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Low Molecular Weight Protein-tyrosine Phosphatase Is Involved in Growth Inhibition during Cell Differentiation*

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Low molecular weight protein-tyrosine phosphatase (LMW-PTP) is an enzyme involved in mitogenic signaling and cytoskeletal rearrangement after platelet-derived growth factor (PDGF) stimulation. Recently, we demonstrated that LMW-PTP is regulated by a redox mechanism involving the two cysteine residues of the catalytic site, which turn reversibly from reduced to oxidized state after PDGF stimulation. Since recent findings showed a decrease of intracellular reactive oxygen species in contact inhibited cells and a lower tyrosine phosphorylation level in dense cultures in comparison to sparse ones, we studied if the level of endogenous LMW-PTP is regulated by growth inhibition conditions, such as cell confluence and differentiation. Results show that both cell confluence and cell differentiation up-regulate LMW-PTP expression in C2C12 and PC12 cells. We demonstrate that during myogenesis LMW-PTP is regulated at translational level and that the protein accumulates at the plasma membrane. Furthermore, we showed that both myogenesis and cell-cell contact lead to a dramatical decrease of tyrosine phosphorylation level of PDGF receptor. In addition, we observed an increased association of the receptor with LMW-PTP during myogenesis. Herein, we demonstrate that myogenesis decreases the intracellular level of reactive oxygen species, as observed in dense cultures. As a consequence, LMW-PTP turns from oxidized to reduced form during muscle differentiation, increasing its activity in growth inhibition conditions such as differentiation. These data suggest that LMW-PTP plays a crucial role in physiological processes, which require cell growth arrest such as confluence and differentiation.

The control of protein tyrosine phosphorylation *in vivo* is regulated by the coordinated and competing actions of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs)[1] (1). PTPs have been identified in eukaryotes, prokaryotes, plants, and virus and comprise a large family of enzymes, that rival protein-tyrosine kinases in structural diversity and complexity (2). PTPs may either antagonize or potentiate protein-tyrosine kinase-induced signaling *in vivo* and have been implicated in fundamental physiological processes such as growth and proliferation, differentiation, and cytoskeletal functions (3). Most growth factors, when bind to their receptors, activate an intracellular cascade of events involving tyrosine phosphorylation of the receptor itself and of several other substrates, phosphotyrosine-dependent recruitment of multiple signal transducers to the cell membrane and, eventually, the delivery of mitogenic stimuli (4). With some exceptions, PTPs exert a general inhibitory effect on this intracellular cascade by attenuating the intensity of phosphorylation signals initiated by activated receptors and accelerating their extinction (2).

LMW-PTP is an 18-kDa enzyme widely distributed in mammalian tissues (5). Previous findings demonstrated that PDGFr is an *in vivo* substrate of LMW-PTP and that this protein is involved in the control of signaling pathway triggered by PDGF activation (6). In particular, LMW-PTP participates in the control of *myc* expression interfering with Src pathway and in the regulation of *fos* activation through an extracellular signal-regulated kinase-independent pathway mediated by the STAT proteins (7). Recent data showed that two differently regulated pools of LMW-PTP are present in NIH3T3 cells: cytoplasmic and cytoskeleton-associated fractions. The cytoplasmic pool of protein binds and dephosphorylates PDGFr, while the cytoskeletal-associated LMW-PTP is involved in PDGF-mediated cytoskeletal rearrangement controlling the phosphorylation level of p190RhoGAP, which in turn regulates Rho activity (8).

A peculiar feature of all PTPs is their sensitivity to redox regulation. In fact, they are easily inactivated by oxidation of a critical cysteine residue located in the catalytic site, representing a potential target for both exogenous and endogenous reactive oxygen species (9). Recently, we demonstrated that LMW-PTP has a peculiar and reversible redox regulation in response to PDGF stimulation. Exogenous or endogenous H_2O_2 inhibits LMW-PTP activity, consequently increasing the tyrosine phosphorylation level of its substrates PDGFr and p190RhoGAP. Furthermore, we showed that the reactivation of LMW-PTP is due to reduced glutathione, which probably behaves as electron donor when oxidative stress is removed (10).

In multicellular organisms, cell growth is regulated not only by the availability of growth factors but also by a number of positive and negative co-stimuli delivered by the extracellular matrix and the neighboring cells. Recent observations showed that growth inhibition by cell density operates, at least in part,

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[1] The abbreviations used are: PTPs, protein-tyrosine phosphatases; LMW-PTP, low molecular weight protein-tyrosine phosphatase; PDGF, platelet-derived growth factor; PDGFr, PDGF receptor; DM, differentiating medium; 5′-F-IAA, 5′-iodoacetamide fluorescein; ROS, reactive oxygen species.

through a redox regulation of growth factor signaling. In particular these results demonstrate that the generation of intracellular ROS is decreased in contact-inhibited fibroblasts and suggest a model in which impaired production of ROS and increased PTPs activity impede mitogenic signaling in contact-inhibited cells (11).

On the basis of these results, we have studied whether LMW-PTP expression is regulated by cell density and cell differentiation, both processes requiring growth arrest and cell cycle exit. Herein, we demonstrate that the LMW-PTP intracellular level is up-regulated by cell confluence and differentiation and that, in these conditions, the tyrosine phosphorylation level of PDGFr decreases. In addition, we showed an increased association between LMW-PTP and PDGFr during myogenesis. Furthermore, we demonstrate that LMW-PTP is strongly converted in its reduced form in dense cultures with respect to sparse ones and in mature myotubes in comparison with myoblasts. These findings suggest that LMW-PTP, down-regulating PDGFr mitogenic signaling cascade, plays a role in contact inhibition and cell differentiation.

EXPERIMENTAL PROCEDURES

Materials—Unless specified all reagents were obtained from Sigma. NIH3T3, C2C12, and PC12 cells were purchased from ATCC; human recombinant PDGF-BB was from Peprotech; enhanced chemiluminescence kit and protein A-Sepharose were from Amersham Pharmacia Biotech; nitrocellulose membrane was from Sartorius; TRIzol Reagent was from Life Technologies; anti-PDGFr antibodies and PY20 anti-phosphotyrosine antibodies were from BD Transduction Laboratories; 5′-iodoacetamide fluorescein (5′-F-IAA), anti-fluorescein affinity purified rabbit antibodies, dichlorofluorescein diacetate, and Alexa Fluor™ 488 goat anti-rabbit IgG were obtained from Molecular Probes; MG-132 and goat anti-mouse IgG Texas Red conjugate was from Calbiochem. Collagen S was from Roche Molecular Biochemicals. Rabbit anti-LMW-PTP antibodies were generated in our laboratory.

