Articles

Modulation of STAT5 Interaction with LMW-PTP during Early Megakaryocyte Differentiation

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ABSTRACT: STATs are involved in a variety of cellular processes, including cell proliferation and differentiation. They are activated through tyrosine phosphorylation, which promotes their dimerization via SH2 domains. We have demonstrated previously that in DAMI megakaryoblastic cells LMW-PTP dephosphorylates STAT5, interacting with an essential sequence of nine amino acids in its C-terminal region. Here we characterize STAT5 tyrosine phosphorylation and its interaction with LMW-PTP during early phorbol-12-myristate-13-acetate-induced megakaryocyte differentiation; these processes show clear dependence on STAT5 threonine phosphorylation. Since protein kinase C inhibition prevents phorbol-12-myristate-13-acetate-induced STAT5 threonine phosphorylation and association with LMW-PTP, it follows that these processes depend on protein kinase C activity. By using a Thr757/Val mutant of STAT5 we also demonstrate that the 757 serine/threonine conserved residue, which is in the STAT5A region involved in the interaction with LMW-PTP, is essential for such an association, though its phosphorylation is not necessary.

STATs[1] are latent cytoplasmic transcription factors that are activated by a variety of cytokines, such as activation upon tyrosine phosphorylation by Janus kinases (JAKs). Following dimerization via SH2 domains, STATs migrate into the nucleus, where they interact with specific DNA sequences to stimulate transcription (*1*).

The STAT family consists of seven members (1, 2, 3, 4, 5A, 5B, and 6) in mammals, which show a great degree of homology except for their C-terminal region, the transactivation domain or TAD, whose variability determines the specificity in the induction process of gene expression. This region contains the DNA interaction domain and the tyrosine residue whose phosphorylation is critical for STATs dimerization. Moreover, it was hypothesized that this region in STAT5 is responsible for the interaction with a phosphotyrosine phosphatase that is capable of dephosphorylating it (*2*). We accordingly demonstrated that LMW-PTP associates with and dephosphorylates murine STAT5A, interacting with its 749−757 C-terminal region (corresponding to region 750−758 of human protein) (*3*). Existence of a regulatory mechanism capable of modulating the interaction of the two proteins may be hypothesized, since otherwise STAT5 would constantly be under the negative control of LMW-PTP.

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[1] Abbreviations: LMW-PTP, low molecular weight protein tyrosine phosphatase; STAT, signal transducer and activator of transcription; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; FCS, fetal calf serum; BCA, bicinchoninic acid solution; PBS, phosphate-buffered saline.

STATs transcription factors are involved in a variety of cellular processes, including cell proliferation, differentiation, and prevention of apoptosis. STAT5 activation appears to be fundamental for the survival of cells as they stop replicating and become committed to differentiation, probably by inducing the transcription of the antiapoptotic Bcl-family genes (*4*, *5*). In this respect, the orchestrated activation of PKC and subsequently of MAP kinases and STAT5 represents a typical feature of megakaryocyte differentiation, triggered either by physiological or pharmacological agonists (*6*, *7*).

Since STAT5 is reportedly activated constitutively in proliferating DAMI megakaryoblastic cells (*8*), the transition to PMA-induced differentiation may represent a delicate phase in STAT5 regulation. We report here our results concerning the relation between STAT5 activation and its association to LMW-PTP at the onset of megakaryocyte differentiation, and we provide evidence of a regulatory threonine phosphorylation of STAT5 modulating the interaction of the two proteins.

MATERIALS AND METHODS

Cell Culture. Growth and differentiation of DAMI human megakaryoblastic cells have been described previously (*9*). Cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated foetal calf serum (FCS) (complete medium). NIH/3T3 murine fibroblasts overexpressing LMW-PTP were obtained and described as in ref (*10*). They were cultured in DMEM supplemented with 10% FCS.

