re-suspended in 2 ml/10 cm dish of DMEM containing 0.2% BSA. Resuspended cells were maintained in suspension with gentle agitation for 30 minutes at 37°C and then directly seeded in pre-coated dishes treated overnight with 10 μg/ml of human fibronectin in phosphate buffered saline (PBS), then washed twice in PBS and blocked for two hours with 2% BSA in PBS.

PTP activity assay

The PTP activity was measured as previously reported (Bucciantini et al., 1999). Briefly, 1.5×10^6 cells were collected in 300 μl of 0.1 M sodium acetate pH 5.5, 10 mM EDTA, 1 mM β-mercaptoethanol then sonicated for 10 seconds. The lysates were clarified by centrifugation, and 50 μl was used in the PTP activity assays. We measured PTP activity either by using p-nitrophenylphosphate or ^{32}P-autophosphorylated PDGF-r. In the first case, the cellular lysates were mixed with 250 μl of 10 mM p-nitrophenylphosphate at 37°C for 30 minutes. The production of p-nitrophenol was measured colorimetrically at 410 nm. The results were normalised on the basis of total protein content. Quantification of PTP activity using ^{32}P-autophosphorylated PDGF-r as the substrate was achieved by mixing the cellular lysates with equal amounts of immunoprecipitated ^{32}P-

labeled PDGF-r and incubating at 37°C for 15 minutes. Immunoprecipitated PDGF-r was labeled with [γ-^{32}P]ATP (3000 Ci/mmol) in 100 μl of the kinase buffer (see kinase activity assay) containing 100 nM PDGF-BB for 30 minutes at 30°C. The residual radioactivity on PDGF-r was assessed by SDS-PAGE analysis. The autoradiogramm of the dried gel was analysed by densitometric scanning (Chemi-Doc Biorad) and normalisation of the data was achieved by an anti-PDGF-r immunoblot of the analysed samples.

Measurement of intracellular ROS

NIH3T3 cells were plated in 24-well plates (Corning) at a density of 2×10^4 cells per well in standard culture medium. 24 hours later the cells were serum starved for an additional 24 hours. PDGF at 30 ng/ml was added for the indicated time followed by a 5 minute incubation with 20 μg/ml DCF-DA, an oxidation-sensitive fluorescent dye. Cells were then detached from the substrate by trypsinisation and analysed immediately by flow cytometry using a Becton Dickinson FACSCAN flow cytometer equipped with an Argon laser lamp (FL-1; emission, 480 nm; band pass filter, 530 nm) (Pani et al., 2000).

PDGF-r internalisation by trypsinisation

PDGF-r internalisation was determined by measuring the percentage of receptors that were resistant to trypsinisation according to the protocol of Ceresa et al. (Ceresa et al., 1998). Briefly, cells were serum starved for 24 hours and then stimulated with 30 ng/ml of PDGF-BB at 37°C. The cells were washed twice with PBS and incubated on ice for 8 minutes with ice-cold, 20 mM sodium acetate, pH 3.7. The cells were washed with ice-cold PBS and incubated with trypsin (1 mg/ml in PBS) in ice for 30 minutes with occasional rocking. The reaction was stopped by addition of soybean trypsin inhibitor (1 mg/ml). Cells were then washed and solubilised in lysis buffer for 10 minutes at 4°C. The cell lysates were immunoprecipitated with anti-PDGF-r antibodies and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The trypsin-resistant 190 kDa PDGF-r is the endosomic receptor. Total PDGF-r amount was evaluated in parallel in non-trypsinised cells and the ratio of the two values has been calculated as 10%.

Inhibition of clathrin-mediated endocytosis

In order to inhibit PDGF-r endocytosis, NIH3T3 cells were incubated in hypertonic medium (medium supplemented with sucrose 0.2 M) for 15 minutes at 37°C as reported previously (Lukacs et al., 1997). Cells are then treated with PDGF-BB 30 ng/ml and subjected to subsequent analysis.

PDGF-r kinase activity assay

Cell lysates in lysis buffer were subjected to immunoprecipitation with anti PDGF-r subunit antibodies (Santacruz Biotec.). The immunoprecipitated proteins were washed twice in lysis buffer and twice in 50 mM Tris-HCl, pH 7.4, containing 1 mM sodium orthovanadate and finally resuspended in 50 mM Hepes at pH 7.4, 10 mM $MnCl_2$. The reaction was started with the addition of [^{32}P]ATP (3000 Ci/mmol) (20 μCi) to all samples, which were then incubated at 4°C for 10 minutes. The beads were then washed once with 50 mM Tris-HCl, pH 7.4, and finally resuspended in 20 μl of Laemmli's sample buffer, boiled for 5 minutes and separated by a 7.5% SDS-PAGE (Sorkin et al., 1993). The autoradiogram was scanned using Chemidoc Quantity One software (Biorad). Normalisation was achieved by an anti-PDGF-r immunoblot using a small amount of each of the analysed samples. In addition the PDGF-r kinase activity was assayed using an exogenous substrate, glutathione S-transferase-PLC-γ (DeMali et al., 1997). Immunoprecipitates were incubated in the presence of 20 mM PIPES, pH 7.0, 10 mM $MnCl_2$, 20 μg/ml aprotinin and 10 μCi of [γ^{32}-P]ATP (3000 Ci/mmol) for 10 minutes at 30°C in the presence or absence of 0.5 μg of glutathione *S*-transferase-PLCγ1. The fusion protein included amino acid residues 550-850 of rat PLCγ1. The reaction was stopped by adding an equal volume of 2×sample buffer (10 mM EDTA, 4% SDS, 5.6 mM 2-mercaptoethanol, 20% glycerol, 200 mM Tris-HCl, pH 6.8 and 1% bromphenol blue). The samples were then incubated for 3 minutes at 95°C, spun and resolved on 7.5% SDS-polyacrylamide gel electrophoresis, and the radiolabeled proteins were detected by autoradiography. Normalisation was achieved by an anti-PLCγ1 immunoblot using a small amount of each of the analysed samples.

Phosphatidylinositol 3-kinase assay

The PI3K assay was performed as described elsewhere (Marra et al., 1995; Chodhury et al., 1991). Briefly, serum-starved cells were incubated with 30 ng/ml of PDGF for 10 minutes and then lysed in RIPA buffer. Equal amounts of proteins were immunoprecipitated using PY20 anti-phosphotyrosine antibody (Santacruz Biotec.). After washing, the immunobeads were resuspended in 50 μl of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.5 mM EGTA. 0.5 μl of 20 mg/ml phosphatidylinositol was added, mixed and incubated at 25°C for 10 minutes. 1 μl of 1 M $MgCl_2$ and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) were then added simultaneously and incubated at 25°C for an additional 10 minutes. The reaction was stopped by the addition of 150 μl of a mixture of chloroform, methanol and 37% HCl (relative volumes 10:20:0.2). The samples were extracted with chloroform and dried. Radioactive lipids were separated by thin-layer chromatography using chloroform, methanol, 30% ammonium hydroxide and water (relative values 46:41:5:8). After drying, the plates were autoradiographed. The radioactive spots corresponding to phosphatidylinositol phosphate were scraped and counted in a liquid scintillation counter.

Cell cycle analysis

Cytofluorimetric analysis was performed according to Liu et al. (Liu et al., 1995). Briefly, 2×10^5 cells were seeded into 6 cm dishes the day before analysis to obtain exponentially growing cultures. Cells were rinsed twice with cold PBS and harvested after trypsinization in 1 ml of 50 mg/liter propidium iodide. Analysis was performed in a Becton Dickinson FACScan using the Lysis II and Cell Fit cell analysis software according to the manufacturer's procedure. Cell cycle rate evaluation was performed on five independent dnLMW-PTP overexpressing clones (n=4 for each clone).

Results

Time course of PDGF-r downregulation

For this study we chose PDGF-r as a model of tyrosine kinase receptors and the NIH3T3 cell line for their high level of endogenous receptor expression. First of all we report the time course of tyrosine phosphorylation of PDGF-r in NIH3T3 cells upon stimulation with 30 ng/ml of PDGF-BB (Fig. 1A,B). In our experimental conditions, the tyrosine phosphorylation level reaches its maximum 10 minutes after stimulation and decreases within 2 hours. The decrease in PDGF-r tyrosine phosphorylation level would be achieved either by PTP-dependent dephosphorylation or by a decrease of receptor number, owing to receptor degradation. The internalisation and trafficking of ligand-activated cell-surface receptor has long been reported as the main downregulation mechanism for many receptors (Leof, 2000). The ligand-bound receptors are internalised in clathrin-coated veshicles and are either recycled back to the membrane or sorted to lysosomes for degradation

