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Research Article

Down-regulation of platelet-derived growth factor receptor signaling during myogenesis

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Abstract. Cell differentiation is often associated with a block in the cell cycle. Growth factor signaling has been reported to be impaired in differentiated cells, due to the withdrawal of growth factors or to transcriptional down-regulation of their receptors. Our proposal is that the down regulation of growth factor signaling may be achieved through an alternative pathway: the decrease of growth factor receptor activation and the ensuing inhibition of intracellular pathways leading the cell to division. Here we report that platelet-derived growth factor receptor (PDGFr) signaling is down-regulated during muscle differentiation, although its expression level remains unchanged. PDGFr signaling inhibition is achieved through a decrease in the receptor tyrosine phosphorylation level, in particular of Tyr716, Tyr751, Tyr857 and Tyr1021, leading to down-regulation of intracellular signaling pathways. Furthermore, during myogenesis, the espression level of several phosphotyrosine phosphatases (PTPs) increases and most of them shift toward the reduced/activated state. We propose a causal link between the down-regulation of PDGFr tyrosine phosphorylation and the increases in PTP specific activity during myogenesis.

Key words. Myogenesis; PDGF signaling; phosphotyrosine phosphatase; reactive oxygen species; redox signaling.

The development of skeletal muscle is a multistep process that involves the determination of pluripotential mesodermal cells to give rise to myoblasts, withdrawal of the myoblasts from the cell cycle and their differentiation into muscle cells, and, finally, growth and maturation of skeletal muscle fibers [1]. At the molecular level, muscle differentiation needs the expression of the skeletal muscle-specific helix-loop-helix MyoD family of proteins, which includes MyoD, myogenin, myf-5 and myocyte regulatory factor 4, and the myocyte enhancer-binding factor 2.
Although the temporal sequence of muscle transcription factor activation is well characterized, little is known about the involvement in myogenesis of the intracellular signaling cascades generated by several growth factors. Growth factors, such as basic fibroblast growth factor, insulin-like growth factor-I (IGF-I) and transforming growth factor-β, are involved in satellite cell proliferation and differentiation [2, 3]. In particular, basic fibroblast growth factor is mitogenic for muscle cells but can also act as a negative regulator of myogenesis by inhibiting the expression and/or activity of muscle-specific transcription factors [4]. Recently, IGF-I and platelet-derived growth factor and PDGF-BB were demonstrated to use distinct signaling pathways to maintain myoblast viability. Indeed, while IGF-I leads to a sustained stimulation of the phosphatidylinositol 3-kinase (PI-3K)/Akt cascade and only a transient activation of the mitogen-activated protein kinase (MAPK) pathway, PDGF-BB causes sus-

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tained stimulation of MAPKs and only transient induction of Akt [5].

Despite evidence that the PDGF receptor (PDGFr) cascade is involved in cell differentiation, there are few reports on PDGFr signaling during muscle differentiation. PDGFr down-regulation has been demonstrated to be an important mechanism for reducing PDGF responsiveness in terminally differentiated 3T3-L1 cells. Indeed, while pre-adipocytes express amounts of α and β PDGFr mRNA and protein similar to those expressed in other murine 3T3 fibroblasts, the expression of both receptor transcripts is greatly reduced in differentiated 3T3-L1 cells [6]. Accordingly, only PDGF signaling is inhibited during adipogenesis, while the insulin pathway is unaffected by terminal differentiation. This effect has been reported mainly for the PI-3K/Akt pathway by the group of Summers et al. [7].

There is evidence that PDGF may act by increasing the number of myoblasts involved in muscle regeneration following muscle trauma, by stimulating proliferation and by inhibiting differentiation of myogenic cells [8]. The same group demonstrated that treatment of C2 myoblasts with PDGF-BB can modulate the transition toward the myogenic lineage [9]. Furthermore, recombinant human PDGF-BB has been reported to act as a mitogen for L6 myoblasts and also as a powerful inhibitor of myogenic differentiation. Indeed, treatment of myoblasts with PDGF-BB increased the rate of DNA synthesis and stimulated cell proliferation, while in differentiation medium, PDGF-BB prevented the fusion of confluent myoblasts and suppressed biochemical differentiation [10].

Receptor tyrosine kinases are a large family of transmembrane-spanning proteins that, upon ligand binding, convey signals from the extracellular environment through the activation of their intrinsic kinase activity. Binding of PDGF to the extracellular domain of its receptor induces receptor dimerization, allowing transphosphorylation of adjacent dimerized receptors on specific tyrosine residues within the intracellular region. These phosphorylated tyrosines provide docking sites for recruitment of cytosolic signaling proteins [11] which initiates intracellular signaling events involving the synthesis of second messenger molecules, activation of small G proteins and the protein phosphorylation cascade and, finally, gene transcription. Among the SH2 domain-containing proteins which interact with the receptor phosphotyrosines are the GTPase-activating protein of Ras, namely RasGAP [12], phospholipase Cγ-1 (PLCγ-1) [13], the p85 subunit of PI-3K [14] and the Src family members associating with tyrosines 579 and 581 in the juxtamembrane region of human β-PDGFr [15, 16]. These signaling pathways mediate biological responses to PDGF through cell cycle progression [17].

The tyrosine phosphorylation level of a receptor tyrosine kinase depends on a balance between its intrinsic tyrosine kinase activity and the coordinated action of phosphotyrosine phosphatases (PTPs). Among PTPs which dephosphorylate PDGFr, we include low molecular-weight PTP (LMW-PTP) [18], Src homology phosphatase 1 [19], Src homology phosphatase 2 [20] and density-enhanced phosphatase 1 [21]. Some of these proteins undergo a reversible negative regulation by hydrogen peroxide which is produced after the stimulation of different tyrosine kinase receptors, such as PDGFr, epidermal growth factor receptor and insulin receptor [22].

In a previous paper, we showed that PDGFr tyrosine phosphorylation greatly decreases in mature myotubes with respect to myoblasts. In these conditions, there is an increase in LMW-PTP which is probably one of the PTPs involved in the down-regulation of β-PDGFr phosphorylation in myotubes [23].

Here, we demonstrate, using a murine muscle C2C12 cell model, that β-PDGFr signaling is greatly inhibited in mature myotubes with respect to myoblasts. In addition, we show that phosphorylation of specific phosphotyrosines of the receptor, such as Tyr751, Tyr716, Tyr857 and Tyr1021, decreases during muscle differentiation, leading to down-regulation of the main mitogenic downstream pathways, namely PI3K/Akt and MAPK routes in differentiated cells. We demonstrate that PDGFr down-regulation is accompanied by a raise in PTP specific activity due to both an increase in the expression level of some PTPs and/or a shift toward the active/reduced form of PTPs, in agreement with the great decrease in reactive oxygen species (ROS) production during myogenesis. These experiments suggest that commitment to the muscle lineage needs the down-regulation of the signaling pathways triggered by β-PDGFr and that PTPs play a relevant role in this inhibitory mechanism.

Materials and methods

Materials

Unless specified all reagents were obtained from Sigma. The C2C12 cell line was a gift from Dr M. Massimino from the University of Padua; human recombinant PDGF-BB and epidermal growth factor were from Peprotech; the Enhanced Chemio-Luminescence kit, protein A-Sepharose, γ-[^{32}P]ATP and Sepharose G-50 column were from Amersham Pharmacia Biotech; the PVDF membrane was from Millipore; anti-PDGFr, anti-Akt, anti-phospho-Akt, anti-phospho-Tyr857, anti-phospho-Tyr1021, anti-p85PI-3K and anti-vinculin antibodies were from Santa Cruz; anti-PY20 antibodies were from BD Transduction Laboratories; anti-MAPK and anti-phospho-MAPK antibodies were from Cell Signaling; phospho-Tyr716 antibodies were from Upstate Biotechnology Inc. while those against phospho-Tyr751 were a generous gift of A. Kazlauskas (Schepens Eye Research Institute, Harvard

Medical School, Boston, Mass.); 5-(and 6)-carboxy-2′,7′-dichloro-dihydrofluorescein diacetate (carboxy-H_2DCFDA) was from Molecular Probes; fetal calf serum was from Eurobio; horse serum was from Gibco BRL.