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Plasmids and Transfection. pXM-Thr-stat5 and pXM-Val-stat5 plasmids, coding for wild type or Thr757/Val mutant ovine STAT5A, were kindly provided by M. Vore (Graduate Center for Toxicology, University of Kentucky, Lexington, KY) (*11*). Transient transfection of NIH/3T3 cells was performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer instructions. A 10 or 20 μg amount of each plasmid was used to transfect cells in a 60-mm Petri dish, in DMEM without FCS. After 4 h the medium volume was doubled with DMEM containing 20% FCS (final serum concentration: 10%). The cells were lysed 24 h post transfection.

Antibodies and Reagents. Rabbit polyclonal anti-LMW-PTP antibodies were generated in our laboratory (*12*). Rabbit polyclonal anti-STAT5 (C17 and H134), as well as monoclonal anti-phosphotyrosine antibodies (PY20) and rabbit polyclonal anti-PKCα (C20), was obtained from St. Cruz Biotech. Rabbit polyclonal anti-phosphothreonine antibodies and peroxidase-conjugated anti-rabbit antibodies and Chemilucent (chemiluminescent detection substrate) were obtained from Chemicon. The no-cross-reactivity of this anti-pThr antibody toward pSer and pTyr was tested by the producer and confirmed by others (*13*). Peroxidase-conjugated anti-mouse antibodies and bicinchoninic acid solution (BCA) were obtained from Pierce. Alexafluor 488-labeled chicken anti-rabbit IgG antibodies were obtained from Molecular Probes; propidium iodide was obtained from Fluka. PKC inhibitor Ro 31-8220, protein A-Sepharose, and PMA were obtained from Sigma. 750−758 STAT5A peptide, corresponding to the sequence GEFDLDETM, and the corresponding phosphopeptide (GEFDLDE[pT]M) were synthesized by GenScript Corporation, (Piscatway, NJ). One-step IP Western blot kit was obtained from GenScript, too.

Cell Lysis, Immunoprecipitation, and Western Blotting. Cells were washed twice with phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1 mM sodium vanadate, 20 mM NaF, 10 mM sodium pyrophosphate, 10 μg/mL leupeptin, 20 μg/mL aprotinin, 1 mM PMSF) for 30 min on ice. When required, cells were treated with 100 nM PMA or 2 μM Ro 31-8220 for various lengths of time prior to lysis.

For immunoprecipitation, lysates were clarified at 10 000*g* for 10 min, and 200 μg of proteins, determined with the BCA method, was incubated with specific antibodies (1 μL of anti-LMW-PTP, 5 μL of anti-STAT5) for 1 h on ice. Immunocomplexes were collected on 20 μL of protein A-Sepharose o/n at 4 °C, washed with PBS, and eluted with boiling Laemmli buffer (66 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10 mM EDTA, 10% (w/v) glycerol, and bromophenol blue). They were analyzed through 13.5% SDS-PAGE and immunoblotting with specific antibodies. Immunorecognition with different antibodies on the same membrane was preceded by the stripping procedure. The immunocomplexes were detected using the ChemiDoc system (Bio-Rad) after the use of Chemilucent substrate. Densitometric analysis of bands was performed with the QuantiScan software (Bio-Rad).

Immunofluorescence Analysis. The cells were plated on glass cover slips and after 48 h were stimulated with 100 nM PMA, with or without 2 μM Ro 31-8220, or left untreated. Untreated cells and Ro 31-8220-treated cells mostly remained suspended, so they were cytocentrifuged in order to facilitate the analysis. Cells were washed with PBS, fixed with 3% *p*-formaldheyde, permeabilized with 0.1% Triton X-100, and incubated overnight at 4 °C with rabbit anti-LMW-PTP (1:200) or anti-STAT5 (H134; 1:200) antibodies. The cells were then stained with Alexafluor 488-labeled anti-rabbit; propidium iodide was used to visualize nuclei. Cells stimulated for 1 h with PMA were also used in order to determine the specificity of anti-rabbit antibodies, omitting primary antibodies from the overnight incubation step. Images were obtained using a Leica SP5 laser scanning confocal microscope.