Cell Culture—C2C12 cells were routinely cultured in growing medium consisting of Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum in 5% CO_2 humidified atmosphere. To induce myogenic differentiation of C2C12 cells, subconfluent cultures were switched from growth medium to differentiating medium (DM) consisting of Dulbecco's modified Eagle's medium supplemented with 1% horse serum. PC12 cells were cultured in RPMI supplemented with 10% inactivated horse serum and 5% fetal calf serum, in 5% CO_2 humidified atmosphere. To induce differentiation PC12 cells were plated on 5 $\mu g/cm^2$ collagen S and cultured in complete medium supplemented with 30 ng/ml of nerve growth factor β. For MG-132 experiments, 5×10^5 C2C12 cells were seeded and the day after 50 μM MG-132 were added for the indicated times.

Immunoprecipitation and Western Blot Analysis—Undifferentiated and differentiated cells were cultured as described above. For PDGF stimulation, cells were serum starved for 24 h before receiving 30 ng/ml PDGF-BB. Cells were then lysated for 20 min on ice in 500 μl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Lysates were then clarified by centrifugation. 25 μg of total proteins were used in each Western blot experiment, while for the immunoprecipitation (performed overnight at 4 °C) we adjusted each sample at 1 mg/ml concentration in 1 ml of final volume, using 2 μg/ml anti-PDGFr antibodies and 5 μg/ml anti-LMW-PTP antibodies, respectively. Immunocomplexes were collected on protein A-Sepharose, separated by SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose. 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 h at room temperature, probed first with specific antibodies and then with secondary antibodies conjugated with horseradish peroxidase, washed, and developed with the enhanced chemiluminescence kit.

Northern Blot Analysis—Total mRNA from control and differentiated C2C12 cells were prepared using TRIzol Reagent. RNA samples were resolved in a formaldehyde-agarose gel and transferred to a nitrocellulose membrane. Northern blot analysis was performed according to Maniatis *et al*. (12). The murine LMW-PTP cDNA was used as probe for hybridization of the filter (13).

Measurement of Intracellular ROS—5×10^5 C2C12 cells were seeded in a 10-cm dish. For differentiation, C2C12 cells were cultured in DM. Medium was then replaced with red phenol-free Dulbecco's modified Eagle's medium and after 1 h, 5 μg/ml dichlorofluorescein diacetate, an oxidant-sensitive fluorescent dye, was added. Cells were then detached from the substrate by trypsinization and analyzed immediately by flow cytometry using a Becton Dickinson FACSCAN flow cytometer equipped with Argon laser lamp (FL-1; emission, 480 nm; band pass filter, 530 nm).

In Vivo 5′-F-IAA Labeling—C2C12 cells were lysed in RIPA buffer at pH 7.5 and 5′-F-IAA (from freshly prepared stock) was added to a final concentration of 5 μM. The lysates were maintained 10 min at 37 °C for the labeling step and then were treated for immunoprecipitation with anti-LMW-PTP antibodies. Redox state was evidenced by anti-fluorescein immunoblot.

LMW-PTP Assay—The PTP activity was measured as previously reported (14). Briefly, control and differentiated C2C12 cells were lysated in RIPA buffer and LMW-PTP was immunoprecipitated from lysates. Immunoprecipitates were then resuspended in 100 μl of 0.1 M sodium acetate, pH 5.5, 10 mM EDTA. PTP activity assay was performed adding 100 μl of 10 mM *p*-nitrophenyl phosphate at 37 °C for 1 h. The production of *p*-nitrophenol was measured colorimetrically at 410 nm. The results were normalized on the basis of LMW-PTP content.

Confocal Microscopy—6×10^4 C2C12 cells were grown on glass coverslips in growth medium. After 24 h to induce differentiation C2C12 cells were treated with DM for 6 days. Cells were then washed with phosphate-buffered saline and fixed in 3% paraformaldehyde for 20 min at 4 °C. Fixed cells were permeabilized with three washes with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) and then blocked with 5.5% horse serum in TBST for 1 h at room temperature. Cells were then incubated with specific primary antibodies, diluted 1:100 in TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) overnight at 4 °C. Cells were then washed once with TBST and once with TBST with 0.1% bovine serum albumin and incubated with secondary antibodies (diluted 1:100) for 1 h at room temperature in TBST with 3% bovine serum albumin. After extensive washes in TBST cells were mounted with glycerol plastine and observed under a laser scanning confocal microscope (Bio-Rad MRC 1024 Es, Hercules, CA). A series of optical sections (512×512 pixels) were taken through the depth of the cells with a thickness of 1 μm at intervals of 0.2 μm.

RESULTS

LMW-PTP Expression Is Positively Regulated by Cell Density—Recent data suggest that PTPs may be involved in contact inhibition since cell-contact is relieved by treatment with the PTPs inhibitor orthovanadate (11). Previous results showed that cell density increases the LMW-PTP level in NIH3T3 cell line (15). To address the possibility whether cell confluence may up-regulate LMW-PTP also in other cell types, we analyzed C2C12 myoblasts at different degrees of density. C2C12 cells were plated at different densities (5×10^5 and 1.5×10^6 cells in a 10-cm dish, respectively) and, after 24 h, LMW-PTP level was assayed by Western blot. Results (Fig. 1*A*) show that the LMW-PTP level increases in cells seeded at higher density with respect to cells seeded at lower concentration. These data demonstrate that cell-density positively regulates LMW-PTP expression and that the protein level increases in parallel with cell density in C2C12 cells. The same results have been obtained in PC12 pheocromocytoma cells, where a great increase of LMW-PTP level was observed in confluent cells (Fig. 1*B*) indicating that this effect is very likely a general phenomenon common to different cell types.