Cell culture

C2C12 cells were routinely cultured in growing medium (GM) consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a 5% CO_2-humidified atmosphere. Muscle differentiation was induced by switching subconfluent cultures of C2C12 cells from growing medium to differentiating medium (DM) consisting of Dulbecco's modified Eagle's medium supplemented with 2% horse serum.

Immunoprecipitation and Western blot analysis

For PDGF stimulation, cells were serum starved for 24 h before receiving 30 ng/ml PDGF-BB for the indicated times. Cells were then lysed for 20 min on ice in 1 ml of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton, 2 mM EGTA) and a protease inhibitor cocktail (1 mM AEBSF, 8 µM aprotinin, 20 µM leupeptin, 40 µM bestatin, 15 µM pepstatin A and 14 µM E-64). Lysates were then clarified by centrifugation. Total proteins (25 µg) were used in each Western blot experiment, while for immunoprecipitation (performed overnight at 4 °C), each sample was adjusted to a 1 mg/ml concentration in 1 ml of final volume, using 2 µg/ml of specific antibodies. Immunocomplexes were collected on protein A-Sepharose, separated by SDS-polyacrylamide gel electrophoresis, and transferred onto PVDF membrane. Immunoblots were probed first with specific antibodies in 1% bovine serum albumin, 0.05% Tween 20 in TBS buffer (10 mM Tris, pH 7.5, 100 mM NaCl), and then with secondary antibodies conjugated with horseradish peroxidase, washed and developed with the enhanced chemioluminescence kit.

Measurement of intracellular ROS

Intracellular generation of ROS was performed as previously described [24]. Undifferentiated and differentiated cells were treated for 5 min with dichlorofluorescein diacetate (DCF-DA) to a final concentration of 5 μg/ml. Cells were then washed with PBS buffer and lysed in RIPA buffer.

Lysates were clarified by centrifugation and immediately analyzed by fluorescence spectrophotometric analysis using a Perkin Elmer Fluorescence Spectrophotometer 650-10S equipped with a Xenon Power Supply (excitation wavelength 488 nm, emission wavelength 510 nm). The results obtained were normalized on total protein content.

PTP assay

PTP activity was measured as previously reported [25]. Briefly, undifferentiated and differentiated cells were lysed in RIPA buffer. PTP activity was assessed by adding 100 µl of lysate to 1 ml of 5 mM p-nitrophenylphosphate at 37 °C for 20 min. The production of p-nitrophenol was measured colorimetrically at 410 nm. The results were then normalized on the basis of protein content.

In-gel phosphatase assay

For detection of PTP activity, we prepared a 10% SDS-polyacrylamide gel containing 105 cpm/ml of [^{32}P]-labeled substrate as described previously [26]. Briefly, 0.3 mg of substrate (poly-[glutamate:tyrosine]) was dissolved in kinase buffer (150 mM NaCl, 2 mM $MgCl_2$, 12 mM Mg acetate, 0.02% Triton, 5% glycerol, 50 mM Hepes pH 7.4, 0.1 M ATP) and incubated with 200 µCu of γ-[^{32}P]ATP, 2.5 U epidermal growth factor receptor and 500 ng epidermal growth factor for 18 h at 37 °C. The substrate was then precipitated by adding an equal volume of 20% trichloroacetic acid. After centrifugation at 12,000 g for 10 min, the pellet was resuspended in 100 µl of 2 M Tris pH 7.2, and then purified via a Sepharose G-50 column equilibrated with imidazole 50 mM pH 7.2. Cells were lysed in 25 mM sodium acetate, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 100 µg/ml catalase and protease inhibitor cocktail as described above. In the modified version of the assay, used to detect oxidized PTPs, 10 mM iodoacetic acid was added to the samples, after degassing the buffer [27]. Total proteins (15 µg) were then loaded onto the radiolabeled gel. After electrophoresis, gels were sequentially washed for the indicated times at room temperature in about 200 ml of the following buffers: buffer 1 (overnight): 50 mM Tris pH 8.00 and 20% isopropanol; buffer 2 (twice, for 30 min): 50 mM Tris pH 8.00 and 0.3% β-mercaptoethanol; buffer 3 (90 min): 50 mM Tris pH 8.00, 0.3% β-mercaptoethanol, 6 M guanidine hydrochloride and 1mM EDTA; buffer 4 (three washes for 1 h each): 50 mM Tris pH 7.4, 0.3% β-mercaptoethanol, 1mM EDTA and 0.04% Tween 20; buffer 5 (overnight): 50 mM Tris pH 7.4, 0.3% β-mercaptoethanol, 1mM EDTA, 0.04% Tween 20 and 4 mM dithiothreitol.

Gels were then stained with Coomassie brillant blue, destained in 40% methanol and 10% acetic acid, dried and analyzed using a Cyclone system (Perkin Elmer).

Results

PDGFr phosphorylation level is decreased during myogenesis

In a previous paper, we reported that tyrosine phosphorylation of PDGFr decreases in myotubes with respect to myoblasts [23]. Here, we investigated the time course of PDGFr tyrosine phosphorylation in control and differentiated cells. Undifferentiated and 6-day differentiated C2C12 cells were stimulated with PDGF for different

times (10 min, 45 min, 2 h, 4 h, 6 h and 8 h). Total lysates were used in a western blot analysis using anti-phosphotyrosine antibodies. The membrane was then reprobed with anti-PDGFr antibodies for normalization and with anti-vinculin antibodies to check equal loading. The results showed that the intensity of the receptor activation peak (10 min after stimulation) greatly decreases in differentiated with respect to control cells (fig. 1). In addition, in myotubes, PDGF-r activation decreased with slower kinetics with respect to myoblasts. Hence, these findings demonstrate that myogenesis greatly affects the activation of PDGFr upon stimulation.

Binding of PDGF to its receptor leads to dimerization and autophosphorylation on specific tyrosine residues which act as docking sites for downstream signaling molecules. We were interested to study the specific phosphorylation of different PDGFr tyrosines in a time course experiment using undifferentiated and 6-day differentiated C2C12 cells. In particular, we analyzed the phosphorylation of Tyr716 (fig. 2A), Tyr751 (fig. 2B) and Tyr1021 (fig. 2C), which act as docking sites for different downstream signalling proteins, and Tyr857 (fig. 2D) which is the PDGFr regulatory tyrosine, located in the activation loop. Cells were stimulated with PDGF for different times (10 min, 45 min and 2 h). Total lysates were then transferred to a membrane that was probed with specific anti-phosphotyrosine antibodies. Blots were then reprobed with anti-PDGFr antibodies for normalization. The results show that tyrosine phosphorylation of each specific analyzed residue decreased in myotubes with respect to myoblasts. In fact, each phospho-tyrosine revealed a maximum activation at 10 min after PDGF stimulation both in control and differentiated cells, while the intensity of this phosphorylation greatly decreased in myotubes with respect to myoblasts. Together, these results demonstrate that activation of all tested specific tyrosine residues on PDGFr decreases in differentiated cells with respect to control ones. These observations are clearly in agreement with the lower total tyrosine phosphorylation level of the receptor already observed in myotubes with respect to myoblasts (fig. 1).