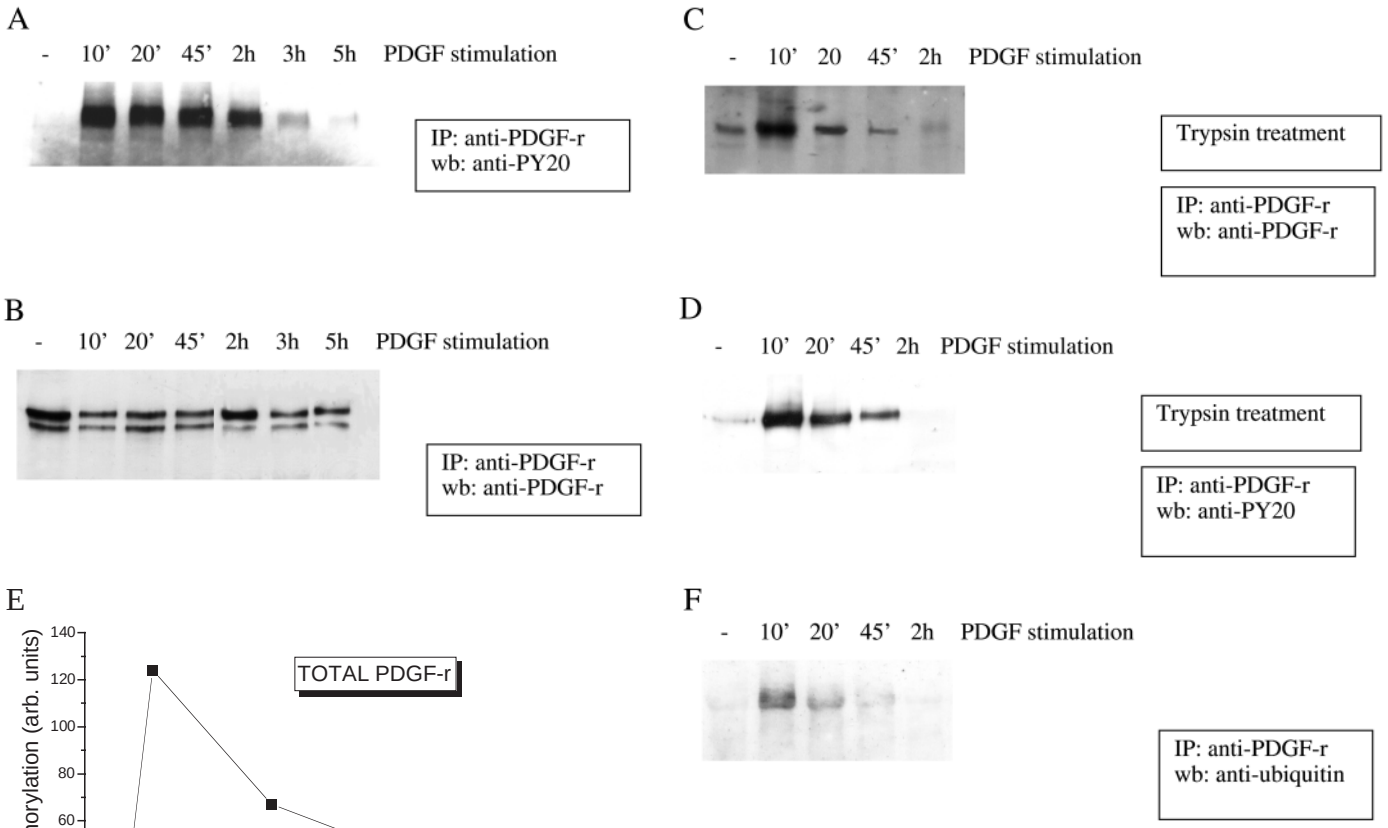


Fig. 1. PDGF-r tyrosine phosphorylation, internalisation and ubiquitination during a time-course agonist stimulation. 1×10^6 NIH3T3 cells were serum starved for 24 hours and then stimulated with 30 ng/ml PDGF-BB. PDGF-r was immunoprecipitated from lysates. (A) An anti-phosphotyrosine immunoblot is shown. The blot was stripped and probed again with anti PDGF-r antibody (B). PDGF-r internalization was evaluated by trypsin treatment as reported in the Materials and Methods. In C, an anti-PDGF-r immunoblot is shown and in D the anti-phosphotyrosine stripping and reprobing is reported. In E, the plots of the normalised tyrosine phosphorylation level of total PDGF-r and endosomic PDGF-r are reported (n=4). In F, the immunoprecipitated total PDGF-r was probed with anti-ubiquitin antibodies.

(Edinger et al., 1999). It has been reported that the internalisation of tyrosine kinase receptors depends on tyrosine phosphorylation of the receptor cytoplasmic domain and that this phosphorylation mainly targets activated receptors to lysosomal degradation (Baker et al., 1990; Lohse, 1993). We analysed the level of receptor internalisation by trypsin treatment of agonist-stimulated cells. This method allows a direct analysis of receptor endocytosis by means of trypsin-directed proteolysis of the membrane-exposed receptor. The endosomic receptors are in fact the only ones retaining the original molecular weight, whereas the receptor molecules, which remain at the cell surface, are cleaved by trypsin. Our results (Fig. 1C) indicate that receptor internalisation shows a kinetic activity similar to receptor tyrosine phosphorylation, with a maximum 10 minutes after stimulation and then a rapidly decrease. Tyrosine phosphorylation of the endosomic receptor (Fig. 1D) increases within 10 minutes of the stimulation, in agreement with previous observation by other groups (Joly et al., 1995; Baker et al., 1990; Lohse, 1993). The level of tyrosine phosphorylation of the internalised receptor does not decrease as rapidly as that of the membrane-bound receptor, instead it remains high for at least 2 hours. Fig. 1E shows the plot of the phosphorylation level of total PDGF-r compared with that of the endosomic receptor.

Furthermore, it has been reported that PDGF-r downregulation could be achieved by proteasome-mediated degradation of the ubiquitinated receptor (Miyake et al., 1998).

We analysed the time course of PDGF-r ubiquitination in PDGF-stimulated NIH3T3 cells and the results are shown in Fig. 1F. Again, PDGF-r labelling with ubiquitin follows a kinetic pattern similar to its tyrosine phosphorylation, showing a maximum 10 minutes after stimulation and slowly decreasing thereafter.

Furthermore, in order to study the fate of PDGF-r upon stimulation in NIH3T3 cells, we analysed the amount of total PDGF-r using a time course experiment with PDGF stimulation. The result (Fig. 2A) showed that the receptor level decreases by about 30% within 10 minutes of stimulation and then increases to 90% of the level seen in unstimulated cells (as indicated by densitometric analysis). Hence, it is likely that the decrease in the total amount of PDGF-r in Fig. 2A during agonist stimulation is only marginally caused by protein degradation. In any case the amount of downregulation of PDGF-r by physical removal from the cell surface and/or degradation is only a modest phenomenon. In order to confirm that the number of receptors that physically remain in the cell is relevant with respect to the amount of receptor eliminated by protein degradation, we analysed the ability of PDGF-r to be restimulated after a first 3 hour long stimulation and the removal of agonist by acid wash. The results (Fig. 2B) indicate that after the first treatment lasting 3 hours, PDGF-r is completely restimulated and shows a tyrosine phosphorylation level almost comparable with the original. A PDGF dose-response curve in NIH3T3 indicates that 3 ng/ml of the growth factor saturates the response. Using 30 ng/ml of PDGF in both treatments, we can exclude the possibility that the second increase in tyrosine phosphorylation of PDGF-r was caused by a limiting dose of PDGF during the first stimulation. Taken together these data suggest that the physical degradation of PDGF-r during agonist stimulation is only a marginal phenomenon and that the main part of the receptor is still able to be activated after the decrease in its tyrosine phosphorylation.

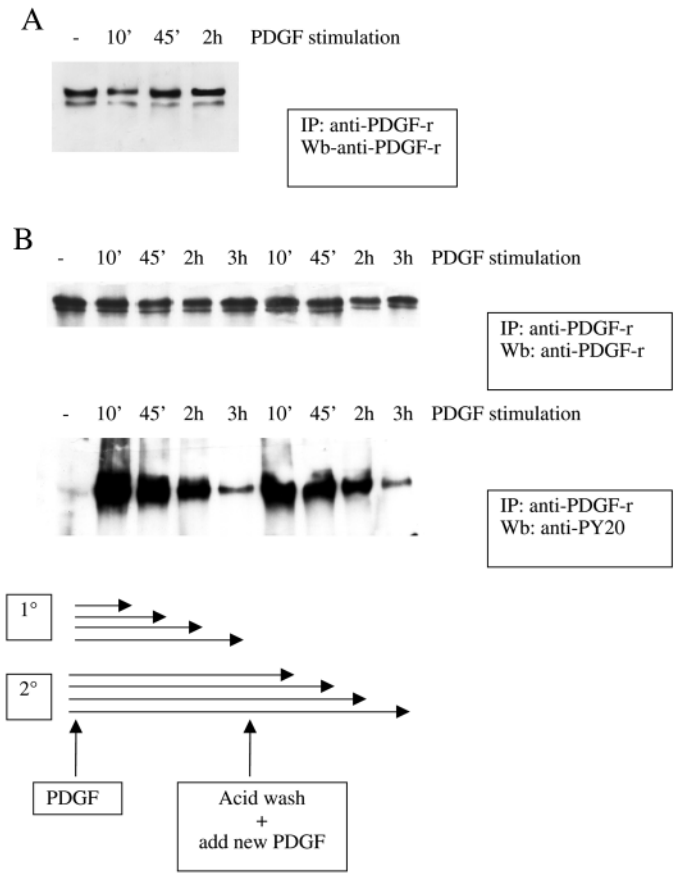


Fig. 2. Amount of PDGF-r upon agonist stimulation that is able to be restimulated. 1×10^6 NIH3T3 cells were serum starved for 24 hours and then stimulated with 30 ng/ml PDGF-BB. PDGF-r was immunoprecipitated from lysates. (A) Anti-PDGF-r immunoblot. (B) Cells were stimulated with PDGF-BB during two time courses for 10 minutes, 45 minutes, 2 hours and 3 hours for the first set, and for 3 hours, before being washed and restimulated again for 10 minutes, 45 minutes, 2 hours and 3 hours for the second set. The antiphosphotyrosine immunoblot and the anti-PDGF-r normalisation are shown.