Tyrosine Phosphorylation Level of PDGFr Decreases in Dense Cultures—Impaired growth factor signaling and increased PTP activity have been described by several authors as a characteristic of contact-inhibited cells (16, 17). Since PDGFr is an *in vivo* LMW-PTP substrate, we wanted to study the phosphorylation level of this receptor in C2C12 dense cultures. C2C12 cells were plated at different densities (5×10^5 and 1.5×10^6 cells in a 10-cm dish, respectively). After 24 h PDGFr was immunoprecipitated and Western blot analysis was first performed with anti-phosphotyrosine antibodies and then with anti-PDGFr antibodies. Band intensities of both anti-phospho-

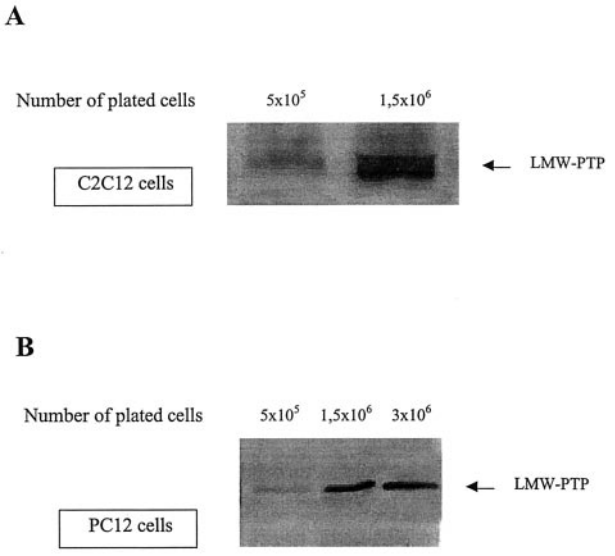

FIG. 1. **LMW-PTP expression level in sparse and dense cultures.** *A*, 5×10^5 and 1.5×10^6 C2C12 cells were seeded in 10-cm dishes. After 24 h LMW-PTP level was assayed by Western blot analysis. *B*, 5×10^5, 1.5×10^5 and 3×10^6 PC12 cells were seeded in 10-cm dishes. After 24 h LMW-PTP expression level was assayed by Western blot analysis. The same results were obtained in three independent experiments.

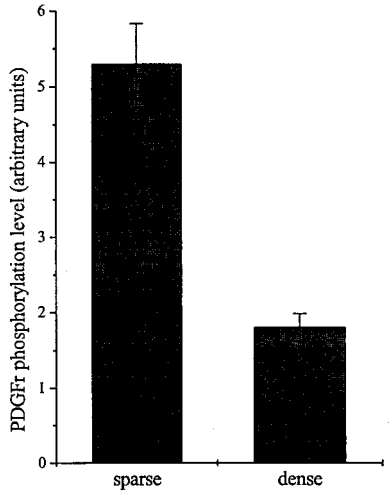

FIG. 2. **Tyrosine phosphorylation level of PDGFr in sparse and dense cultures.** 5×10^5 and 1.5×10^6 C2C12 cells were seeded in 10-cm dishes. Cells were serum starved for 24 h and then stimulated with 30 ng/ml PDGF-BB for 10 min. PDGFr was immunoprecipitated and an anti-phosphotyrosine immunoblot was performed. The membrane was then reprobed with anti-PDGFr antibodies for normalization. These results are the mean of six independent experiments.

tyrosine and anti-PDGFr immunoblots were quantitated by densitometric analysis. The ratio between these two values is reported in Fig. 2 as PDGFr phosphorylation level. Results clearly demonstrate that the PDGFr phosphorylation level decreases in C2C12 dense cultures with respect to sparse ones (although the PDGFr expression is comparable in control and differentiated cells). These findings suggest that the increase of LMW-PTP observed in these conditions could be responsible for this dephosphorylation.

LMW-PTP Expression Is Positively Regulated by Cell Differentiation—Cell differentiation is a coordinated process that includes cell cycle exit and the expression of unique genes to specify tissue identity. In particular, myogenesis requires cell-cell contact to permit the fusion of individual myoblasts into multinucleate myotubes. Since we have demonstrated that cell confluence enhances the intracellular LMW-PTP level, we wanted to assess whether muscle differentiation influences LMW-PTP expression. In particular, we performed a time course evaluation of LMW-PTP level during myogenesis. We cultured C2C12 cells in DM for 2, 4, and 6 days and then we assayed the level of LMW-PTP by Western blot analysis (Fig. 3*A*). Muscle differentiation of C2C12 cells was evaluated by analyzing the expression of caveolin 3 as a marker for myogenic differentiation (Fig. 3*B*). Results show that, for the analyzed period, the amount of LMW-PTP greatly increases only after 2 days of differentiation and reaches the maximum level observed after 6 days of differentiation. Furthermore, these findings indicate that the LMW-PTP increase is an early event in muscle differentiation. Hence, myogenesis, as well as cell confluence, induces LMW-PTP increase. To assess whether this phenomenon is common to other models of cell differentiation, we performed a time course evaluation of LMW-PTP level during neurogenesis. We choose neuronal differentiation since it does not require cell-cell contact as myogenesis does. PC12 cells were differentiated for different times (2, 4, 5, 6, and 7 days, respectively) and a Western blot, using anti-LMW-PTP antibodies, was performed. In agreement to what was observed during muscle differentiation, we revealed a similar increase of LMW-PTP, as shown in Fig. 3*C*. In particular, the protein level increases after 2 days of differentiation in comparison with control cells and reaches a maximum after 5 days. Neuronal differentiation of PC12 cells was assayed by the analysis of the morphological changes occurring during cell differentiation. Fig. 3*D* shows 5 days differentiated PC12 cells: it is evident the loss of cells rounded shape and an extensive neurite outgrowth with respect to control ones. On the basis of these results, we conclude that both muscle and neuronal differentiation causes an induction of the intracellular LMW-PTP level.

Tyrosine Phosphorylation Level of PDGFr Decreases during Myogenesis—To address the question whether the increased level of LMW-PTP observed during muscle differentiation leads to PDGFr dephosphorylation, we studied the receptor phosphorylation level during myogenesis. Lysates from control and differentiated C2C12 cells were immunoprecipitated using anti-PDGFr antibodies and immunocomplexes were probed with anti-phosphotyrosine antibodies in a Western blot analysis. The membrane was then reprobed with anti-PDGFr antibodies for normalization. Densitometric analysis of the bands of two immunoblots was performed and the ratio between these two values is reported in Fig. 4*A* as PDGFr phosphorylation level. Results demonstrate that tyrosine phosphorylation of PDGFr is higher in undifferentiated cells, in comparison to differentiated C2C12 cells. On the basis of these results, we conclude that tyrosine phosphorylation of PDGFr decreases during muscle differentiation.