PDGFr downstream signalling pathways are inhibited during myogenesis

Since our findings indicate a significant decrease in the phosphorylation level of specific PDGFr tyrosines during myogenesis, our interst focused on the study of two main mitogenic pathways starting from PDGFr, namely PI-3K

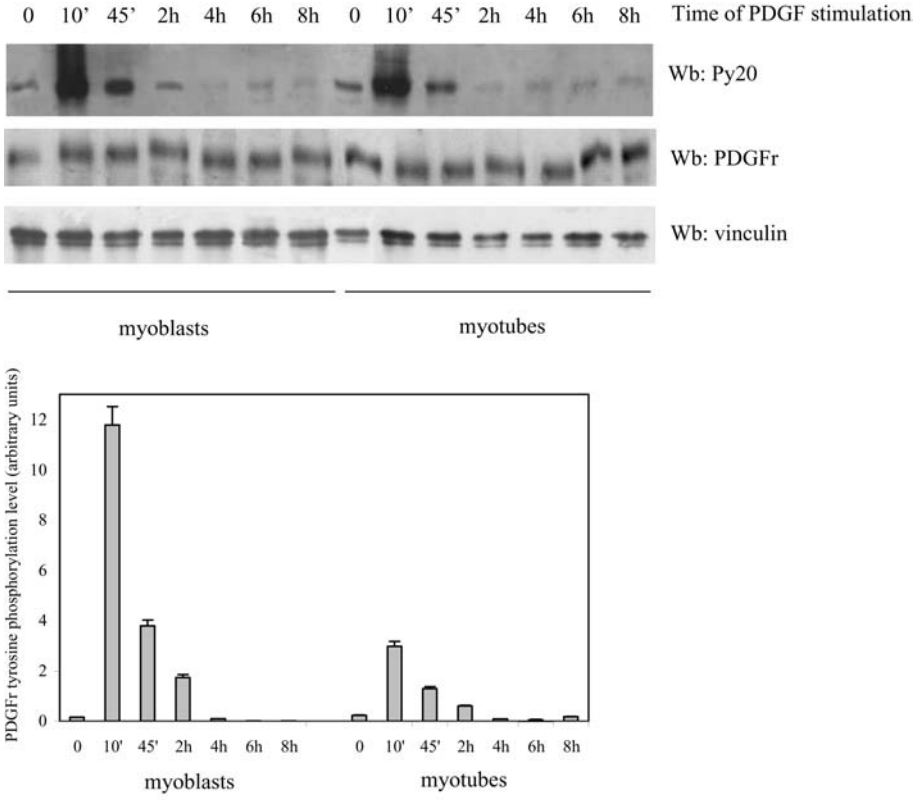


Figure 1. Total PDGFr phosphorylation level in myoblasts and myotubes. Undifferentiated and 6-day differentiated cells were serum starved for 24 h and then stimulated with 30 ng/ml PDGF-BB for the indicated times. Lysates were used for a Western blot analysis. The membrane was then treated first with anti-PY20 antibodies and then stripped and reprobed with anti-PDGFr antibodies and anti-vinculin antibodies for normalization. Densitometric analysis of the bands obtained from three independent experiments of anti-PY20 and anti-PDGF-r immunoblots was performed and the ratio between the two values is reported in the histogram.

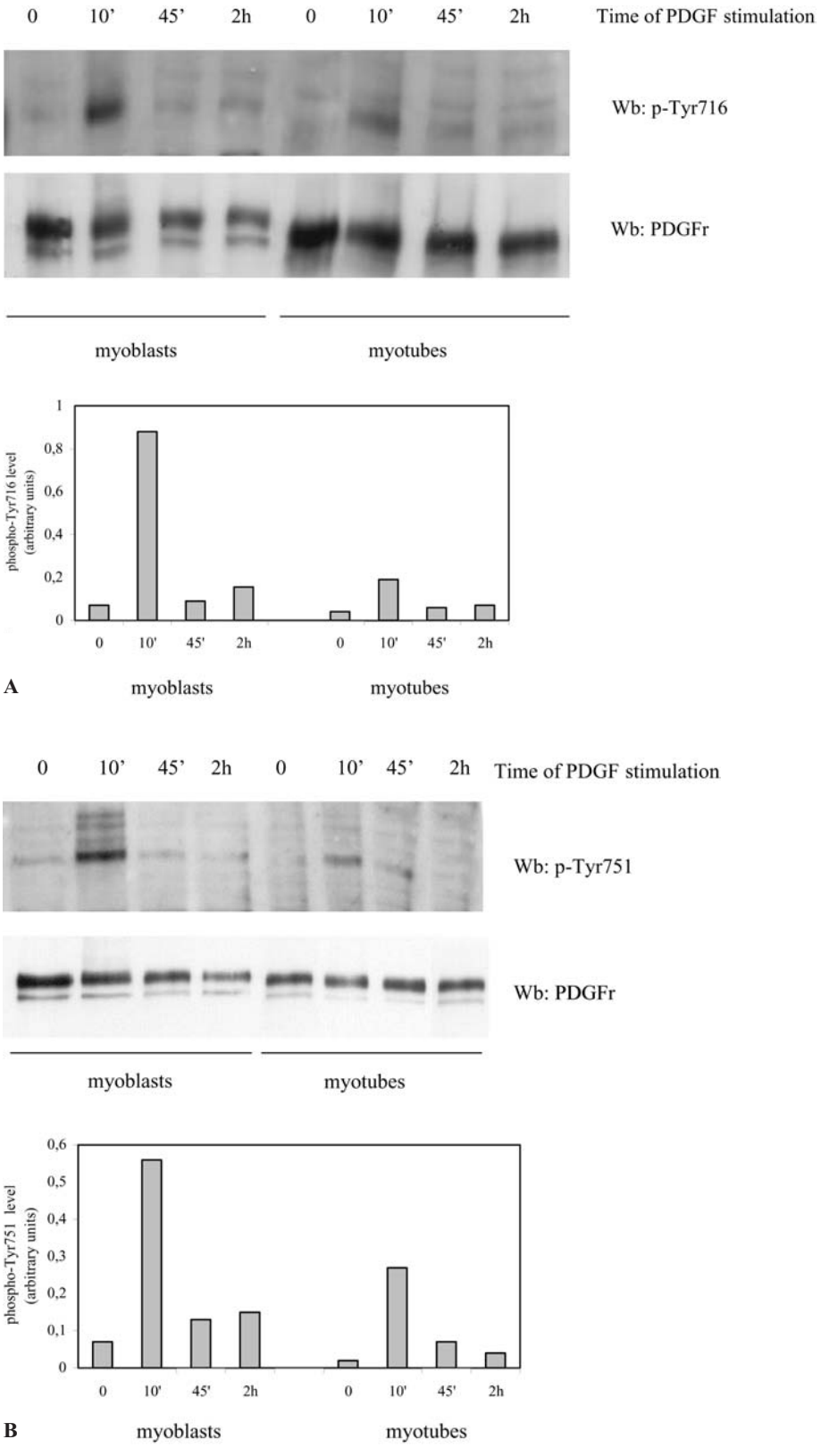
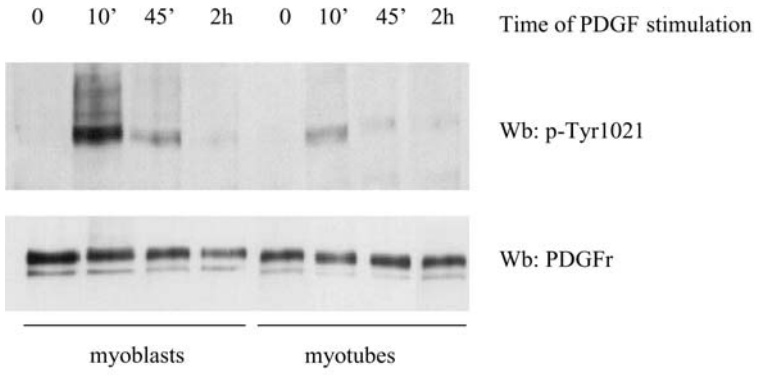
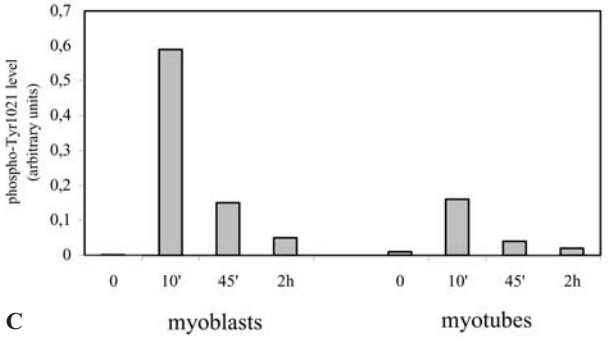


Figure 2. PDGFr phosphorylation level of specific tyrosine residues. Undifferentiated and 6-day differentiated cells were serum starved for 24 h before stimulation with 30 ng/ml PDGF-BB for the indicated times. A western blot analysis was performed with cell lysates. The membrane was probed with specific antibodies against phospho-Tyr716 (*A*), phospho-Tyr751 (*B*), phospho-Tyr1021 (*C*) and phospho-Tyr857 (*D*). After reprobing the membranes with anti-PDGFr antibodies for normalization, a densitometric analysis of the two bands was performed. This result is representative of at least three independent experiments.