Relevance of different downregulation mechanisms in PDGF-r tyrosine phosphorylation

Evaluation of the time course of each receptor downregulation mechanism would allow us to discover which one is the most relevant for mitotic regulation. Unfortunately, receptor dephosphorylation, receptor internalisation and ubiquitination have a similar time course, each having a maximum activity 10 minutes after stimulation and a reduced activity thereafter. The use of specific inhibitors of each mechanism could allow us to elucidate which is the most potent in terms of PDGF-r downstream signalling inactivation. In particular, we used pervanadate in order to block PTP activity in NIH3T3 cell upon PDGF treatment and MG-132 to selectively block ubiquitin-mediated protein degradation. The block in clathrin-mediated endocytosis of PDGF-r was achieved by treatment with a hypertonic medium containing 0.2 M sucrose (Lukaks et al., 1997). The tyrosine phosphorylation level of total PDGF-r is shown in Fig. 3A; we demonstrate that only the block in dephosphorylation with pervanadate leads to a long lasting hyperphosphorylation of the receptor. The block in clathrin-mediated endocytosis leads to only a small delay in the kinetic activity of receptor activation, but not an upregulation, whereas the block in proteasomal degradation is ineffective in receptor switching off. On the contrary, PTP inhibition by pervanadate does not affect the tyrosine phosphorylation of the endosomic receptor (Fig. 3B), which was analysed after trypsin treatment. MG132 proteasome degradation inhibitor is efficient in increasing the amount of ubiquitinated, but not degraded, PDGF-r. (Fig. 3C, right panel). Similarly, hypertonic medium pretreatment of cells almost completely blocks PDGF-r ligand-induced internalisation, as shown by trypsin treatment (Fig. 3C, left panel). The inability of the block in receptor internalisation and proteasomal degradation to affect the kinetics of PDGF-r activation suggests that amongst the different downregulation mechanisms analysed the most significant is phosphotyrosine dephosphorylation, although receptor internalisation retains a minor role. In addition, it is likely that PTP dephosphorylation has preferential membrane-associated PDGF-r pools as targets.

Relevance of the different downregulation mechanisms in PDGF-r signalling

By means of experiments similar to those described in the

previous section, we evaluated the activation of the main early signal transduction pathways elicited by PDGF, namely the PI3K and the mitogen activated protein kinase (MAPK) pathways, during selective blocking of the downregulation mechanisms of PDGF-r. In particular, we were interested in clarifying the role of receptor internalisation in MAPK activation. In fact there are conflicting data on this point: receptor internalisation is generally considered to be a downregulation mechanism for receptor signalling (Joly et al., 1995), but the endosomic receptor seems to mainly mediate the activation of Shc and Grb2 (Ceresa et al., 1998) and thus to have an intrinsic positive role in signal transduction, at least for insulin receptor. We selectively blocked ubiquitin-mediated degradation of PDGF-r (with MG132), receptor internalisation (with hypertonic medium) or receptor dephosphorylation (with sodium pervanadate). Lysates from these cells, stimulated or not with PDGF, were used to evaluate MAPK activation (Fig. 4A). The results indicate that MAPK activation is considerably reduced by the inhibition of PDGF-r internalisation, whereas it is unaffected by the other treatments. Hence, we propose that neither receptor ubiquitin-mediated degradation nor PDGF-r dephosphorylation are relevant for MAPK activation, as none of the treatments has a positive effect on signalling pathways. On the contrary, receptor internalisation seems to act positively on MAPK activation pathway, as its inhibition by hypertonic medium leads to a dramatic reduction in MAPK activation. In order to exclude the possibility that 0.2 M sucrose suppresses MAPK pathway activation independently from PDGF-r internalisation, we checked its specificity in inhibiting a PDGF-free system, namely integrin-induced MAPK activation (Fig. 4B). The results confirm that the 0.2 M treatment specifically inhibits PDGF-induced MAPK activation, and it is particularly effective on integrin-induced MAPK activation. These data on PDGF-r are in agreement with others for different growth factor receptors, for which it has been demonstrated that the internalised receptor selectively signals in the MAPK pathway (Ceresa et al., 1998; Chow et al., 1998).

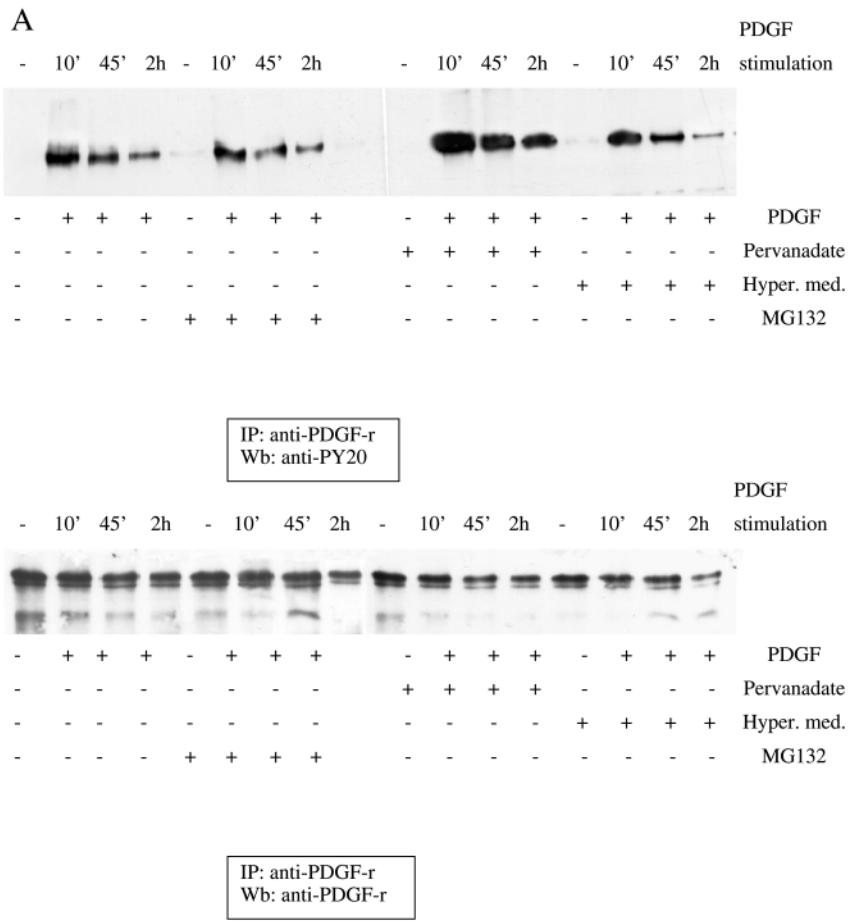
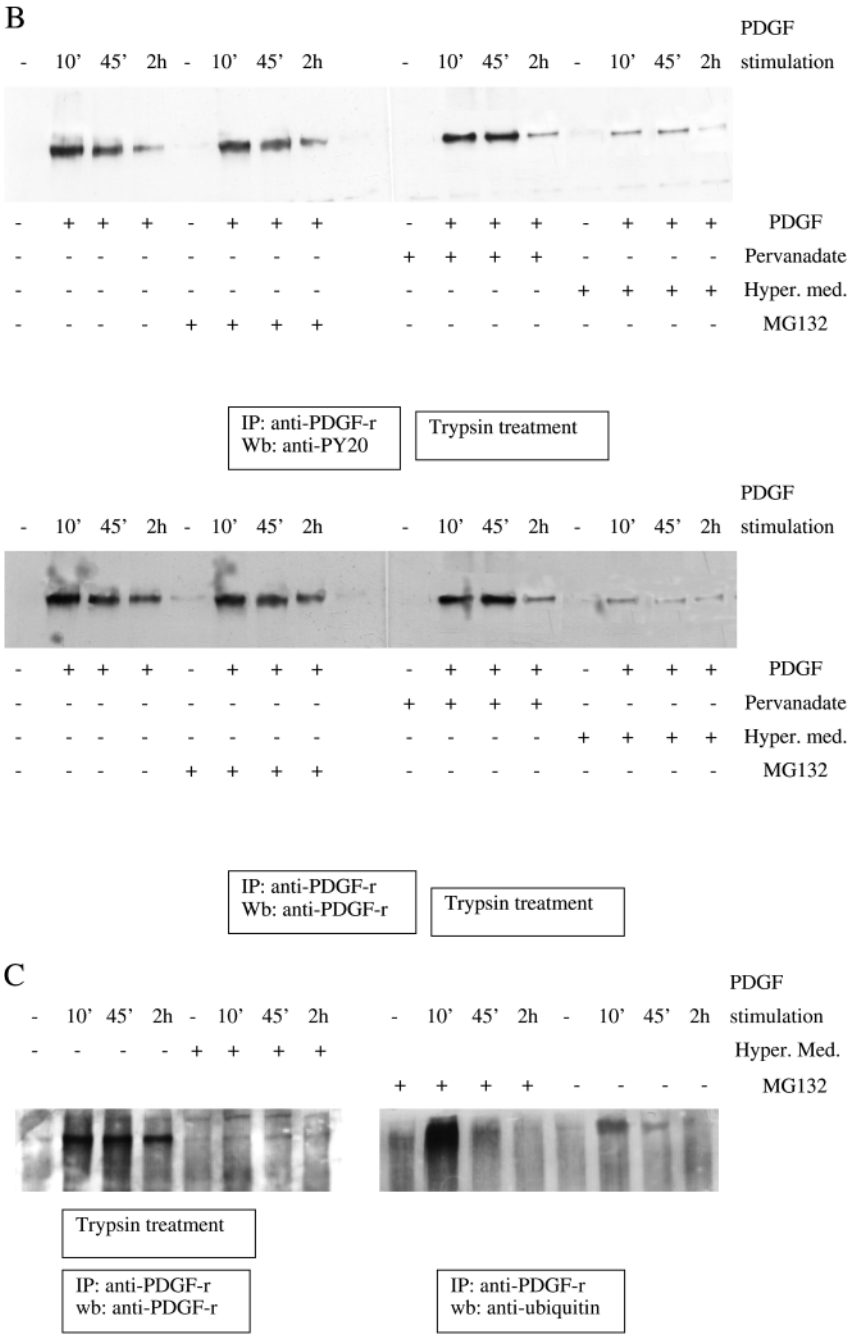