Since LMW-PTP levels increase during myogenesis, we wanted to study LMW-PTP association with its substrate PDGFr. Control and 6 days differentiated C2C12 cells, stimulated with or without PDGF for 10 min, were lysed and an immunoprecipitation was performed using anti-PDGFr antibodies. Treatment of the membrane with anti-LMW-PTP antibodies reveals that the association of LMW-PTP with PDGFr, which depends on receptor stimulation, increases in differentiated cells, in comparison to control, as shown in Fig. 4*B*. This result is in agreement with the increasing amounts of LMW-PTP observed during myogenesis.

Regulation of LMW-PTP Expression in C2C12 Cells—To verify if a transcriptional regulation is responsible for the increase of LMW-PTP expression during myogenesis, we performed Northern blot analysis. Equal amounts of total RNA of control and differentiated C2C12 cells was transferred to nitrocellulose

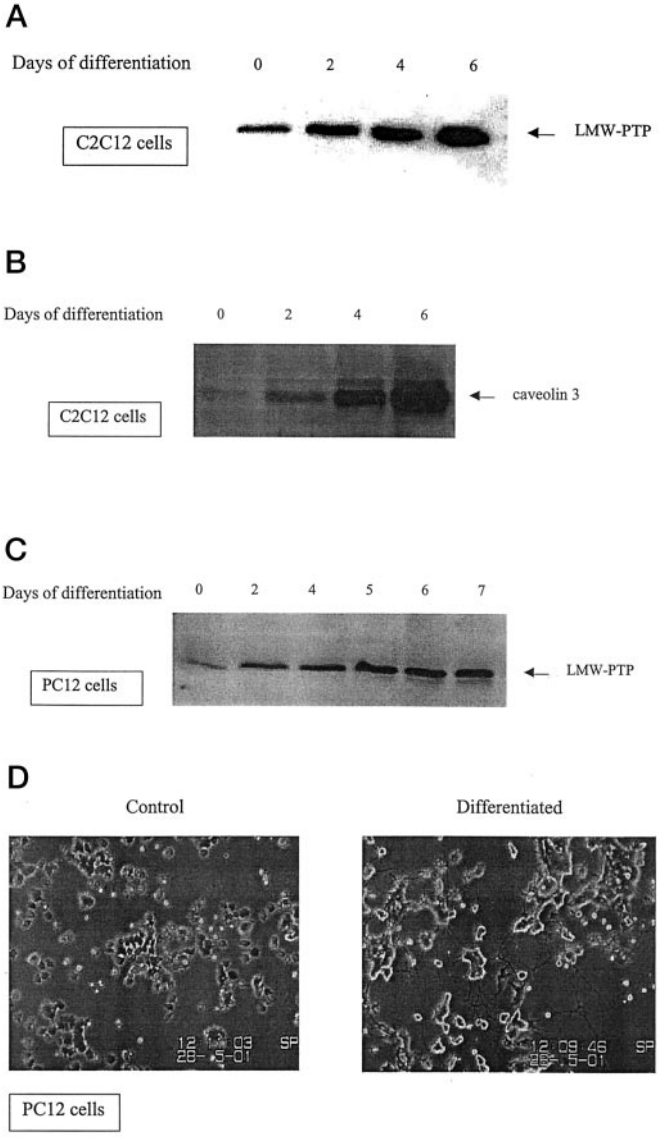

FIG. 3. **Up-regulation of LMW-PTP during differentiation.** *A*, time course of LMW-PTP expression during myogenesis. C2C12 cells were treated with or without DM for the indicated times. To assay the amount of LMW-PTP a Western blot analysis was performed. *B*, caveolin 3 expression during myogenesis. An immunoblot anti-caveolin 3 was performed with the same lysates. The amount of caveolin 3 was used as a marker of differentiation for C2C12 cells. *C*, time course of LMW-PTP expression during neurogenesis. PC12 cells were treated or not with DM for the indicated times and then a Western blot was performed to assay LMW-PTP amount. *D*, neuronal differentiation of PC12 cells. Control and 5 days differentiated PC12 cells are shown. In the figure point 0 indicates undifferentiated cells. The same results showed in this figure were obtained in four independent experiments.

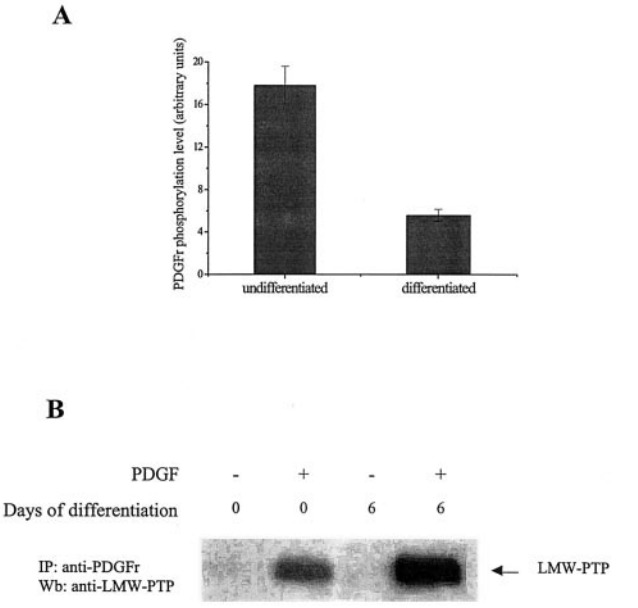

FIG. 4. **PDGFr tyrosine phosphorylation level and association of LMW-PTP with PDGFr during myogenesis.** *A*, PDGFr tyrosine phosphorylation level in myotubes. Control and 6 days differentiated C2C12 cells were serum starved for 24 h and then stimulated with 30 ng/ml PDGF-BB for 10 min. PDGFr was immunoprecipitated and membrane was probed first with anti-phosphotyrosine antibodies and then with anti-PDGFr antibodies. Densitometric analysis of the bands obtained in the two blots was performed. The ratio between these two values is reported in the histogram. These results are the mean of six independent experiments. *B*, LMW-PTP/PDGFr association in myotubes. C2C12 cells were treated with or without DM for 6 days, serum starved for 24 h, and then stimulated or not with 30 ng/ml PDGF-BB for 10 min. Anti-PDGFr immunocomplexes were then probed with anti-LMW-PTP antibodies in a Western blot analysis. In the figure point 0 indicates C2C12 undifferentiated cells. The same results were obtained in three independent experiments.

membrane and hybridized using murine LMW-PTP cDNA as probe. The results exclude any transcriptional regulation, since the level of specific mRNA is comparable in control and differentiated C2C12 cells (Fig. 5*A*).