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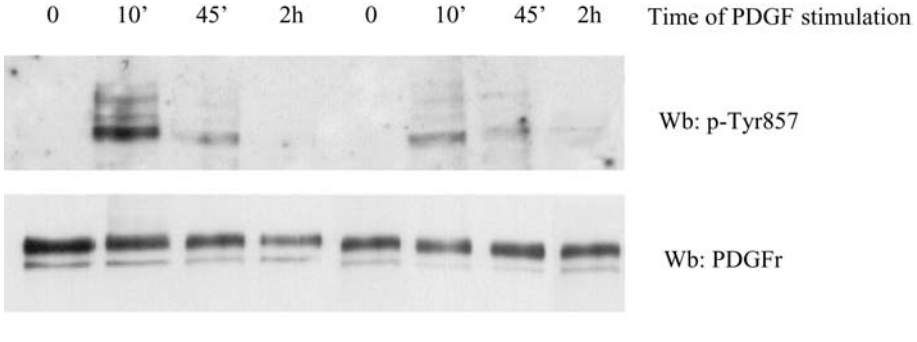
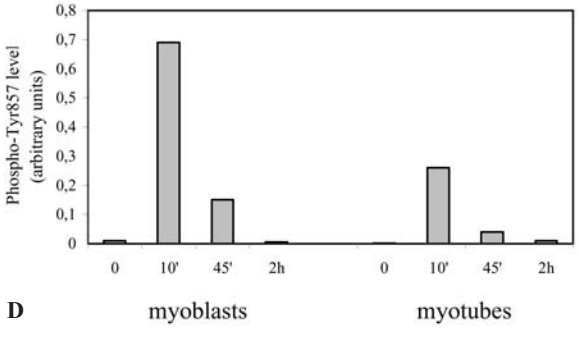




D

Figure 2 (continued)

and MAPK. First, we analyzed PI-3K activity, by means of its association with PDGF-r phospho-Tyr751. Control and 6-day differentiated C2C12 cells were stimulated with PDGF for different times (10 min, 45 min and 2 h). Lysates were used for an anti-PDGFr immunoprecipitation to be probed with anti PI-3K antibodies. Figure 3A shows that binding of PI-3K to PDGFr is reduced in myotubes with respect to myoblasts, although the maximum level of association is observed 10 min after PDGF stimulation both in control and differentiated cells. This finding is in agreement with our observations about the phosphorylation level of the PI-3K docking site, Tyr751, during differentiation (fig. 2B). A second light form of PI-3K appears only in myotubes. This form of PI-3K may be due to a post-translational modification of the p85 subunit of the protein, since the apparent molecular weight of this form is different from that of several isoforms of PI-3K known in mammalian cells [28].

Akt/protein kinase B, a downstream target of PI-3K, is an important component of survival signals in response to several growth factors. To confirm our findings on PI-3K activity, we investigated Akt activation in myoblasts and in myotubes following PDGF stimulation. Control and 6-day differentiated C2C12 cells were stimulated with PDGF for different times (10 min, 45 min, 2 h, 4 h, 6 h and 8 h). Cell lysates were used in a western blot analysis and then treated with anti-phospho-Akt antibodies which recognize the phosphorylated activated form of Akt. The blot was then reprobed with anti-Akt antibodies for normalization. Figure 3B shows that, during myogenesis, Akt activation after PDGF treatment is inhibited. In myoblasts, Akt shows the maximum peak of activation 10 min after PDGF stimulation, while in myotubes, PDGF is able to induce only a very low Akt activation. Notably, in myotubes, the basal activity of Akt is higher with respect to undifferentiated cells, likely due to the commitment of the survival program engaged by differentiated cells.

In addition we studied MAPK activation in control and differentiated cells. These enzymes seem to play a key role during both the early and late stages of skeletal muscle differentiation [29]. To analyze the activation level of MAPKs in response to PDGF in myoblasts and myotubes,

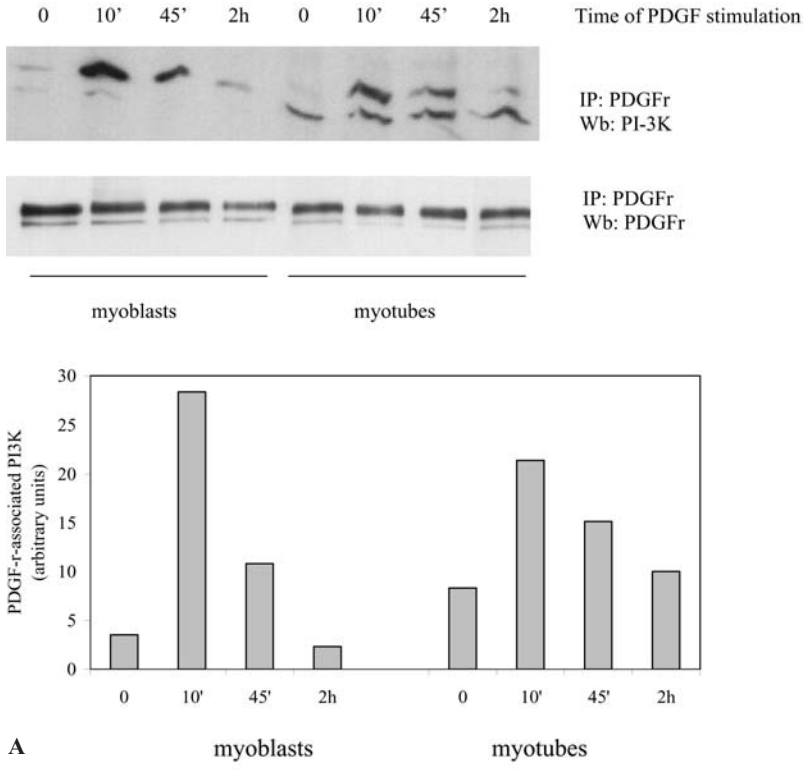
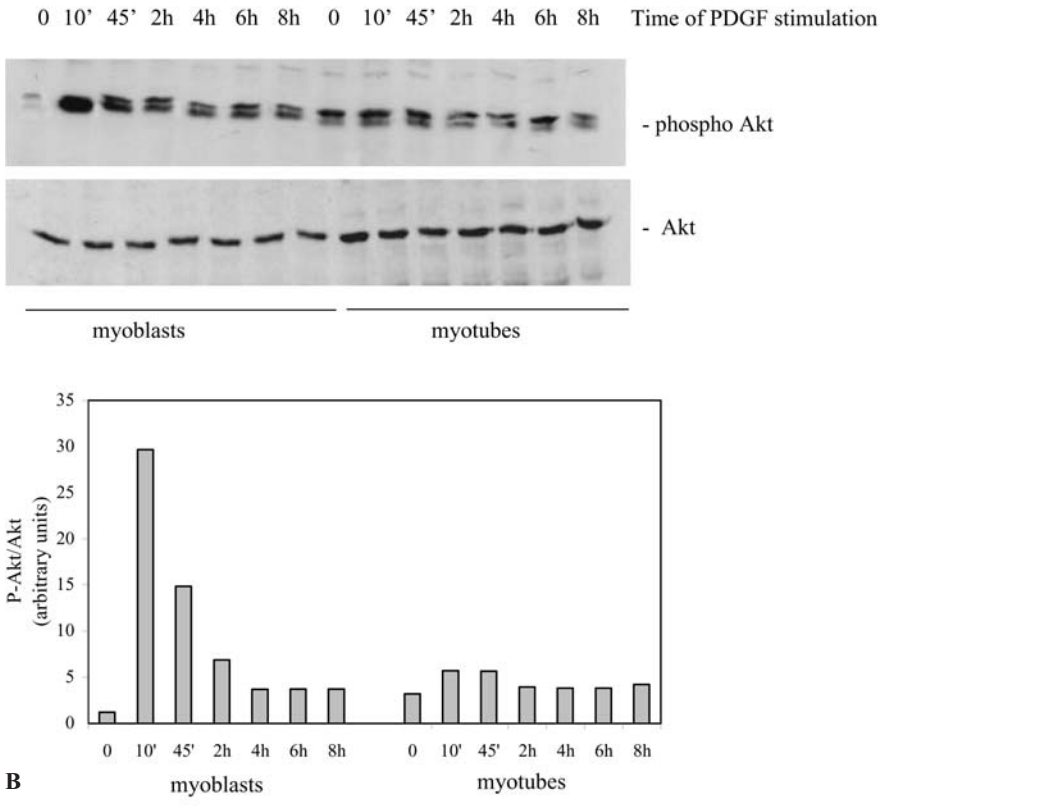