Fig. 3. The effect of PDGF-r downregulation mechanisms on PDGF-r tyrosine phosphorylation. 1×10^6 NIH3T3 cells were serum starved for 24 hours and then stimulated with 30 ng/ml PDGF-BB. (A) The hypertonic medium or 5 μM MG132 was added 30 minutes before stimulation, whereas 0.1 mM pervanadate was added together with the growth factor. PDGF-R was immunoprecipitated from lysates, and an antiphosphotyrosine immunoblot was performed. (B) Cells were treated as in A, and the endosomic PDGF-r pool was analysed by trypsin treatment as reported in the Materials and Methods. PDGF-r was immunoprecipitated from lysates, and an antiphosphotyrosine immunoblot was performed. The blot was stripped and reprobed with anti-PDGF-r antibodies for normalisation. (C) As a control for MG132 inhibition of proteasome-mediated proteolysis of PDGF-r, an anti-PDGF-r immunoprecipitation of PDGF-stimulated cells untreated or treated with MG132 was performed (right). The analysis of the accumulation of ubiquitinated but not the degradation PDGF-r was performed using an anti-ubiquitin immunoblot. The effect of sucrose treatment on the inhibition of clathrin-mediated endocytosis was checked. The endosomic PDGF-r pool was evaluated by trypsin treatment, as reported in the Materials and Methods.

It has been demonstrated that PI3K plays a major role in initiating PDGF-dependent DNA synthesis, whereas other signalling molecules are required only for other PDGF-mediated functions (Rosenkranz et al., 1999). We selectively blocked all PDGF-r downregulation mechanisms, and the lysates from PDGF-treated cells were used for the evaluation of PI3K activity (Fig. 4C). Our data indicate that only in cells treated with pervanadate and PDGF is there an increase in PI3K activity with respect to cells treated with PDGF alone, whereas the other treatments are almost ineffective. Taken together these findings show that the main mitosis-correlated PDGF-induced signal transduction pathway, namely PI3K activation, is influenced only by PTP inhibition. Hence, we propose again that phosphotyrosine dephosphorylation is the most relevant signalling inhibition system, awarding to receptor internalisation a specific and positive role in MAPK signal transduction.

Correlation between PDGF-r tyrosine phosphorylation level and cell cycle rate

In order to analyse the correlation between the tyrosine phosphorylation level of PDGF-r and PDGF-r's main functional role, namely mitosis promotion, we used a dominant-negative PTP to increase the tyrosine phosphorylation level of the



receptor. Dominant-negative PTPs are widely used to study the action of these enzymes (Way and Mooney, 1993; Chiarugi et al., 1995; Kenner et al., 1996; Östmann and Böhmer, 2001) and commonly lead to an increase in tyrosine phosphorylation of their PTP substrates. Many PTPs, such as LMW-PTP (Chiarugi et al., 1995), SHP-1 (Way and Mooney, 1993), DEP-1 (Kovalenko et al., 2000) and others, act on phosphorylated PDGF-r, although in many cases a clear specificity for dephosphorylation sites has not been demonstrated. In this study, we use dominant-negative LMW-PTP (dnLMW-PTP), which we have already reported to greatly increase the tyrosine phosphorylation level of PDGF-r (Chiarugi et al., 1995; Chiarugi et al., 1998). In this work we exclusively use dnLMW-PTP, which is a useful tool to increase the tyrosine phosphorylation level of the PDGF-r. Cells overexpressing dnLMW-PTP were stimulated with PDGF, and the tyrosine phosphorylation level of PDGF-r was evaluated in comparison with mock-transfected cells in a kinetic experiment. The results, shown in Fig. 5A, again show that inhibition of PTP action on the activated receptor leads to a long-lasting increase in its tyrosine phosphorylation, a result comparable to that obtained with pervanadate (Fig. 3A).

The relevance of dephosphorylation of PDGF-r for intracellular signalling pathways leading to G1 phase progression was confirmed by means of dnLMW-PTP overexpression. We evaluated the potency of PDGF signalling by cytofluorimetric analysis of cycling cells upon PDGF treatment during the block of each of the receptor downregulation mechanisms. NIH3T3 cells were serum starved for 24 hours and then stimulated with PDGF for 15, 17 and 20 hours. Unfortunately, treatment for a complete cell cycle with pervanadate, hypertonic medium and MG132 does not permit cell survival. The use of dnLMW-PTP allowed us to demonstrate that the difference in tyrosine phosphorylation level of PDGF-r in dnLMW-PTP-expressing cells and mock-transfected ones leads to a more rapid entry into G2/M phase, thus determining a decrease in cell cycle rate (Fig. 5B).

Taken together, these data suggest that the potency of the signal elicited by PDGF-r is proportional to its tyrosine phosphorylation level, which, in turn (Fig. 3A,B), is influenced mainly by PTPs and marginally by receptor internalisation but not by ubiquitination.

PDGF-r tyrosine phosphorylation regulation

The tyrosine phosphorylation level of PDGF-r during the 2 hour time course may be regulated by the rate of phosphorylation and dephosphorylation. In this light, we analyzed the kinase activity of PDGF-r and PTP activity during a 2 hour time course of PDGF stimulation. The results of an in vitro PDGF-r kinase assay using immunoprecipitated PDGF-r from agonist-stimulated cells is reported in Fig. 6A. The kinase activity of PDGF-r shows a slow decrease during the first 2 hours, suggesting that the rate of receptor activity does not remain constant over the 2 hour stimulation with PDGF. Pervanadate treatment of NIH3T3 cells results in a rapid upregulation of PDGF-r kinase activity 10 minutes after stimulation, but not at longer times (Fig. 6A). PDGF-r kinase activity on an exogenous substrate such as PLCγ1 was monitored during pervanadate treatment of cells (Fig. 6B), and results in agreement with the PDGF-r autophosphorylation assay were obtained. This finding suggests a role for PTPs in the regulation of the receptor activity only at very short times of agonist stimulation. Kazlauskas proposes that although the kinase activity of the receptor is