On the basis of these results, we investigated whether the increase of LMW-PTP amount could be due to reduced degradation of the protein during differentiation. In particular, we analyzed the involvement of the proteasome machinery using the selective proteasome inhibitor MG-132. Undifferentiated C2C12 cells were treated for different times (4, 6, 9, and 15 h, respectively) with 50 μM MG-132 and an anti-LMW-PTP immunoprecipitation was performed. Immunoprecipitates were probed with anti-LMW-PTP antibodies in a Western blot analysis. Results (Fig. 5*B*) show that the LMW-PTP level is very similar in all samples, suggesting that the proteasome activity is not involved in post-translational control of LMW-PTP. These results suggest that the marked LMW-PTP increase observed during muscle differentiation is due neither to a transcriptional control nor to an inhibition of proteasome activity, indicating that most likely a translational control is involved.

Redox Regulation of LMW-PTP—Recently, we demonstrated that H_2O_2, endogenously produced after PDGF stimulation, inhibits LMW-PTP activity on both its phosphorylated substrates PDGFr and p190RhoGAP. Furthermore, we showed that the two cysteines present in the catalytic site of the protein are responsible of this redox regulation and that glutathione is probably the electron donor for LMW-PTP reduction/reactivation. Recent observations about the time course of the LMW-PTP redox state in the NIH3T3 cell line upon PDGF stimulation demonstrate that about 80% of LMW-PTP is already oxidized and inactivated 2 min after PDGF stimulation, reaches a maximal inactivation after 10 min, and recovers its activity after 40 min. The same experiment performed on C2C12 cells gave very similar results (10).

In this contest, we wanted to address the question whether LMW-PTP undergoes oxidative regulation during muscle differentiation. In particular, we studied the redox state of the protein in control and 6 days differentiated C2C12 cells with the 5′-F-IAA labeling method. After LMW-PTP immunoprecipitation, the immunoprecipitates were labeled with 5′-F-IAA and used for an immunoblot which was probed with anti-fluorescein antibodies (Fig. 6*A*). The blot was reprobed with anti-LMW-PTP antibodies (Fig. 6*B*) and band intensity of both anti-fluorescein and anti-LMW-PTP immunoblots were quan-

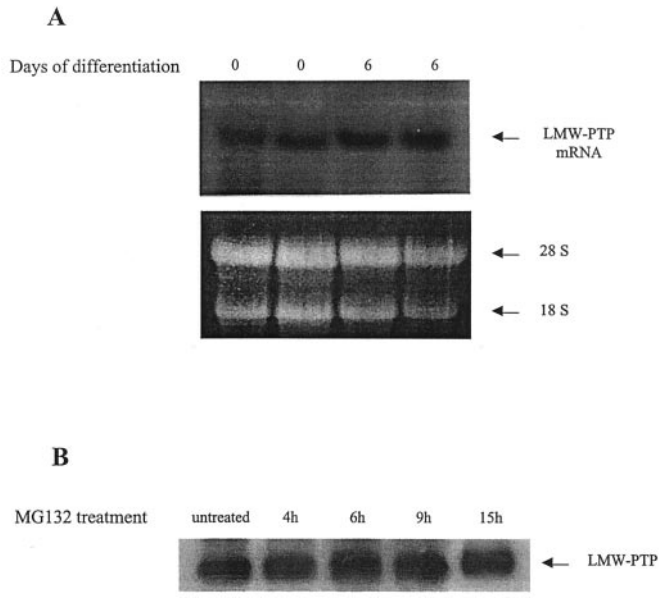

FIG. 5. **Regulation of LMW-PTP expression during myogenesis.** *A*, LMW-PTP mRNA level in control and 6 days differentiated C2C12 cells. Total RNA was used in duplicate in a Northern blot experiment using murine LMW-PTP cDNA as a probe. The specific LMW-PTP transcript is indicated. Ethidium bromide gel confirms equal loading of total RNA in each lane. 18 S and 28 S ribosomal RNA are indicated. Point 0 indicates undifferentiated cells. *B*, proteasome degradation of LMW-PTP in C2C12 cells. C2C12 cells were treated with 50 μM MG-132 for the indicated times. LMW-PTP was immunoprecipitated and immunocomplexes were probed with anti-LMW-PTP antibodies. The same results were obtained in three independent experiments.

titated. The ratio between these two values is reported in Fig. 6*C* as percentage of LMW-PTP reduction. Results clearly show that muscle differentiation leads not only to an increase in the amount of LMW-PTP, but also to an increase of the reduced form of the phosphatase. In fact, LMW-PTP appears more oxidized in control cells in comparison to differentiated cells. In this way, we show that myogenesis causes a shift from the oxidized to the reduced form of LMW-PTP, since a nearly 3-fold increase of the reduced form of the protein is observed during muscle differentiation.

A transient increase in the intracellular concentration of hydrogen peroxide has been reported as an important signaling event upon cell stimulation with epidermal growth factor and PDGF (18). Such an oxidative burst reversibly inhibits PTPs, thereby promoting the propagation of tyrosine phosphorylation signals (19). Recent evidence has shown a direct involvement of cell-cell contacts in the modification of intracellular redox environment in confluent cultures. In particular, epidermal growth factor-induced levels of intracellular oxygen radicals are significantly reduced in fibroblasts plated at high density in comparison to sparse cultures (11). It is likely that the impairment of ROS production is connected with growth inhibition conditions. As both cell-contact and differentiation involve growth inhibition, we studied the generation of intracellular ROS during myogenesis, a differentiation process that requires cell-cell contacts to form myotubes. Control and differentiated C2C12 cells were treated with the ROS-sensitive fluorescent probe dichlorofluorescein diacetate for 1 h and a cytofluorimetric analysis was performed. Results (Fig. 6*D*) show that levels of intracellular ROS are significantly lower in differentiated C2C12 cells in comparison to control ones.