Figure 3. Analysis of the PDGFr downstream signaling pathway. (*A*) PDGFr/PI-3K association in myoblasts and myotubes. Control and 6-day differentiated C2C12 were stimulated with PDGF-BB for the indicated times 24 h after serum starvation. Lysates were immunoprecipitated with anti-PDGFr antibodies and a Western blot analysis was performed. After reprobing the membranes with anti-PDGFr antibodies for normalization, a densitometric analysis of the two bands was performed. (*B*) Activation level of Akt in myoblasts and myotubes. Undifferentiated and 6-day differentiated cells were serum starved for 24 h before stimulation with PDGF-BB for the indicated times. Lysates were used for a Western blot experiment. The membrane was incubated first with anti-phospho-Akt antibodies and then with anti-Akt antibodies for normalization. A densitometric analysis of the bands obtained from two blots was performed and the ratio between the two values is reported in the histogram. (*C*) Activation level of MAPKs in myoblasts and myotubes. C2C12 cells were treated as described for AKT. The MAPK activation level was analyzed using specific anti-phospho-MAPK antibodies. Normalization was obtained by reprobing the membrane with anti-MAPK antibodies. This result is representative of at least three independent experiments.



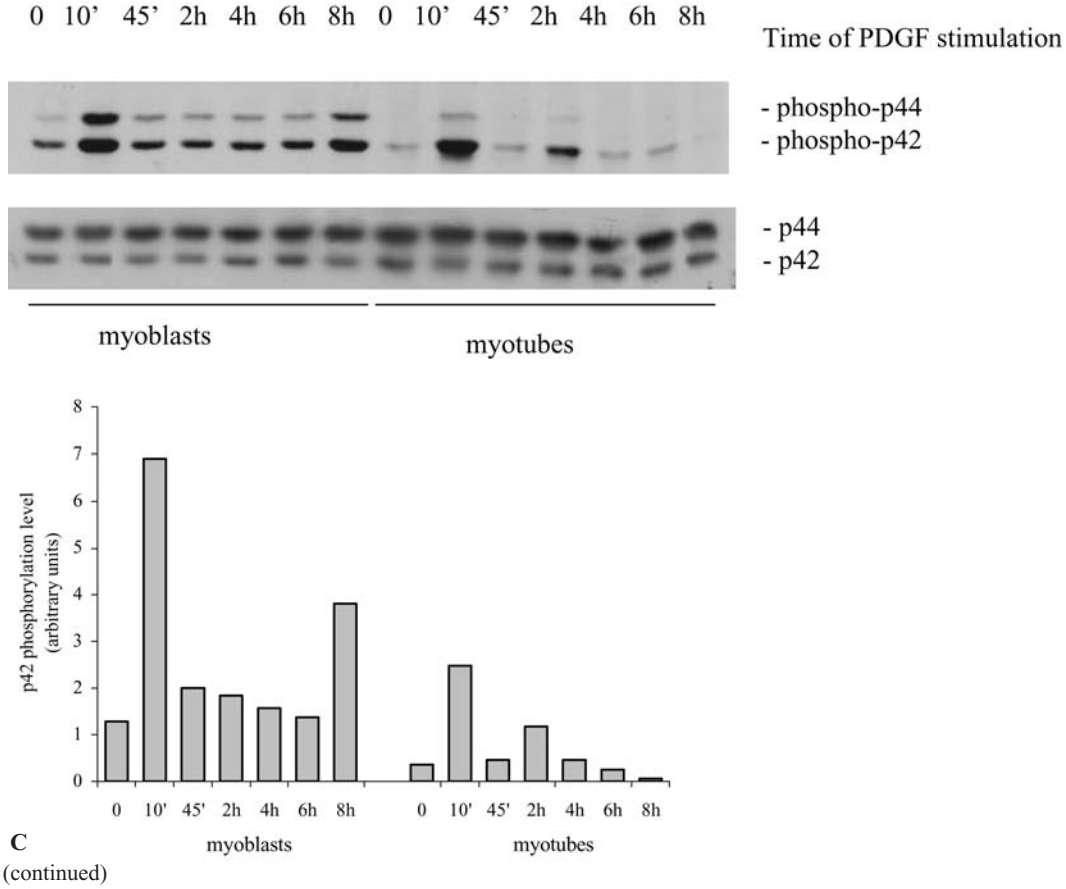


Figure 3 (continued)

control and 6-day differentiated C2C12 cells were stimulated for different times with PDGF (10 min, 45 min, 2 h, 4 h, 6 h and 8 h), and lysates were used in an immunoblot with anti-phospho-p42/p44 MAPK antibodies. After reprobing the membrane with anti-MAPKs for normalization, a densitometric analysis of the two blots was performed. The results showed that MAPKs have two peaks of activation both in myoblasts and myotubes (fig. 3C). p42 and p44 were both maximally activated 10 min after PDGF stimulation, after which, a decrease of activation was observed. Then, a second peak appeared, 8 h after stimulation in myoblasts and only 2 h after stimulation in myocytes. These findings clearly demonstrate that during differentiation, MAPK signaling is down-regulated and that the kinetics of phosphorylation of both p42 and p44 is greatly affected.

In summary, all these experiments underline that the response of mature myotubes to PDGF is severely impaired, the two main proliferation pathways starting from the receptor, namely the PI-3K and MAPK routes, being greatly inhibited.

Myogenesis greatly reduces the ROS content in myotubes

Proliferative signals such as those delivered by the activation of growth factor receptors and G proteins of the Ras family are accompanied by intracellular production of endogenous oxygen species, which are necessary for downstream propagation of mitogenic signaling. ROS, and in particular hydrogen peroxide, operate as key signaling molecules in the pathway triggered by PDGF [30], epidermal growth factor [31], cytokine and antigen receptors [32, 33]. Here, we investigated the relationship between the ROS level during differentiation and PTP inactivation. A decrease in ROS production has been demonstrated in cell-cell contact conditions, which is accompanied by an activation of total PTP activity and a drastic decrease in the tyrosine phosphorylation level [24]. Tyrosine phosphatases are sensitive to redox regulation and are easily inactivated by oxidation of a cysteine residue located in the catalytic site [34]. In particular, protein tyrosine phosphatase 1B [35] is rapidly and transiently inactivated by ROS after epidermal growth factor stimulation, while LMW-PTP is sensitive to a transient inhibition due to ROS production after PDGF stimulation [36]. Recently, we demonstrated that ROS generation is impaired in mature myotubes with respect to myoblasts using the C2C12 murine cells as a model [23]. First, we present here the time course of ROS production during myogenesis. Control and C2C12 cells, treated with differentiating medium for different times (from 1 to 6 days), were used to assay ROS generation using the fluorescent probe DCF-DA. Each value obtained by fluorimetric measurement was normalized on the basis of the protein content in each sample. The results, shown in figure 4A, clearly demonstrate that, already after 1 day of differentiation, ROS production was greatly decreased with respect to control cells, and remains low until the late stages of myogenesis. Furthermore, the evaluation of ROS production in control and 6-day differentiated cells after different times of PDGF stimulation (10 min, 45 min and 2 h) shows that the characteristic ROS burst after agonist administration was totally abolished in myotubes (fig. 4B). This effect is probably due to a reduced responsiveness of the receptor toward its agonist. Altogether, these results demonstrate that myogenesis greatly reduces the ROS content in myotubes, either without agonist treatment or after PDGF stimulation.

PTP specific activity is increased during myogenesis due to both expression and redox up-regulation

Here, we observed a general down-regulation of signaling pathways triggered by PDGFr in myotubes with respect to myoblasts, due to a decrease in tyrosine phosphorylation of the receptor itself. Since the tyrosine phosphorylation level is the result of a balance between tyrosine kinases and tyrosine phosphatase activity, we compared the specific activity of total PTPs between myoblasts and myotubes. Lysates obtained from control and differentiated cells were used in an in vitro PTP assay using p-nitrophenylphosphate as a substrate. The results, shown in figure 5A, indicate an increase in PTP specific activity during myogenesis. The same experiment, performed using control and differentiated cells stimulated with or without PDGF for 10 min, reveals the same increase observed in unstimulated cells, suggesting that this phenomenon is not affected by PDGF treatment.