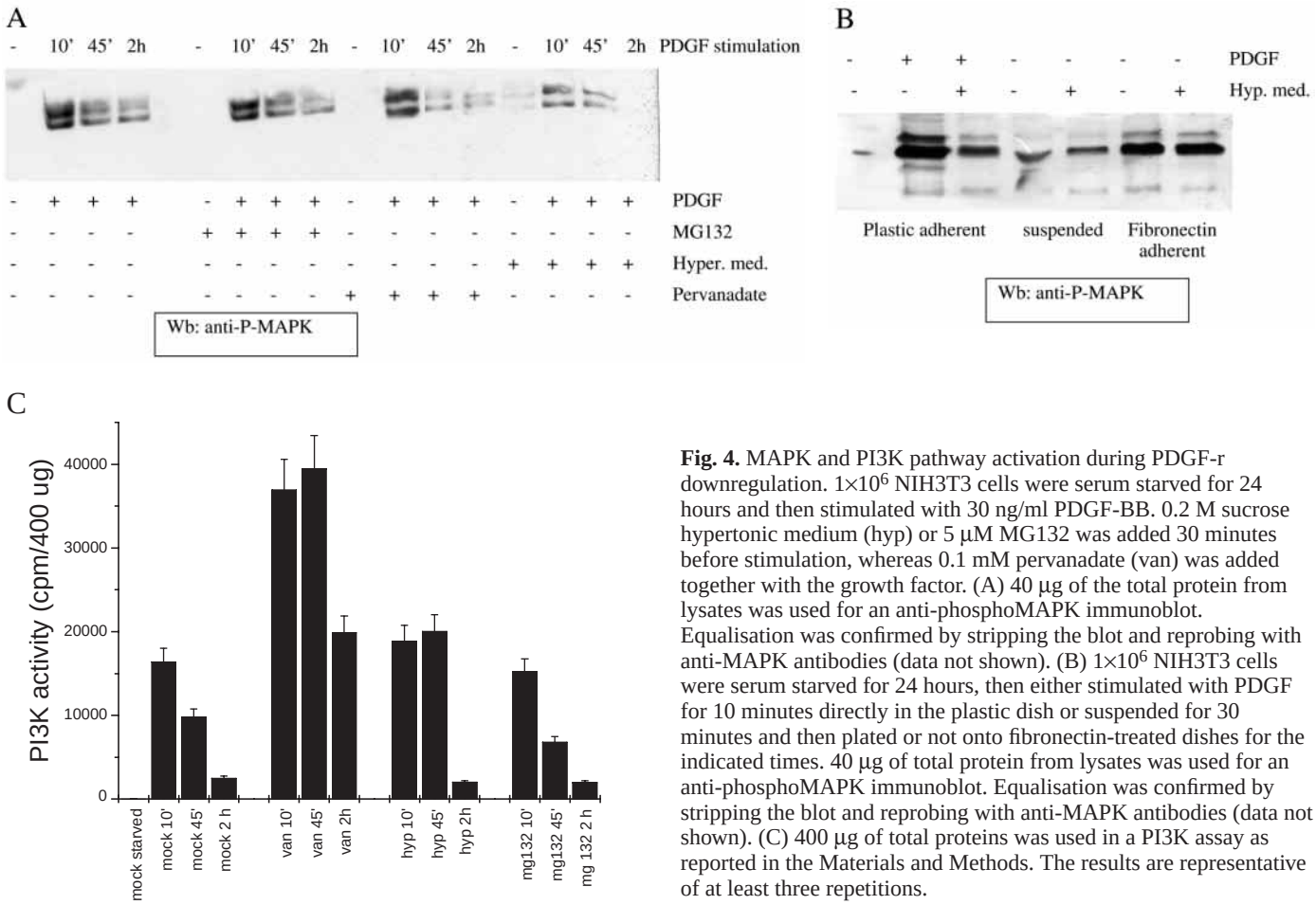


Fig. 4. MAPK and PI3K pathway activation during PDGF-r downregulation. 1×10^6 NIH3T3 cells were serum starved for 24 hours and then stimulated with 30 ng/ml PDGF-BB. 0.2 M sucrose hypertonic medium (hyp) or 5 μM MG132 was added 30 minutes before stimulation, whereas 0.1 mM pervanadate (van) was added together with the growth factor. (A) 40 μg of the total protein from lysates was used for an anti-phosphoMAPK immunoblot. Equalisation was confirmed by stripping the blot and reprobing with anti-MAPK antibodies (data not shown). (B) 1×10^6 NIH3T3 cells were serum starved for 24 hours, then either stimulated with PDGF for 10 minutes directly in the plastic dish or suspended for 30 minutes and then plated or not onto fibronectin-treated dishes for the indicated times. 40 μg of total protein from lysates was used for an anti-phosphoMAPK immunoblot. Equalisation was confirmed by stripping the blot and reprobing with anti-MAPK antibodies (data not shown). (C) 400 μg of total proteins was used in a PI3K assay as reported in the Materials and Methods. The results are representative of at least three repetitions.

regulated within the first 10 minutes of stimulation, other receptor functions persist for longer times (Bernard and Kazlauskas, 1999). We analysed the tyrosine phosphorylation level of PDGF-r Tyr-857 (by means of anti-phospho-Y857-PDGF antibodies), which is required for full activation of the receptor kinase activity (Baxter et al., 1998), in pervanadate-treated cells. The results, shown in Fig. 6C, indicate that the phosphorylation of this residue is extremely transient, reaching its maximum after 10 minutes and completely disappearing thereafter. Hence, it is likely that PTPs affect receptor kinase activity only after 10 minutes, because the phosphorylation of the tyrosine residue required for activation of receptor kinase activity is available only during the first 10 minutes.

PTP activity could be a housekeeping process or a finely regulated phenomenon. In particular, the downregulation of the PDGF-r tyrosine phosphorylation should caused by redox regulation of PTP activity during the stimulation process (Östmann and Böhmer, 2001; Chiarugi et al., 2001). First, we analyzed the PDGF-induced reactive oxygen species (ROS) production in NIH3T3 cells (Fig. 7A), which reaches its maximum 10 minutes after stimulation and rapidly declines within 20 minutes. These preliminary data are consistent with the current hypothesis of a redox regulation of many PTPs by growth factor ROS production (Östmann and Böhmer, 2001). In this light, we analysed the PDGF-r tyrosine phosphorylation level in a kinetic experiment during inhibition of ROS production. For this purpose we treated cells either with catalase (Fig. 7B), which reduces the intracellular H_2O_2 concentration, or with diphenyl-iodide (DPI) (Fig. 7C), a strong and specific inhibitor of NADPH oxidase. The results indicate that the inhibition of ROS production leads to a dramatic decrease in tyrosine phosphorylation of PDGF-r. In addition, we checked that neither catalase nor DPI influenced the tyrosine kinase activity of the receptor (data not shown). We further verified that DPI and catalase treatments greatly reduced the redox inactivation of PTPs during PDGF stimulation: for this purpose we use pNPP (Fig. 7D) and an in vitro phosphorylated PDGF-r (Fig. 7E) as substrates. It is likely that the decreased ROS concentration in both catalase and DPI-treated cells leads to a general upregulation of PTPs, which are not downregulated via oxidation, thus remaining fully active on phosphorylated PDGF-r.

On the basis of these data, we suggest that the tyrosine phosphorylation level of PDGF-r is regulated positively by the increase in its tyrosine kinase activity only during the first 10 minutes, and the activity rapidly decreasing thereafter. By contrast, PDGF-r phosphorylation level is negatively controlled by PTPs, which act on both its tyrosine kinase activity and on the general tyrosine phosphorylation level.

PDGF-r tyrosine phosphorylation is regulated in both of its distinct phases of signaling

Recently, it has been demonstrated that prolonged and continuous exposure to growth factors is required to commit

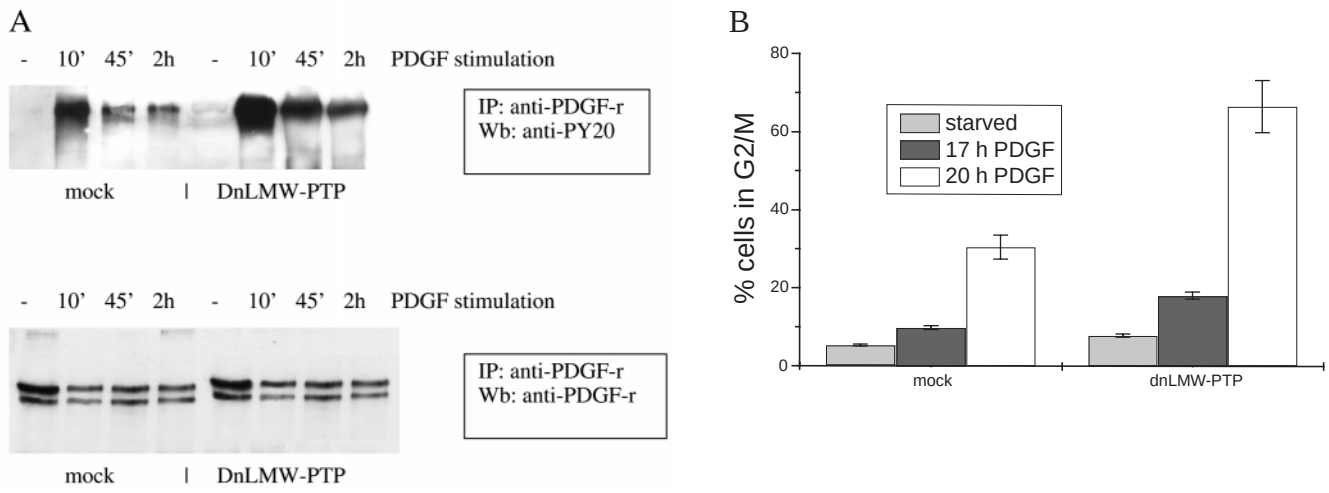


Fig. 5. Cell cycle analysis in dnLMW-PTP-expressing cells. (A) 2×10^5 dnLMW-PTP- or mock-transfected NIH3T3 cells were serum starved for 24 hours and then stimulated with 30 ng/ml PDGF-BB for the indicated times. Cells were then lysed and stained with propidium iodide for cytofluorimetric cell cycle analysis with Cell FIT (Becton Dickinson) software. The results are representative of several repetitions (n=4 for each of five independent clones). (B) 1×10^6 dnLMW-PTP- or mock-transfected NIH3T3 cells were serum starved for 24 hours and then stimulated with 30 ng/ml PDGF-BB. PDGF-R was immunoprecipitated from lysates, and an antiphosphotyrosine immunoblot was performed. The blot was stripped and reprobed with anti-PDGF-r antibodies for normalisation. The results are representative of at least three experiments.

cells to the cell cycle (Jones and Kazlauskas, 2001). In particular, the requirement for continuous exposure to mitogens can be replaced by two short pulses of growth factor: one for 30 minutes at time zero and the second at 8 hours. These extremely important data give rise to the hypothesis that the PDGF receptor may remain tyrosine phosphorylated for a very long time, as PDGF-activated signalling molecules continue to signal up to 8 hours. This hypothesis is supported by the data of Bernard et al., which demonstrated that the phosphorylation of the different tyrosines in PDGF-r is temporal and spatial controlled (Bernard and Kazlauskas, 1999), thus suggesting that although the kinase activity of the receptor is regulated within the first 10 minutes of stimulation, other receptor functions, and in particular PI3K activation, persist for longer times. We analysed the tyrosine phosphorylation level of PDGF-r in NIH3T3 cells in a 0-9 hour time course experiment. The results, reported in Fig. 8A, suggest that PDGF-r remains tyrosine phosphorylated for up to 8 hours, although it reaches its maximum level after 10 minutes and then remains at lower levels. It is likely that although after a very short time the receptor becomes phosphorylated at nearly all its tyrosines, fewer tyrosines remain phosphorylated after a longer time, thus giving a general anti-phosphotyrosine immunoblot signal lower than at 10 minutes.