Since oxidized PTPs are not functional anymore, we assayed LMW-PTP activity during myogenesis. LMW-PTP was immunoprecipitated from undifferentiated and differentiated C2C12 cells treated with DM for 2, 4, and 6 days. LMW-PTP immunoprecipitates were used to measure PTP activity of endogenous LMW-PTP. Results (Fig. 6*E*) clearly demonstrate that LMW-PTP activity increases during myogenesis, since after 6 days of differentiation the activity is about 4-fold greater in comparison to the undifferentiated cells. These data strongly suggest that the increase of LMW-PTP activity is probably due to the observed decrease of ROS during muscle differentiation. Taken together these data suggest that the decrease of intracellular ROS observed during myogenesis could be responsible for the maintenance of LMW-PTP in its reduced form.

LMW-PTP Localization in Myotubes—To study the localization of LMW-PTP in mature myotubes we performed a confocal microscopy analysis. Confocal imaging and pseudocolors attribution (Fig. 7, *A* and *C*) confirms that LMW-PTP increases during myogenesis as observed by Western blot analysis. Muscle differentiation was checked by anti-caveolin 3 immunostaining (Fig. 7*B*), which confirms the differentiation stage of C2C12 cells, on the basis of the high level of expression of caveolin 3 in myotubes. Control samples probed with secondary antibodies alone confirms the specificity of the signal (data not shown).

Protein relocalization has been recognized as a mechanism of protein function regulation, since it may allow the localization of a protein neighbor to its substrate. Confocal imaging demonstrates that LMW-PTP is subjected to a relocalization in myotubes. In particular, LMW-PTP shows cytoplasmatic distribution in myoblasts, while it accumulates along plasma membrane in myotubes. These data were confirmed by pseudocolors attribution to C2C12 differentiated cells. In fact, Fig. 7*C* shows a plasma membrane accumulation of LMW-PTP in myotubes in comparison to undifferentiated cells. These findings suggest that muscle differentiation causes both an increase and a relocalization of LMW-PTP in differentiated cells, in agreement with the increased association between this phosphatase and the membrane-associated PDGFr.

DISCUSSION

Contact-induced growth inhibition is a characteristic feature of normal cells grown in monolayer. Although the molecular events involved in this process remain largely unknown, the involvement of protein-tyrosine phosphatases in contact inhibition was supposed. Previous findings showed that total PTP activity is increased in lysates or membrane fractions derived from high density cultures (20, 21). The receptor-like protein-tyrosine phosphatase DEP-1 (density-enhanced phosphatase-1) increases gradually with cell density in WI-38 and AG1518 cells (22). Furthermore, a decreased tyrosine phosphorylation of PDGF-β and epidermal growth factor receptors are shown in cells of dense culture in comparison to sparse ones. These data suggest that this effect may be caused by an increased PTP activity toward the receptors in dense cultures, since this effect was reduced by pretreatment of cells with PTP inhibitors (11).

On the basis of these observations, we studied if LMW-PTP expression is influenced by cell density in C2C12 and PC12 cells. Results (Fig. 1, *A* and *B*) clearly show that in both cell lines, cell contact inhibition up-regulates LMW-PTP intracellular level, since the amount of the protein is greater in dense cultures with respect to sparse cells. In a previous work we demonstrated that LMW-PTP dephosphorylates *in vivo* PDGFr, thus regulating the mitogenic signaling cascade after PDGF stimulation (7, 8). The evaluation of PDGFr phosphorylation level in dense and sparse cultures showed that it appears more dephosphorylated in contact inhibited C2C12 cells in comparison to sparse cultures (Fig. 2). It is likely that the reduced PDGFr activation in contact-inhibited cells, observed in these conditions, is due, at least in part, to the increase of