To elucidate the involvement of PTPs in muscle differentiation, we performed an in-gel assay for PTP activity together with a modified version of this technique. These two methods allowed us to analyze both the profile of PTP expression during myogenesis (by the in-gel assay) and the redox state of PTPs during this process (using the modified in-gel assay). Control and differentiated C2C12 cells were lysed in lysis buffer containing or not iodoacetic acid (IAA). Only the PTPs, which presented their catalytic cysteines in the reduced form, are irreversibly inactivated by alkylation with IAA, while in vivo-oxidized PTPs are not subjected to this modification. Lysates were then loaded on a modified polyacrylamide gel in which a radiolabeled substrate of PTPs (poly-glutamate:tyrosine) had been incorporated before polymerization. After SDS-PAGE, the gel was washed with specific buffers to allow the complete renaturation and reduction of proteins. In the modified in-gel assay, where the samples have been treated with IAA, only PTPs that were oxidized in vivo, and hence not alkylated, can regain their activity and hence remove the labeled phosphate from the substrate, creating the white bands seen

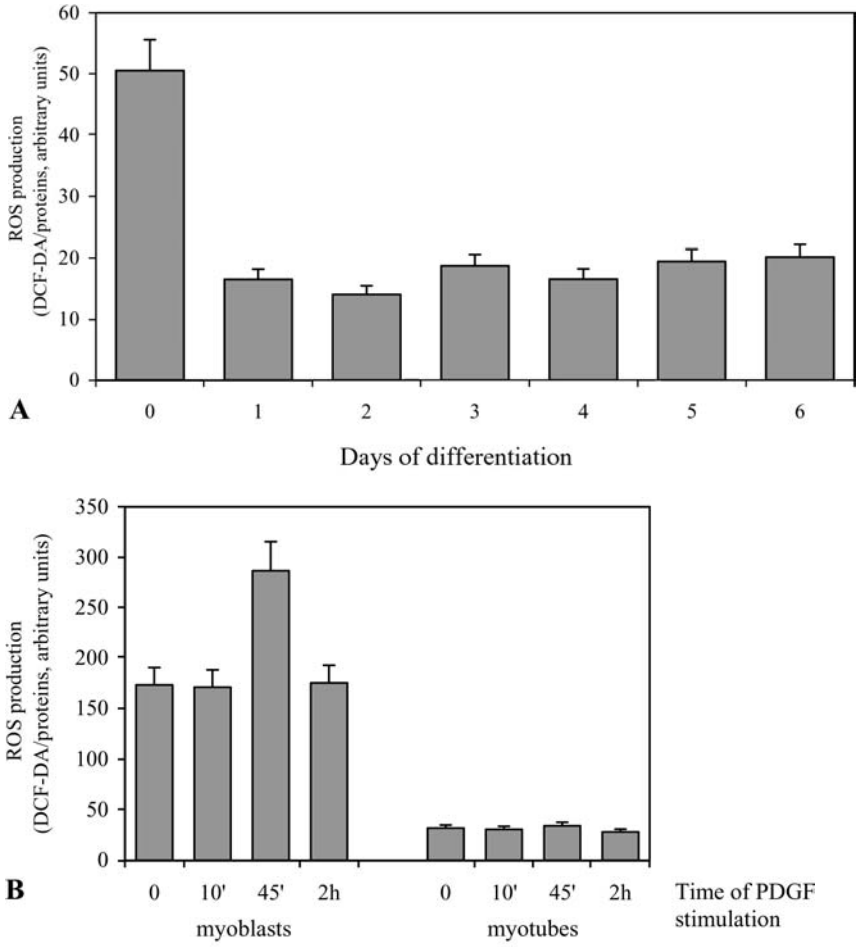


Figure 4. ROS generation during myogenesis. (*A*) ROS production during muscle differentiation. C2C12 cells were cultured with DM medium for the indicated days. ROS production at different stages of myogenesis was performed adding DCF-DA (5 µg/ml) to the medium for 5 min. After lysis, a fluorimetric analysis was performed. Values were normalized on the basis of the protein content. (*B*) ROS generation in myoblasts and myotubes after PDGF stimulation. Undifferentiated and 6-day differentiated C2C12 cells were stimulated with 30 ng/ml PDGF-BB for the indicated times. ROS production was assayed as described above. Values were normalized on the basis of the protein content. Data are the mean of three independent experiments.

after autoradiography. On the other hand, samples not treated with IAA show the complete profile of expression of PTPs, as all PTPs renaturate and regain catalytic activity.

Figure 5B shows the results obtained with the unmodified in-gel assay. Myogenesis induced a general increase in PTP expression, already detectable at an early stage of differentiation and becoming more evident after 6 days. In particular, we distinguished seven bands, indicated as A–G (ranging from about 32 to 110 kDa), whose expression differentially increased during muscle differentiation. While bands A and E were unaffected by differentiation, bands B, C, F and G rose notably during myogenesis.

The results obtained with an in-gel modified assay are shown in figure 5C. We observed the presence of six bands (indicated as B–G) corresponding to PTPs in the oxidized form. During muscle differentiation, bands E and G disappeared after 4 and 6 days of differentiation, demonstrating that these proteins completely shift towards their reduced form during myogenesis. PTPs corresponding to bands B, C and D also increased the amount of their reduced form during muscle differentiation, considering that the bands in figure 5C should be normalized on the basis of total PTP content (fig. 5B). PTPs corresponding to band A and band E in figure 5B, which are not visible in the modified in-gel assay (fig. 5C), were likely PTPs not undergoing redox control under our conditions. These results show that induction of differentiation affects not only the overall expression profile of PTPs, but also their redox state, promoting a shift toward the reduced active form. These findings contribute to explaining the increase in PTP specific activity observed in myotubes with respect to myoblasts, a phenomenon that may be responsible for the down-regulation of PDGFr signaling.