In order to study PDGF-r downregulation during prolonged growth factor stimulation, we first analysed the amount of PDGF-r in a 0-9 hour time course experiment. Fig. 8B shows that the level of total PDGF-r decreases at 10 minutes, probably because of degradation, but then remains almost constant for the next 9 hours. These preliminary data suggest that the long-lasting downregulation of PDGF-r is not mediated by protein degradation either lysosomal or proteasomal. The role of PTPs in the long-lasting PDGF-r downregulation can be elucidated by pervanadate-treated cells or by dnLMW-PTP-expressing cells. Unfortunately 8-9 hours of pervanadate treatment is not compatible with cell life. By contrast, dnLMW-PTP expression gives rise to interesting results: the tyrosine phosphorylation level of PDGF-r is greatly influenced by LMW-PTP inhibition both at short and longer times (Fig. 9A), although it is more pronounced at the shortest times. We suggest that LMW-PTP acts on phosphorylated PDGF-r throughout the time that it remains phosphorylated and not only for the initial phase of signalling.

The main role of the PI3K pathway downstream of the second pulse (8 hours) of PDGF stimulation has been unequivocally demonstrated by Jones et al. (Jones et al., 1999). In fact, synthetic PI3K lipid products can entirely and uniquely mimic the second pulse of PDGF stimulation. To confirm our findings for the role PTP in the later phase of signalling, we analysed the behaviour of the PI3K pathway throughout a 9 hour time course of PDGF stimulation. We analysed the PI3K activity in a time course experiment (Fig. 9B) in cells expressing dnLMW-PTP in comparison with mock-transfected cells. The results reveal that PTP inhibition leads to enhanced PI3K activity in both the immediate (10 minute) and the tardier (5-8 hours) waves of phosphoinositide production. Taken together, these findings suggest that PTPs work throughout the period of PDGF-r phosphorylation, probably acting on the specific residue/s that remains phosphorylated for up to 8 hours.

Discussion

Ligand binding to plasma membrane receptors initiates a series of events culminating in a variety of changes in cellular phenotypes. The specific and precise signalling of tyrosine kinase receptors requires that both the intensity and the duration of the elicited signals be tightly regulated. Although numerous publications have documented the activation pathways of membrane receptors and signalling molecules, relatively few investigations have focused on downregulation mechanisms, which terminate the signal elicited by growth-factor–receptor interaction. The current view reports that

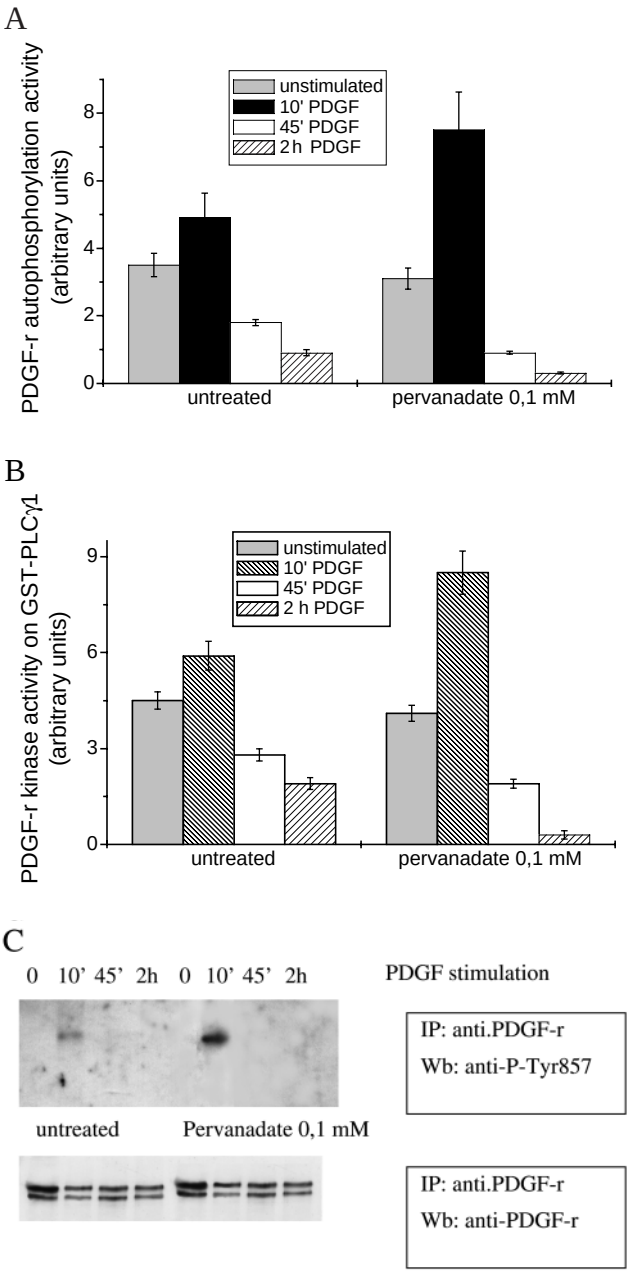


Fig. 6. PDGF-r kinase activity during PTPs inhibition. (A,B) 1×10^6 NIH3T3 cells were serum starved for 24 hours and then stimulated with 30 ng/ml PDGF-BB and with the growth factor together with 0.1 mM pervanadate. PDGF-r was immunoprecipitated from lysates, and an immunokinase assay was performed as reported in the Materials and Methods. PDGF-r autophosphorylation kinase activity is reported in A and PDGF-r kinase activity towards GST-PLCγ1 is reported in B. The ratio between the densitometric analyses of kinase assays and normalization blots (anti PDGF-r or anti-PLCγ1 immunoblots) is shown in both A and B. (C) 1×10^6 NIH3T3 cells were serum starved for 24 hours and then stimulated with 30 ng/ml PDGF-BB and with the growth factor together with 0.1 mM pervanadate. PDGF-r was immunoprecipitated from lysates and an anti-phospho-Tyr857 immunoblot was performed. Equalisation was checked by stripping the blot and reprobing with anti-PDGF-r antibodies. The results are the means of at least three experiments.

receptor endocytosis is the main downregulation mechanism (Leof, 2000). Perhaps the first evidence supporting a clearing function for the endocytotic system was the finding that failure of downregulation of epidermal growth factor receptors results in increased signalling and in some cases enhanced tumorigenesis (Wells et al., 1990). Although there is some evidence favouring growth factor signalling in the absence of significant endocytic activity, there is now a similar body of literature supporting a role for endosomes in either initiating or extending the signal elicited at the plasma membrane (Kranenburg et al., 1999; Vieira et al., 1996; Burke et al., 2001). Clathrin-coated vesicles derived from agonist-mediated endocytosis could be recycled back to the plasma membrane or targeted to the lysosomal compartment. The real downregulation of growth factor receptors induced by endocytosis could be mediated by lysosomal degradation of the endocytic veshicle. C-Cbl-mediated ubiquitination of activated PDGF-r also terminates signalling by marking active receptors for degradation (Joazeiro et al., 1999; Miyake et al., 1999). The ubiquitinated receptors are targeted to the proteasomal machinery for destruction. In *Caenorhabditis elegans* the *c-cbl* homologue gene has been genetically defined as a negative regulator of RTKs (Yoon et al., 1995), and in mice pre-B lymphomas and myeloid leukemias have been correlated with the viral oncogene *v-cbl* (Langdon et al., 1989). Furthermore c-Cbl overexpression enhances the ubiquitination and degradation of PDGF-r (Miyake et al., 1998). Finally, RTK downregulation could be achieved by the orchestrated action of PTPs, which terminate the output signals by dephosphorylating the activated receptors (Östmann and Böhmer, 2001). Among the PTPs that act on RTK, we include LMW-PTP (Chiarugi et al., 1995), SHP-1 (Way and Mooney, 1993), SHP-2 (Qi et al., 1999), DEP-1 (Kovalenko et al., 2000), PTP1B (Flint et al., 1997) and some others. It appears that there is no selectivity amongst PTPs, that is, many PTPs can interact with more than one RTK and vice versa (Östmann and Böhmer, 2001), although cell- and tissue-specific expression of PTPs and RTK may provide a level of selectivity.