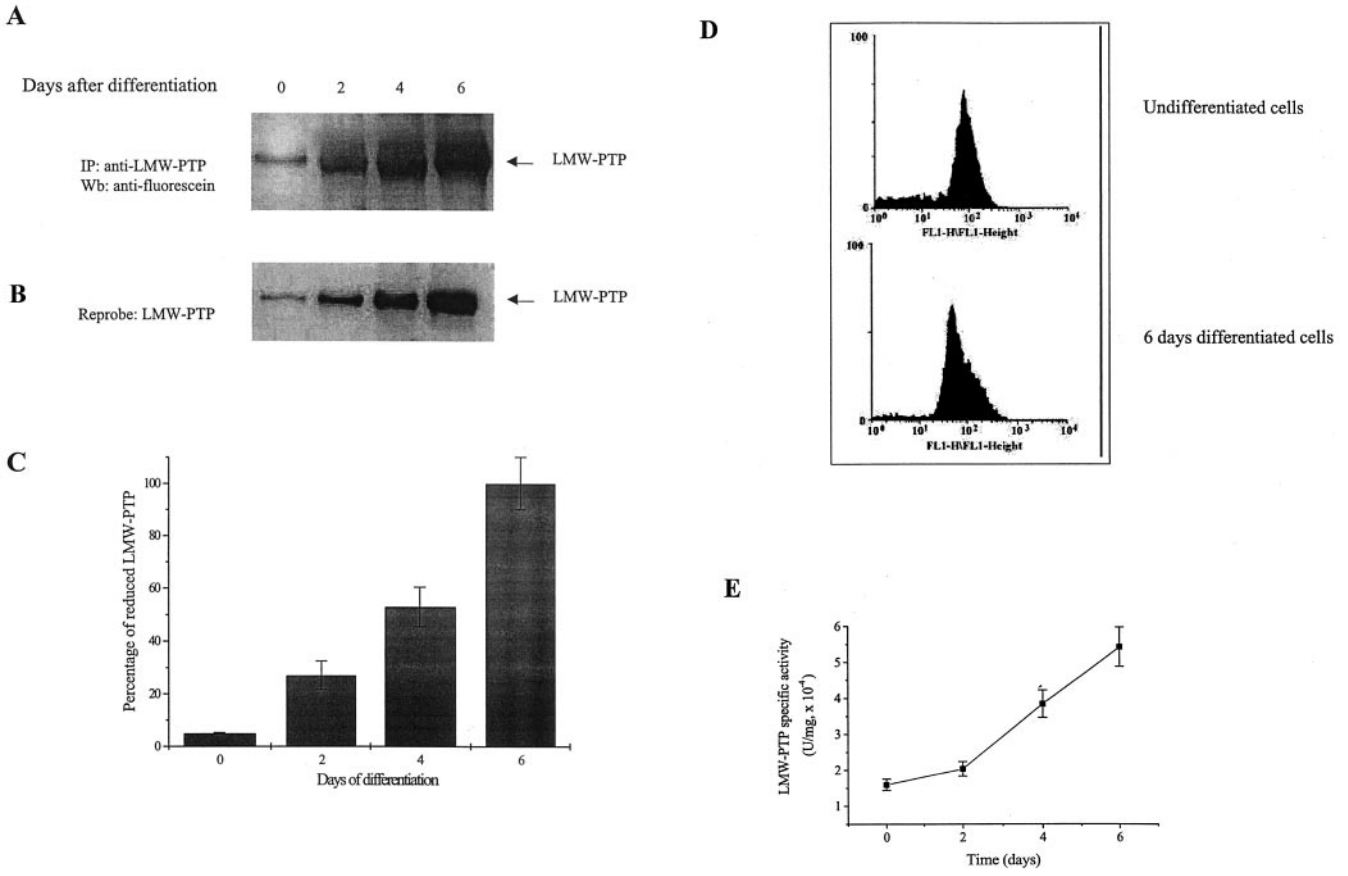

FIG. 6. **LMW-PTP redox state and intracellular ROS level during myogenesis.** *A*, LMW-PTP redox state in myotubes. LMW-PTP was immunoprecipitated from control and differentiated (for the indicated times) C2C12 cells. Immunocomplexes were treated with 5′-F-IAA as indicated under "Experimental Procedures." An anti-fluorescein immunoblot was then performed. Point 0 indicates undifferentiated cells. *B*, normalization was obtained by reprobing the blot with anti-LMW-PTP antibodies. *C*, percentage of reduced LMW-PTP. Band intensity of the two blots described were quantitated by densitometric analysis and the ratio of the two values was calculated. In the *ordinate* is reported the percentage of reduced form of LMW-PTP taking as 100% the reduction value of LMW-PTP after 6 days of differentiation. These results were obtained by five independent experiments. *D*, ROS intracellular level in myotubes. Undifferentiated and 6-day differentiated C2C12 cells were treated with dichlorofluorescein diacetate (*DCF-DA*) and a cytofluorimetric analysis was performed. *E*, LMW-PTP activity during myogenesis. LMW-PTP was immunoprecipitated from control and differentiated C2C12 cells at the indicated times and a PTP activity assay was performed using PNPP. These data are obtained by five independent experiments.

LMW-PTP. It has been reported that many PTPs, in addition to LMW-PTP, can regulate PDGFr tyrosine phosphorylation level, including DEP1, PTP1D, PTP1C, and PTPα. In particular, DEP1 displays site-selectively dephosphorylation of PDGFr for Tyr-763, -771, and -778 and not affecting the Tyr-857 regulatory site (23), while PTP1D dephosphorylates preferentially Tyr-771, -751, and -750 (24). Although it is possible that PDGFr dephosphorylation is an orchestrated cooperation among several PTPs, we point out that the only two PDGFr interacting PTPs, which have been recognized to increase in growth inhibition, are DEP1 and, herein, LMW-PTP. Cell-to-cell adhesion and cell-to-substrate adhesion are key processes in the organization of multicellular organisms. Cell adhesion must be sufficiently strong to maintain tissue architecture, and, at the same time, able to be promptly reduced to allow cells to move within tissues for morphogenesis, regeneration, and cell division. Furthermore, some differentiation processes, such as myogenesis, require cell-cell contacts. Cell differentiation is a physiological process, which is accompanied by cell cycle arrest and transcriptional activation of tissue-specific genes. In particular, myogenesis requires cell-cell contact in order to permit the fusion of individual myoblasts into multinucleate myotubes. Since we demonstrated that LMW-PTP is up-regulated in dense cultures, we studied whether cell differentiation regulates the protein expression. In particular, we compare myogenesis and neurogenesis, as differentiation models, taking into account that only muscle differentiation requires cell-cell contact to progress, while neurogenesis is independent from the formation of contacts between cells. Both myogenesis and neurogenesis are characterized by the initiation and maintenance of cell cycle exit, despite optimal cell growth conditions (22). Our results (Fig. 3, *A* and *C*) clearly show that cell differentiation, as well as cell confluence, positively regulates LMW-PTP expression, since the protein increases greatly either during myogenesis or neurogenesis.

The increase of LMW-PTP during muscle differentiation is most likely under translational control, as indicated by mRNA constant level and the absence of ubiquitin-mediated proteasomal degradation (Fig. 5, *A* and *B*). In addition, LMW-PTP is subjected to a relocalization to plasma membrane in response to differentiating stimuli, suggesting that this relocalization promotes the interaction of LMW-PTP with PDGFr (Fig. 7, *A* and *C*). In fact, we demonstrate that cell differentiation increases the LMW-PTP/PDGFr association in comparison with control cells, resulting in a strong decrease of tyrosine phosphorylation level of the receptor (Fig. 4, *A* and *B*). These data clearly demonstrate that LMW-PTP expression is controlled both by cell-cell contact and cell differentiation, suggesting that LMW-PTP is involved in the delivery of the antiproliferative signals connected to growth inhibition. Little is known about