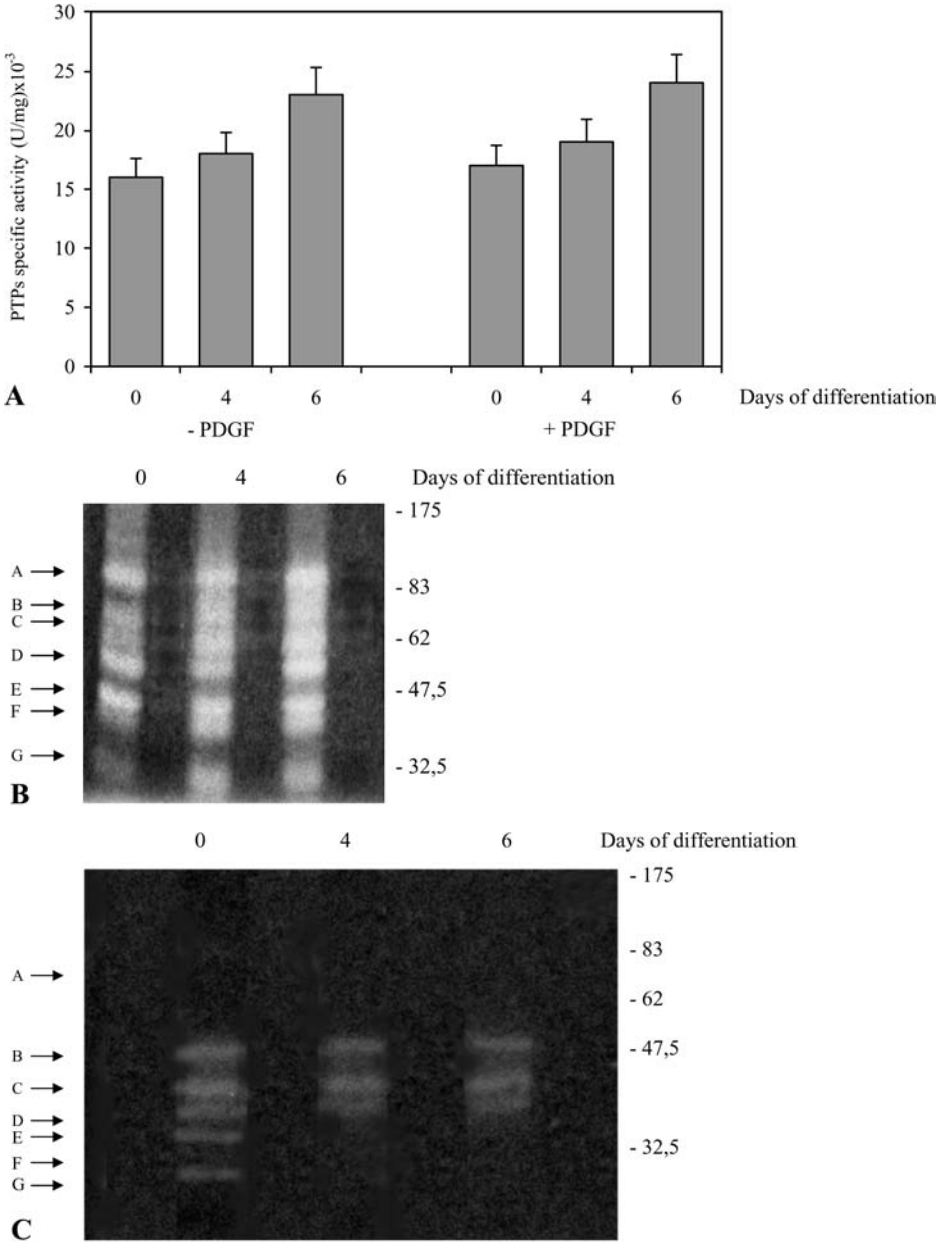


Figure 5. Analysis of PTPs during myogenesis. (*A*) Analysis of PTP specific activity during muscle differentiation. C2C12 cells were differentiated for the indicated times and then stimulated or not with 30 ng/ml of PDGF-BB for 10 min. A PTP assay was performed using p-nitrophenylphosphate as a substrate. Results were normalized on the basis of the total protein content. These data are the mean of three independent experiments. (*B, C*) In-gel phosphatase assay. C2C12 cells were differentiated for the indicated times. PTP activities were analyzed from whole-cell lysates in an in-gel phosphatase assay, using [^{32}P]poly-(Glu:Tyr) as the gel-embedded substrate. After electrophoresis, SDS was removed from the gel, and proteins were renatured. The gel was then dried and PTP activities were visualized using a Cyclone system (Perkin Elmer). (*B*) Expression profile of total PTPs at different stages of differentiation. (*C*) Results obtained with the modified version of the PTP in-gel assay. The same results were obtained in four independent experiments.

Discussion

Myogenesis is a coordinated process which requires the withdrawal of growth factors to allow induction of differentiation, leading to the fusion of single cells into multinucleate myotubes and the expression of muscle-specific transcription factors.

In this paper, we demonstrated that (i) the PDGFr total tyrosine phosphorylation level and the amount of activation of some of its specific tyrosines decrease with myogenesis, (ii) some of the PDGFr downstream signaling pathways, namely PI-3K and MAPK, are inhibited during muscle differentiation and (iii) myotubes show a general increase in PTP activity due to a higher ex-

pression of several PTPs and a redox regulation of most of them.

The total PDGFr tyrosine phosphorylation level is greatly decreased in differentiated cells in comparison to undifferentiated ones (fig. 1). Accordingly, some phosphotyrosine residues, which acts as docking sites for downstream signaling proteins (Tyr716, Tyr751, Tyr1021 and Tyr857) show similar reductions (fig. 2A–D). These observations strongly suggest that the PDGFr activation level is greatly reduced during muscle differentiation. Of note is that the kinetics of PDGFr down-regulation are different in differentiated myotubes with respect to growing myoblasts.

Down-regulation of PDGFr, through a transcriptional control, was demonstrated in adipogenesis [6]. The results presented here suggest that during myogenesis, the down-regulation of the PDGFr pathway is obtained through a decreased phosphorylation level of the receptor itself. This may represent a novel mechanism which permits undifferentiated cells to cut off the mitogenic pathway triggered by PDGFr and to induce the myogenic program. Recently, Chiarugi et al. [37] demonstrated that the ability of PDGFr to promote mitosis is proportional to its tyrosine phosphorylation level. The results presented here are in agreement with those findings. In fact, since a differentiating program needs inhibition of the mitogenic signaling pathway, the PDGFr tyrosine phosphorylation level plausibly decreases during myogenesis. Myogenesis affects only the receptor phosphorylation level, since the amount of protein is unchanged in control and differentiated cells.

Analysis of the two main downstream signaling pathways originating from PDGFr again shows that these pathways are inhibited in myotubes. In particular, we investigated the activation level of MAPKs and PI-3K/Akt cascades after PDGF stimulation (fig. 3B, C). These findings are in agreement with results obtained with PI-3K. In fact, Akt is a downstream substrate of PI-3K which, after growth factor stimulation, is recruited to the receptor and activated, resulting in the production of Phosphatidyl Inositol Phosphate (PIP_3). PI3K activation induces the recruitment of Akt to the membrane, where it is phosphorylated and activated on threonine 308 and serine 473. We demonstrated that myogenesis greatly decreases the association of PI-3K with PDGFr (fig. 3A), probably resulting in a lower activation of PI-3K, which is unable to fully activate its downstream substrates.

Both PI-3K [38] and MAPK [39] cascades are important for cell growth. Furthermore, the contribution of both Akt and MAPK pathways in the induction of muscle differentiation has already been reported. As far as MAPKs are concerned, some experiments have shown that cells decide for differentiation or proliferative responses purely on the basis of the duration of MAPK activation. Studies performed on PC12 neuronal cells showed that stimulation with empidermal growth factor generated a transient activation of MAPKs, which is associated with proliferating signals, while treatment of cells with nerve growth factor led to sustained activation of MAPKs and differentiation [40, 41]. This model of MAPK activation, based on experiments with neuronal cells, is further supported by recent findings. In particular, Sarbassov et al. [42] demonstrated that myogenic differentiation of C2C12 cells was also associated with sustained activation of MAPKs. Furthermore, Bennett et al. [29] suggested a function for MAPKs during the early and late stages of skeletal muscle differentiation. In particular, they showed that, after the removal of growth factors, p42 is inactivated concomitantly with up-regulation of muscle specific genes in C2C12 myoblasts. At this stage, endogenous MAPK phosphatase 1 increases, suggesting that the rise in this phosphatase may be a mechanism whereby cells exit from the cell cycle and induce myogenesis [43].

There are several results demonstrating an essential role for the PI-3K/Akt pathway in myogenesis. Kaliman et al. [44] reported that treatment of the L6E9 muscle cell line with two PI-3K inhibitors, such as wortmannin and LY294002, abolishes the ability of myoblast to form myotubes, without affecting myoblasts proliferation, elongation or alignment [44]. Furthermore, overexpression of a constitutively active form of PI-3K was demonstrated to strongly enhance myogenic differentiation, increasing the size of the myotubes and inducing elevated levels of muscle-specific proteins. In contrast, inhibition of PI-3K with LY294002, or the use of PI-3K dominant negative mutants, causes opposite effects, suggesting PI-3K as an upstream mediator for the expression of muscle-specific genes [45]. This role of PI-3K as a survival factor for differentiating cells may validate our findings concerning a differential effect of muscle differentiation on PI-3K signaling with respect to other signaling pathways (figs. 2B, 3A, B). PI-3K has been reported to play a key role in the mitogenic signaling cascade triggered by PDGFr. In fact, Valius and Kazlauskas found that PDGF-induced DNA synthesis requires mainly the activation of PI-3K [38]. In contrast, other signaling molecules including Src and PLCγ contribute to PDGFr chemotaxis. The results presented here concerning PI-3K down-regulation during myogenesis are in agreement with these previous findings. In fact, since the differentiation process requires the cutting off of intracellular mitogenic signaling, inhibition of the PI-3K-induced mitogenic cascade after PDGF stimulation may be a key event for induction of the myogenic program. Together, the results presented here indicate down-regulation of the mitogenic signaling cascade generated by PDGFr through a lower activation of the receptor itself.