The aim of this work was to compare the different downregulation mechanisms acting on PDGF-r. In particular, we wanted to distinguish between the efficiency of transient molecular modifications of PDGF-r leading to the downregulation of membrane-bound receptors or the destruction of the PDGF-r molecule by one of the possible proteolysis mechanisms. The data presented herein, using tyrosine kinase receptors as a model, suggested that the kinetics of tyrosine phosphorylation of PDGF-r are comparable with its ligand-induced internalisation and with its ubiquitination (Fig. 1A,C,D). The coexistence of both the peak of activation of the signal (i.e. the tyrosine phosphorylation of the PDGF-r) and of the maximum of endocytosis and ubiquitination could support the hypothesis of a signalling role for both mechanisms instead of them being termination systems. By contrast, ligand-induced PDGF-r degradation, either obtained by clathrin-mediated endocytosis and lysosomal delivery or ubiquitin-elicited proteasomal proteolysis, is actually a system to limit the availability of transducing receptors. Our data suggest that PDGF-r destruction is a marginal phenomenon occurring either for a

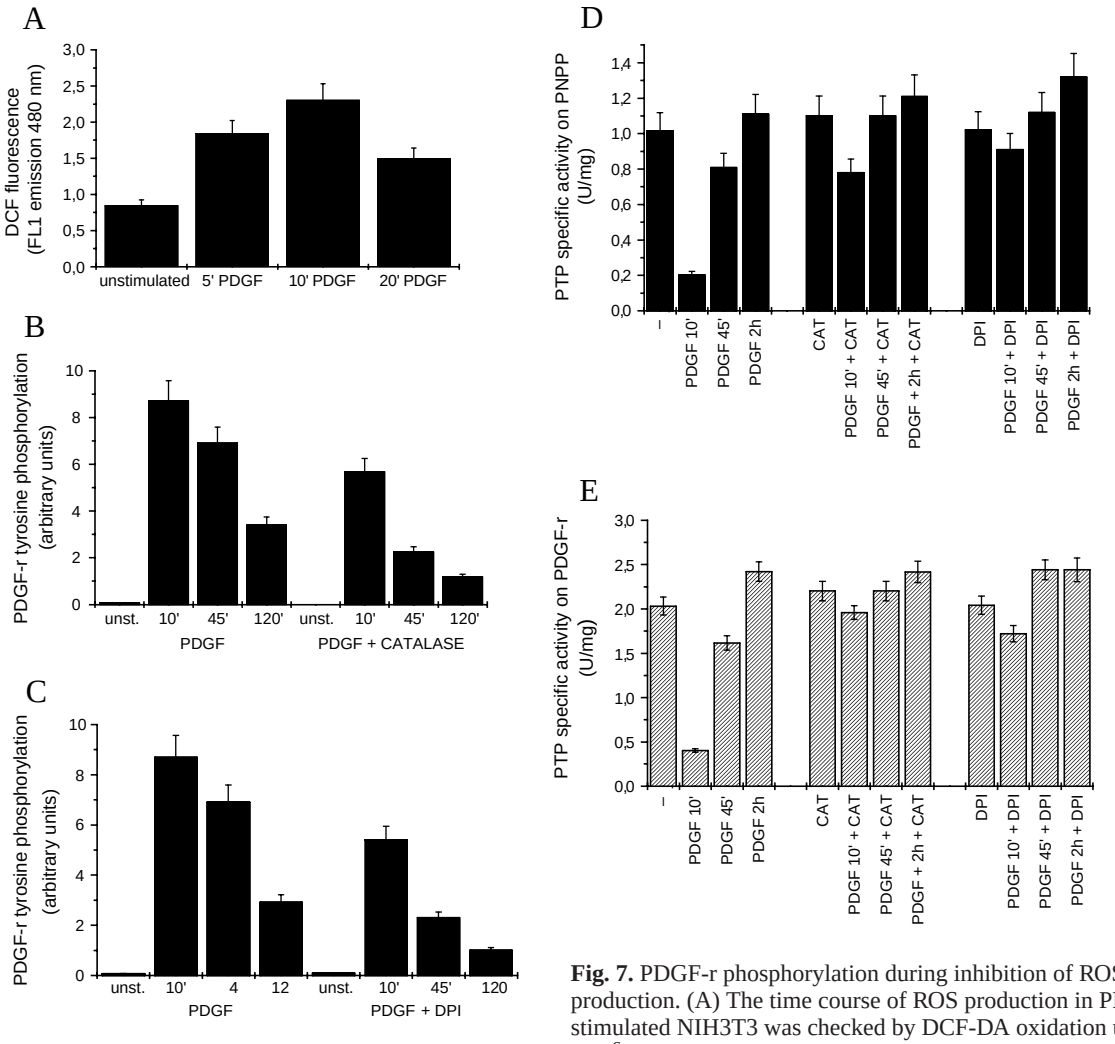


Fig. 7. PDGF-r phosphorylation during inhibition of ROS production. (A) The time course of ROS production in PDGF-stimulated NIH3T3 was checked by DCF-DA oxidation using cytofluorimetric analysis as reported in the Materials and Methods. (B,C) 1×10^6 NIH3T3 cells were serum starved for 24 hours, pretreated or untreated with 1 μg/ml catalase (B) or 50 μM DPI (C) and then stimulated with 30 ng/ml PDGF-BB. PDGF-r was immunoprecipitated from lysates, and an antiphosphotyrosine immunoblot was performed. Equalisation was checked by stripping the blot and reprobing with anti-PDGF-r antibodies. The ratios of the densitometric analyses of anti-phosphotyrosine and anti-PDGF-r signals are reported in B and C. (D,E) PTP assay of lysates from PDGF-stimulated cells. Cells were pretreated or untreated with DPI or catalase for 30 minutes and then stimulated for the indicated times with PDGF-BB. PTP activity on PNPP or on in vitro ^{32}P-autophosphorylated PDGF-r is shown in D and E, respectively. The results are representative of at least three independent experiments.

short time (Fig. 2A) or for longer, lasting growth factor stimulation (Fig. 9B). We evaluated the reduction in the amount of total PDGF-r during agonist treatment, and found it to be reduced by 30%: this decrease could not account for the strong decline in PDGF-r tyrosine phosphorylation (Fig. 1A) and of intracellular signalling pathway activation (Fig. 3A, Fig. 4A,B). In addition, we demonstrated that the main part of the receptor remains available for further stimulation (Fig. 2B). Our data could not completely exclude a possible refilling of membrane-exposed receptors with newly synthesised PDGF-r, thus contributing to the total amount of PDGF-rs. It has been reported that upon agonist stimulation, PDGF-r expression is repressed during the G1 phase by the c-Myc transcription factor (Oster et al., 2000). For this reason we suppose that receptor refilling could contribute only marginally to receptor availability after agonist stimulation. The use of selective inhibitors for anyone of the PDGF-r signalling termination systems point towards PTPs as the main regulators of PDGF-r tyrosine phosphorylation level, although they do not exclude a minor role for receptor endocytosis. Inhibition of ubiquitination has almost no effect on PDGF-r tyrosine phosphorylation, although blocking of clathrin-mediated endocytosis causes a small delay in PDGF-r activation. Furthermore, our data suggest that ligand-induced PDGF-r internalisation retains its role in MAPK pathway signal transduction. These data are in agreement with previous reports on inhibition of clathrin-mediated insulin receptor endocytosis (Ceresa et al., 1998), which demonstrate a selectivity in attenuation of Shc tyrosine phosphorylation and MAPK activation. In addition, Burke has recently demonstrated that epidermal growth factor signalling is regulated by endocytosis and intracellular trafficking (Burke et al., 2001). Taken together these data suggest that the modifications that PDGF-r undergoes during agonist stimulation do not involve protein destruction (i.e. lysosome delivery after internalisation or ubiquitin-mediated proteolysis is not needed) but leave the

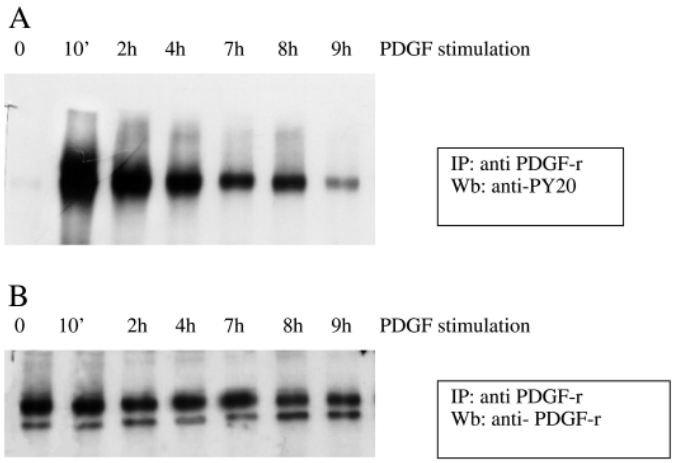


Fig. 8. PDGF-r and PDGF-r phosphorylation persist for many hours. 1×10^6 NIH3T3 cells were serum starved for 24 hours then stimulated with 30 ng/ml PDGF-BB for the indicated times. PDGF-r was immunoprecipitated from lysates and an antiphosphotyrosine immunoblot was performed (A). The blot was stripped and reprobed with anti-PDGF-r antibodies (B).