Competition Experiment. DAMI cells were stimulated for 15 min with 100 nM PMA and lysed in RIPA buffer. A 400 μg amount of total proteins was incubated with 100 μg of BSA or 100 μg of STAT5 peptide or the same amount of STAT5 phosphopeptide for 30 min on ice. Immunoprecipitation was then undertaken, adding 2 μL of anti-LMW-PTP antibodies and shaking for 2 h at 4 °C. Immunocomplexes were collected on 30 μL of Protein A-Sepharose for 45 min at 4 °C, an aliquot of the immunodepleted lysate was recovered, and Sepharose beads were washed three times with PBS and eluted in boiling Laemmli buffer. Immunodepleted lysates and eluates were analyzed by means of 13.5% SDS-PAGE and western blotting, with anti-LMW-PTP and anti-STAT5 antibodies, using the one-step IP Western blot kit.

RESULTS

DAMI megakaryoblastic cells differentiate in response to PMA treatment, acquiring characteristic morphological traits: they adhere to the culture surface and increase considerably in size as polyploidization takes place.

Several authors have reported an early participation of STAT5 during cell differentiation (*15*, *16*). We therefore sought to determine its activation, involving tyrosine phosphorylation, during the first phases of DAMI differentiation. Cells were plated onto culture dishes in complete medium and after 48 h, and 100 nM PMA was added. The cells were lysed at regular intervals, and STAT5 was immunoprecipitated so as to determine, through western blotting, its tyrosine phosphorylation. As seen in Figure 1, STAT5 tyrosine phosphorylation remains undetectable during the first 20 min of PMA stimulation and begins to increase after 45 min.

Having demonstrated that LMW-PTP interacts with STAT5 in undifferentiated cells (*3*), we considered whether LMW-PTP is involved in the timing of STAT5 activation during the early phase of PMA-induced megakaryocyte differentiation. Accordingly, we first investigated the time course of the interaction of LMW-PTP and STAT5.

Cells were again stimulated with PMA for increasing time periods; they were lysed and underwent immunoprecipitation with anti-LMW-PTP or anti-STAT5 antibodies. Western blot analysis with anti-STAT5 antibodies showed that there was a substantial increase in STAT5 coimmunoprecipitation with LMW-PTP after 15 min of treatment, relative to control unstimulated cells. In contrast, after 1 h of PMA treatment, a dissociation of the two proteins was evident (Figure 2, left panels). We did not investigate the reciprocal coimmunoprecipitation since we had already verified that the antibody directed against the STAT5 C-terminus (which must be used

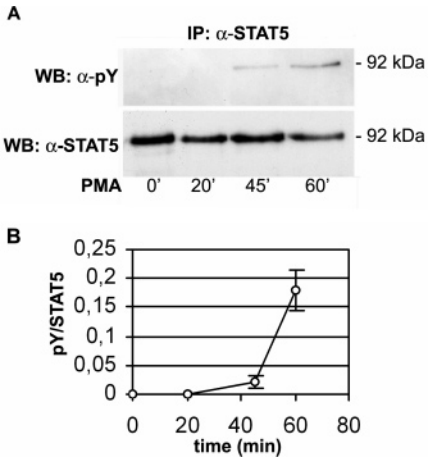

FIGURE 1: STAT5 phosphorylation during stimulation of DAMI cells with PMA. A: Cells were treated with 100 nM PMA for up to 1 h. Immunoprecipitates obtained with anti-STAT5 antibodies were analyzed by western blotting with anti-pY antibodies and, following membrane stripping, with anti-STAT5 antibodies. B: Densitometric analysis of bands was performed and the pY/STAT5 ratio was calculated. Data represents means ± SEM for $n = 3$.