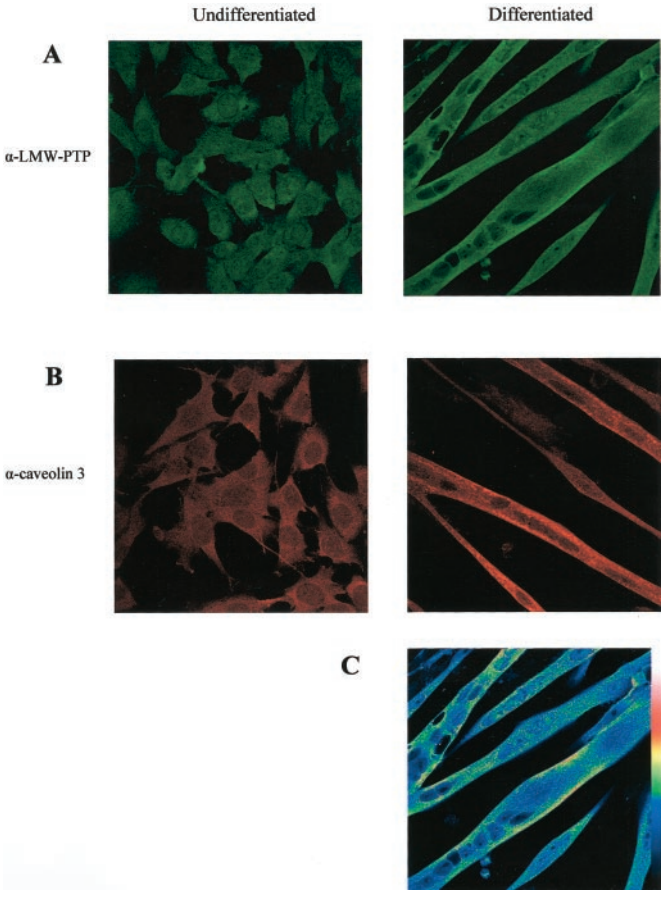

FIG. 7. **Confocal imaging of differentiated C2C12 cells.** C2C12 cells were seeded as described under "Experimental Procedures." *A*, LMW-PTP localization in myotubes. Undifferentiated and 6-day differentiated cells were treated with anti-LMW-PTP antibodies and then with Alexa Fluor™ 488 goat anti-rabbit secondary antibodies. *B*, expression of caveolin 3 in myotubes. Differentiation level of C2C12 was assayed by the expression of the caveolin 3. Control and 6-day differentiated cells were incubated with anti-caveolin 3 antibodies and then with goat anti-mouse IgG Texas Red conjugate secondary antibodies. *C*, pseudo colors attribution to differentiated cells. The green fluorescence intensity is visualized in pseudo colors, increasing from *blue* to *white* (palette bar to the right of *C*), and is superimposed to the phase contrast image (in *black*). The same results were obtained by three independent experiments.

the involvement of PDGF in cell differentiation. Previous results indicate that PDGF can regulate myoblasts proliferation and differentiation *in vitro*, suggesting that PDGF has a role in increasing the number of myoblasts during skeletal muscle regeneration by stimulating proliferation and/or inhibiting differentiation. In particular, a role of PDGF in the growth of satellite muscle cells, supporting the regeneration of adult muscle under stress and wound or trauma has been proposed (25). Recently, a marked decrease in the expression of PDGFr mRNA and cell surface protein levels following hormonal induction of differentiation was demonstrated in 3T3-L1 fibroblasts. This fact suggests that down-regulation of growth factor receptors might be one mechanism whereby differentiating cells become desensitized to the biological action of growth factors (26). In addition, a shift from PDGF-β toward PDGF-α in myotubes with respect to myoblasts, suggesting a role of the specific growth factor receptor isoforms in the muscle differentiation process has been observed (27).

The role of PTPs in the down-regulation of mitogenic signals has long been accepted. Many PTPs have been found associated with cell differentiation, as rVH6 or MKP1 (28, 29), PRL-1 (30), PTP-β2 (31), DEP1 (23), and PTP20 (33). Little is known about the role of these phosphatases with the exception of rVH6 and MKP1, which dephosphorylate specifically the activated MAPKs. The only PTP which acts on activated PDGFr among those mentioned above, is DEP1, which increases in dense cultures but not in differentiated cells, and displays site selectivity dephosphorylation of PDGFr (21, 23). Thus, DEP1 is likely to have a modulatory function on the activated receptor, rather than a general down-regulation role for the mitogenic signal. We recently reported that LMW-PTP acts on phosphorylated PDGFr directly on the Tyr-857, in the kinase activation loop (32), thus determining a general attenuation of PDGF signaling. In this light, the role of LMW-PTP in the down-regulation of PDGFr signaling, in cell contact inhibition and cell differentiation, could be different and wider with respect to DEP1.

The biochemical mechanisms underlying the intracellular delivery of antiproliferative signals are poorly understood in comparison to the well established signaling cascades leading to mitogenesis. Recent observations on the signaling properties of reactive oxygen species have opened a new perspective in the correlation between oxidative stress and cancer. Recent results demonstrate a novel mechanism of negative control of cell growth by cell-cell contact consisting of the reduced production of endogenous ROS and in impaired redox signaling by growth factor receptors. In particular, it has been demonstrated that growth arrest induced by cell confluence is associated, at least in part, to a decrease in the levels of intracellular ROS (11). As cell differentiation involves growth inhibition connected to cell cycle exit, we determined the amount of intracellular ROS during myogenesis. Results clearly demonstrate that muscle differentiation induces a great decrease of reactive oxygen species. These data support the hypothesis that the endogenous ROS concentration is an important element, which participates in the arrest of cell growth, either in cell-cell contact or during cell differentiation. Recently, we demonstrated that LMW-PTP is regulated by a redox mechanism, which involves the two cysteine residues of the catalytic site. LMW-PTP is transiently oxidized during PDGF signaling from endogenously produced hydrogen peroxide and is thereafter reduced, thus permitting the rescue of its catalytic activity. LMW-PTP oxidation/inhibition increases receptor activation while the following reduction/reactivation permits the dephosphorylation of PDGFr, thus down-regulating the signal (10). In this light, LMW-PTP redox regulation could be considered as a mechanism of fine tuning PDGFr signaling. Herein, we report that during muscle differentiation the degree of LMW-PTP reduction is enhanced (Fig. 6*A*). In this condition we confirm that the redox state of LMW-PTP is associated with enzymatic activity, as we demonstrated a 4-fold increase of LMW-PTP specific activity in myotubes with respect to undifferentiated cells (Fig. 6*E*).

These data suggest that during differentiation, LMW-PTP behaves as a growth arrest protein, as (i) its cellular content increases by translational regulation, (ii) its specific activity is up-regulated by a reductant environment, and (iii) its association and PDGFr dephosphorylation is therefore enhanced. Recent evidence suggest that also in cell contact inhibition (another condition requiring cell growth arrest) LMW-PTP may play a very similar role, behaving as a growth arrest protein.[2] In fact during cadherin-mediated cell adhesion, LMW-PTP level and its reduction state are increased, as well as during differentiation. All these results indicate that LMW-PTP plays an important role in the down-regulation of the signaling cascade after PDGF stimulation in different growth arrest conditions such as cell contact and differentiation.

[2] M. L. Taddei, P. Chiarugi, P. Cirri, F. Buricchi, T. Fiaschi, E. Giannoni, D. Talini, L. Formigli, G. Raugei, and G. Ramponi, manuscript in preparation.

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Low Molecular Weight Protein-tyrosine Phosphatase Is Involved in Growth Inhibition during Cell Differentiation

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