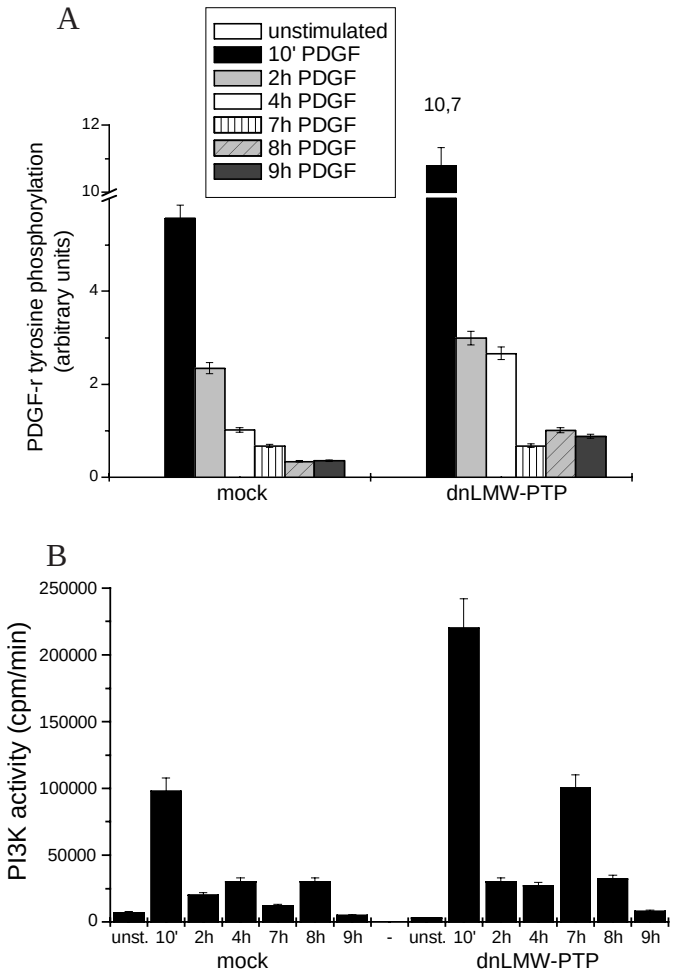


Fig. 9. PTP inhibition affects long lasting PDGF signaling. 1×10^6 NIH3T3 cells were serum starved for 24 hours, then stimulated with 30 ng/ml PDGF-BB for the indicated times. (A) PDGF-r was immunoprecipitated from lysates, and an antiphosphotyrosine immunoblot was performed. Equalisation was checked by stripping the blot and reprobing with anti-PDGF-r antibodies. The ratio between the densitometric analyses of anti-phosphotyrosine and anti-PDGF-r signals is shown. (B) Lysates were used for the PI3K assay as reported in the Materials and Methods.

receptor molecule at the plasma membrane available for further activation.

Receptor tyrosine phosphorylation is a key event in the regulation of many intracellular transduction pathways. SH2-containing proteins involved in signal transduction are recruited to the plasma membrane through their selective interaction with different phosphotyrosines in the activated receptors. The inhibition of PTP action, either achieved with pervanadate treatment (Fig. 3A) or with dnLMW-PTP ectopic expression (Fig. 5A) causes an increase in the tyrosine phosphorylation of PDGF-r. This increase causes an upregulation of the PI3K pathway but not of the MAPK route and leads to an increase in G1 cell cycle phase rate (Chiarugi et al., 1998). The importance of the PI3K pathway for mitotic entry has already been reported by Kazlauskas in 1999 (Rosenkranz et al., 1999). They showed that PDGF-dependent DNA synthesis was strictly dependent on only one of the receptor elicited signalling routes, PI3K. Many others enzymes are essentially required for other PDGF-mediated function, such as chemotaxis. In this light, MAPK activation might depend on signalling from the endosomic receptors, whereas PI3K recruitment and activation and consequently mitotic entry, rely on tyrosine phosphorylation of plasma membrane receptors.

The tyrosine phosphorylation level of a particular RTK is given by the ratio between its intrinsic tyrosine kinase activity and the coordinated activity of PTPs. The receptor tyrosine kinase activity is believed to be controlled by agonist-induced dimerisation of the receptor and by the transphosphorylation of a regulatory tyrosine, Tyr857, located in an activation loop (Kazlauskas and Cooper, 1989). Recent data suggest that inhibition of PTP activity occurs after RTK dimerization and thereby also contributes to receptor tyrosine phosphorylation levels. Oxidation of the active site cysteine residue by H_2O_2 has been identified as a mechanism for negative regulation of PTPs (Lee et al., 1998). Interestingly, H_2O_2 is produced after the stimulation of various RTK, such as PDGF-r, epidermal growth factor receptor (EGF-r), insulin receptor and others (Rhee, 2000). Furthermore, EGF-r stimulation is paralleled by a transient oxidation of PTP1B, and PDGF-r is accompanied by a temporary oxidation of LMW-PTP (Chiarugi et al., 2001). Our analysis of the tyrosine kinase activity of the receptor during inhibition of PTPs by pervanadate suggests that PTPs affect the kinase activity of the receptor only for the first 10 minutes, probably by dephosphorylating the kinase loop tyrosine residue Tyr857. After this time, PTPs no longer influence receptor tyrosine kinase activity, in agreement with previous reports that suggest that Tyr857 is only briefly phosphorylated (Bernard and Kazlauskas, 1999; Rosalind et al., 1996). Inhibition of the production of ROS in PDGF-stimulated cells, either achieved by catalase pre-treatment or inhibition of the NADPH oxidase by DPI, leads to the reduction of the potency of the PDGF-elicited signal, that is, a decrease in the tyrosine phosphorylation level of the receptor. The effects of catalase and DPI indicate that inhibition of PTPs

has an important role in RTK signalling and that the rescue of the catalytic activity of PTPs after oxidation is followed by a dephosphorylation of the activated receptor, thus terminating the signal. In view of this, we propose that during the first 10 minutes after stimulation the receptor tyrosine phosphorylation level increases due to its intrinsic kinase activity and inhibition of PTPs via oxidation. By contrast, during the second phase of the stimulation, the phosphorylation level of the receptor declines as a function of the rescue (via reduction) of PTP activity. This dephosphosphorylation closes the signal elicited from the receptor.

Many studies have shown that in order to enter the S phase of the cell cycle, a serum-arrested fibroblast must be continuously exposed to the growth factor for about 2 hours (Westermark et al., 1990). This means that the cell must remain in presence of the growth factor long after all the signalling events correlated with mitosis have occurred. In fact, some signalling enzymes are not only activated acutely by exposure to growth factor but at a later time as well. Kazlauskas have reported that both PI3K and protein kinase C show two waves of activation, one within minutes of stimulation and one between 3 and 9 hours after the addition of growth factors (Jones et al., 1999; Balciunaite et al., 2000). This late phase of enzymatic activity is required for cell cycle progression. In this context, we suppose that PDGF-r could remain phosphorylated for up to 9 hours in order to elicit both the first and the second wave of enzyme activity. Our results show for the first time that the tyrosine phosphorylation of PDGF-r is a long-lasting phenomenon that reaches a maximum 10 minutes after the stimulation of the receptor, then slowly decreases for 3-4 hours but remains at detectable levels for up to 9 hours. Herein we show that PTPs act as terminators of the PDGF-r signal both in the first phase of PDGF-r activation, when all tyrosine residues are phosphorylated and recruit SH2-containing proteins, and in the second phase, when only a few tyrosines remain phosphorylated and the long-lasting signals take place. In fact, dnLMW-PTP expression causes an increase in both PDGF-r short and long-lasting tyrosine phosphorylation and in PI3K first and second activation waves. Kazlauskas demonstrated that the second phase of PDGF required for S phase entry, at about 8 hours, could be mimicked by synthetic PI3K products (Jones and Kazlauskas, 2001) but not by other second messengers. Therefore, PTPs can act on phosphorylated receptors when they are available, either minutes or several hours after stimulation, thus permitting particular signals to last for a longer time than others. These data are in agreement with a recent paper that reports that for epidermal growth factor receptor the loss of tyrosine dephosphorylation temporally precedes receptor degradation (Burke et al., 2001).

It is likely that PTPs affect RTK tyrosine phosphorylation in either a site-selective manner, that is, modulating preferentially some pathways rather than others, or causing a general phosphotyrosine decrease in content, thus causing a termination signal. Both these situations can be mediated by high site specificity of a particular PTP for the receptor, which acts in the first case on signalling phosphotyrosines or, in the second case, on the regulatory tyrosine in the activation loop of the receptor. Furthermore, our data indicate a preference of PTPs for membrane-exposed receptors, as we report that the endosomal receptor pool remains constantly phosphorylated for up to 2 hours (Fig. 1B-D), suggesting that PTPs may exclude this pool of receptors.

In conclusion we propose a model of PDGF-r downregulation in which the level of ligand-induced internalised tyrosine-phosphorylated receptors does not only play a termination role but also is a positive MAPK activation/transduction signal. PDGF-r protein destruction via lysosomal or ubiquitin-mediated proteolysis plays a marginal role, and the main part of the activated receptor is simply dephosphorylated by the concentrated action of PTPs. These enzymes, which are inhibited by the production of ROS owing to growth factor stimulation and reactivated thereafter by physiological thiols, are crucial for terminating the PDGF-r signal through a time-dependent dephosphorylation of available phosphotyrosines, thus contributing to the dynamic properties of PDGF-r signalling.

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