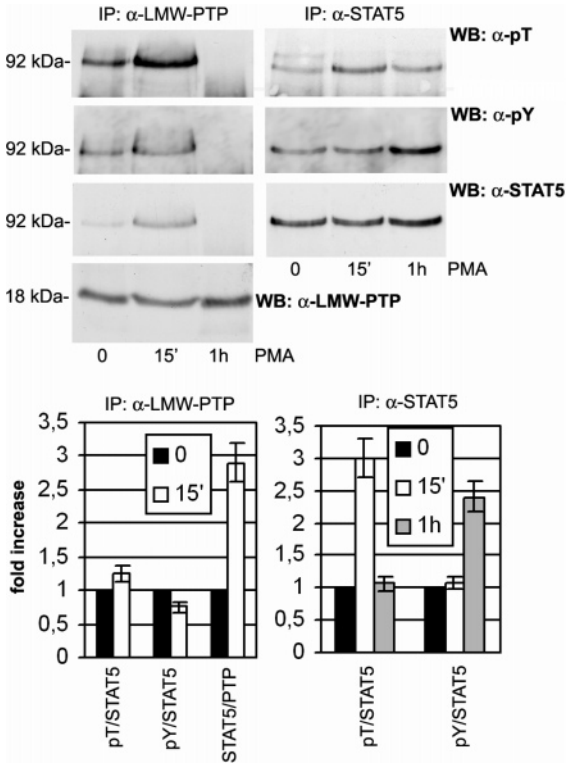

FIGURE 2: Pattern of STAT5 association with LMW-PTP and its threonine- and tyrosine-phosphorylation during PMA treatment. DAMI cells were treated with 100 nM PMA for up to 1 h, and immunoprecipitates were obtained with anti-LMW-PTP or anti-STAT5 antibodies. Western blotting analysis was performed with anti-pT, anti-STAT5, anti-pY, and anti-LMW-PTP antibodies. Following chemiluminescent detection, densitometric analysis of bands was undertaken; the pY/STAT5, pT/STAT5, and STAT5/PTP ratios were calculated and normalized with respect to the value at time zero. Histograms show means ± SEM for $n = 3$.

to immunoprecipitate STAT5) displaced LMW-PTP (*3*). The specificity of STAT5 coimmunoprecipitation with LMW-PTP has been demonstrated previously using these same antibodies (*3*).

Analysis of the tyrosine phosphorylation of total STAT5, which was immunoprecipitated with anti-STAT5 antibodies, clearly showed that it increased after 1 h (Figure 2). Looking at the pY/STAT5 ratio for STAT5 coimmunoprecipitated with LMW-PTP (see Figure 2, bottom left), we observe that it decreased slightly after 15 min of PMA stimulation, concomitantly with the increase in the association of the two proteins. It proved impossible to estimate the pY/STAT5 ratio after 1 h of PMA stimulation, since no STAT5 coimmunoprecipitated with LMW-PTP after prolonged PMA treatment. These data suggest a regulatory event occurring during the first 15 min of PMA stimulation, promoting an increase in STAT5 association with LMW-PTP, which might be involved in maintaining low STAT5 tyrosine phosphorylation.

Since brief PMA treatment is known to activate PKC, we hypothesized that this causes a modulation of STAT5 phosphorylation. Western blot analysis with anti-phosphoserine antibodies did not reveal any significant change in STAT5 phosphorylation (at least during the time course examined; data not shown). In contrast, by performing immunorecognition with anti-phosphothreonine antibodies, we found the threonine phosphorylation of STAT5, which was very faint in unstimulated cells but which increased after 15 min of PMA treatment and subsequently decreased after 1 h (Figure 2, upper panels). This threonine phosphorylation pattern paralleled that of STAT5 association with LMW-PTP. Densitometric analysis of bands revealed that a 3-fold increase in the threonine phosphorylation of total STAT5 was associated to a 3-fold increase in STAT5 coimmunoprecipitation with LMW-PTP (Figure 2, histograms).