There is a large body of literature on PDGFr down-regulation. This could be due to an immediate ligand-induced internalization of ligand-receptor complexes, to receptor degradation by ubiquitin-dependent proteolysis [46] or to a reduction in receptor mRNA expression [47]. Recently,

Chiarugi et al. [37] proposed a new model of βPDGFr down-regulation in which PTPs play a fundamental role. They demonstrated that the main part of the activated receptor is dephosphorylated by the concertated action of PTPs, while PDGFr protein destruction via lysosomal or ubiquitin-mediated proteolysis plays a marginal role [37]. Therefore, PTPs become crucial enzymes for terminating PDGFr signal transduction. Furthermore, many PTPs have been found involved in differentiation, such as MAPK phosphatase 1 [23, 29], phosphatase of regenerating liver-1 [48], PTP-β2 [49], PTP20 [50] and LMW-PTP [23]. On the basis of these results, and because we did not see any difference in the surface exposure of PDGFr during muscle differentiation (data not shown), we investigated the role of PTPs in the down-regulation of the PDGFr phosphorylation level, evaluating the total PTP activity in undifferentiated and differentiated cells. Our findings concerning a rise in PTP activity during myogenesis suggest that these enzymes are involved in the decrease in the PDGFr tyrosine phosphorylation level (fig. 5A). This phenomenon is accompanied by an increase in PTP content in differentiated cells. In fact, a PTP in-gel assay showed that muscle differentiation affects the level of expression of several PTPs, increasing the amount of these proteins (fig. 5B). These results suggest that the myogenic program induces an elevated expression of PTPs, leading to higher PTP activity in differentiated cells with respect to undifferentiated ones. The increase in PTPs may be due to transcriptional control or to translational regulation, as already demonstrate for other PTPs. For example, we previously showed that the expression level of LMW-PTP increases during myogenesis probably through translational regulation [23], while the increase in CD45 in the induction of granulocyte or monocyte differentiation of HL60 leukemia cells [51] and the rise of receptor protein-tyrosine phosphatase α during mouse embryogenesis [52] are due to transcriptional control.

Reversible PTP regulation by ROS has been demonstrated. All PTPs share a common amino acid motif in the active site with a catalytically essential cysteine whose SH group is transformed by ROS to sulfenic acid. These oxidized PTPs are catalytically inactive because they cannot form the cysteinyl-phosphate intermediate during the first step of catalysis. Oxidized/inactivated PTPs can reversibly shift toward their reduced/activated form by the action of the redox cellular system. There is evidence that ligand binding to a variety of cell surface receptors, including those for growth factors and cytokines, induces a transient increase in the intracellular concentration of hydrogen peroxide (H_2O_2) which is able to transiently inactivate PTPs in vivo. PTP-1B is reversibly inhibited after epidermal growth factor [35] and insulin [53] stimulation, while LMW-PTP [36] and Src homology phosphatase-2 [27] are transiently inactivated following PDGF treatment.

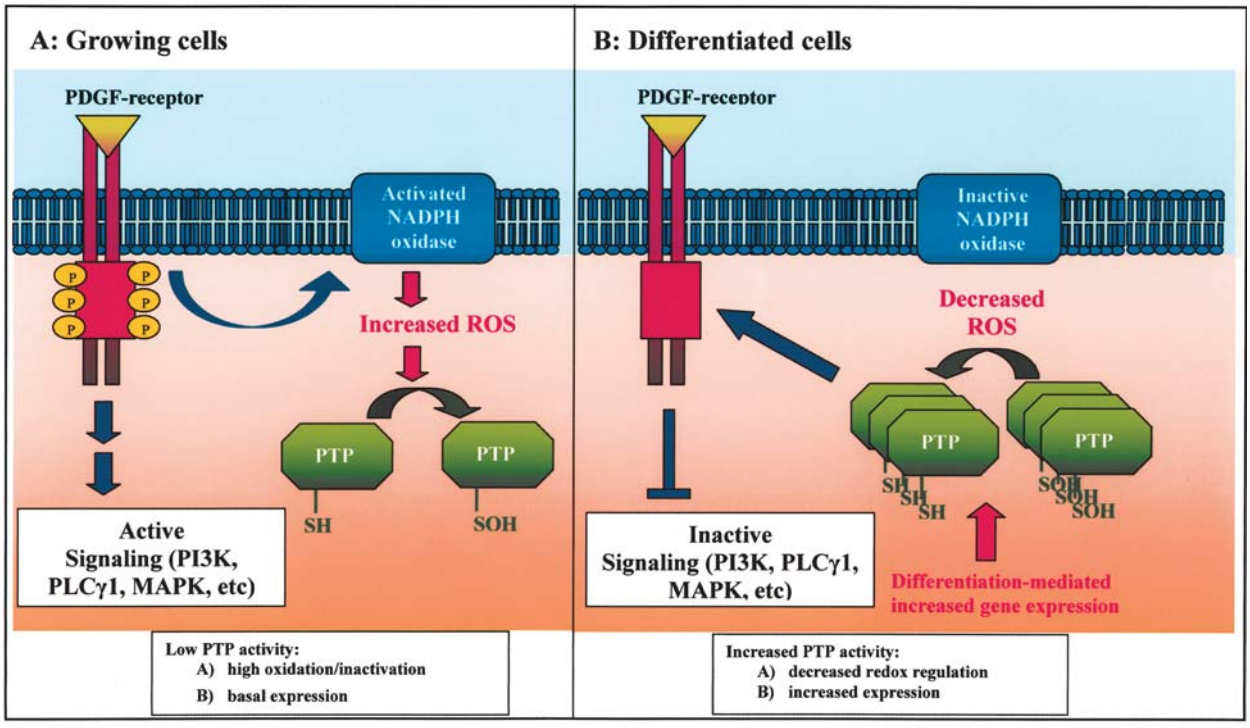


Figure 6. PTP down-regulation of PDGFr in growing and differentiated cells. (*A*) In growing cells, PTPs are expressed at the basal level and are redox down-regulated during PDGFr signaling, thus permitting PDGFr to signal through downstream pathways. (*B*) In differentiated cells, PTP activity is increased through increased expression control and decreased oxidation/inhibition due to decreased ROS levels. The cumulative effect is an increased dephosphorylation of PDGFr and an inhibition of downstream signaling.

Here, we reported that the increase in PTP activity observed in myotubes is due both to an increase in PTP expression levels and to a rise in the reduced/activated form of these enzymes.

In a previous paper, we demonstrated that myogenesis is associated with a decreased generation of ROS [23]. A time course of ROS production at different stages of muscle differentiation clearly shows that the amount of ROS greatly decreases, after only 1 day of differentiation (fig. 4A). Furthermore, we demonstrated that ROS generation is also greatly impaired in myotubes with respect to myoblasts after different times of PDGF stimulation (fig. 4B). The NADPH oxidase complex has been proposed as responsible for growth factor-induced ROS generation. Bae et al. [31] demonstrated that βPDGFr-dependent H_2O_2 generation requires both the intrinsic kinase activity of the receptor as well as the activation of PI-3K. The essential role of PI-3K is to provide PIP^3 for the activation of Rac, a component of the NADPH oxidase complex. Accordingly, we report a reduction of Tyr857 phosphorylation level and a reduced association of PI-3K with PDGFr in myotubes. These two effects may be responsible for the reduced production of ROS after PDGF stimulation in differentiated cells.

Together, the results presented here suggest a novel mechanism which allows undifferentiated cells to cut off the mitogenic signaling pathway and induce the myogenic program (fig. 6). Until now, only the down-regulation of PDGFr through transcriptional control was demonstrated during adipogenesis [6]. During muscle differentiation, the inhibition of the mitogenic cascade generated by PDGFr was obtained through a decreased tyrosine phosphorylation level of the receptor itself, without affecting either the amount of the protein on the membrane exposure of the receptor. We demonstrated that PTPs play a central role in this mechanism, enhancing their specific activity through both an increase in their expression level and changing their redox state. The reduction level of ROS observed in myotubes may allow PTPs to shift from an oxidized/inactivated form toward a reduced/activated state. To identify the PTPs that undergo redox and/or expression control during myogenesis, we are currently approaching this problem by a two-dimensional PTP in-gel assay.

We present a model (fig. 6) that may represent a general mechanism which permits cells to exit the cell cycle and induce differentiation. Further studies are necessary to better understand the intracellular signaling cascade involved in the differentiation process.

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