The initial increase and subsequent decrease in STAT5 threonine phosphorylation and its association with LMW-PTP can result from activation and inhibition of PKC, which take place at around 15 min and 1 h of PMA stimulation, respectively. To demonstrate the direct involvement of PKC in these events, cells were again stimulated with PMA for 15 min, with or without the specific PKC inhibitor Ro 31-8220 (Figure 3A,B). Clearly, Ro 31-8220 prevents any PMA-elicited increase in STAT5 threonine phosphorylation and in its coimmunoprecipitation with LMW-PTP. In this case also, a 2.5 fold increase in STAT5 threonine phosphorylation following 15 min of PMA stimulation was associated with a similar increase in its interaction with LMW-PTP (Figure 3B). No differences were observed in LMW-PTP and STAT5 expression levels (Figure 3A). Furthermore, when cells were treated with PMA for 1 h, a decrease in cellular content of PKCα was evident, relative to untreated cells (Figure 3C) and consistent with PKC downregulation through proteolysis stemming from prolonged PMA stimulation (*17*). Again, no variations in LMW-PTP expression were seen (Figure 3C lower panel).

These data confirm the direct involvement of PKC in STAT5 threonine phosphorylation and further confirm the relation between this phosphorylation and the association of STAT5 with LMW-PTP.

One of the first events taking place during PMA-induced megakaryocyte differentiation is cell adhesion (after 15 min), followed by the acquisition of a spread phenotype (1-4 h). We demonstrated previously that adhesion events promote LMW-PTP redistribution from the cytosol to the cytoskeleton in NIH/3T3 fibroblasts (*18*). We then considered whether changes in cellular architecture could determine changes in the distribution of LMW-PTP and STAT5 during PMA stimulation. Cells were examined after immunofluorescence

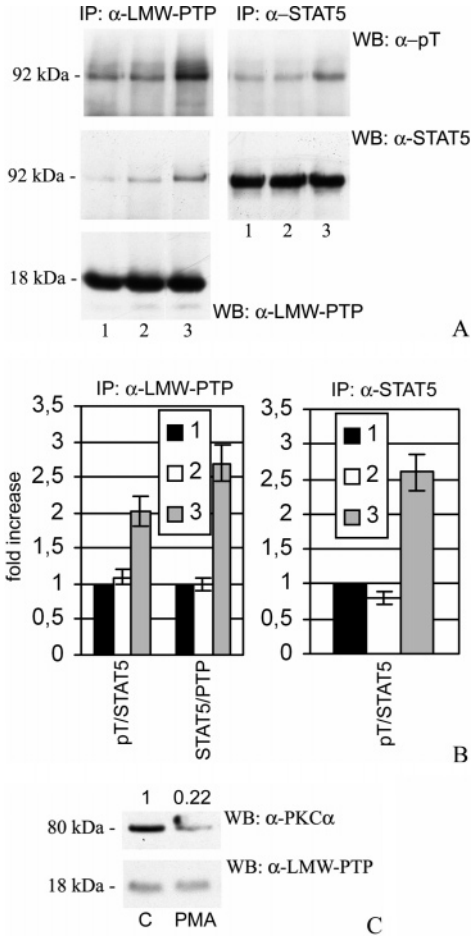

FIGURE 3: PKC inhibition reduces both STAT5 threonine phosphorylation and its association with LMW-PTP. A: Cells were treated with vehicle (lane 1), with 100 nM PMA and 2 μM Ro 31-8220 (lane 2), or with 100 nM PMA alone (lane 3) for 15 min. Immunoprecipitates were obtained with anti-LMW-PTP or anti-STAT5 antibodies and were analyzed by western blotting with the antibodies indicated. B: Densitometric analysis of bands was undertaken, and the pT/STAT5 and STAT5/PTP ratios were calculated and normalized with respect to the control value (lane 1). Histograms show means ± SEM for $n = 3$. C: Prolonged PMA stimulation causes a decrease in the PKC protein level. Cells were treated with 100 nM PMA (PMA) or with vehicle (C) for 1 h. Total lysates were subjected to western blotting with anti-PKCα and anti-LMW-PTP antibodies. The lane numbers represent the intensity of PKCα bands, normalized with respect to the control. This experiment was repeated three times, giving similar patterns.

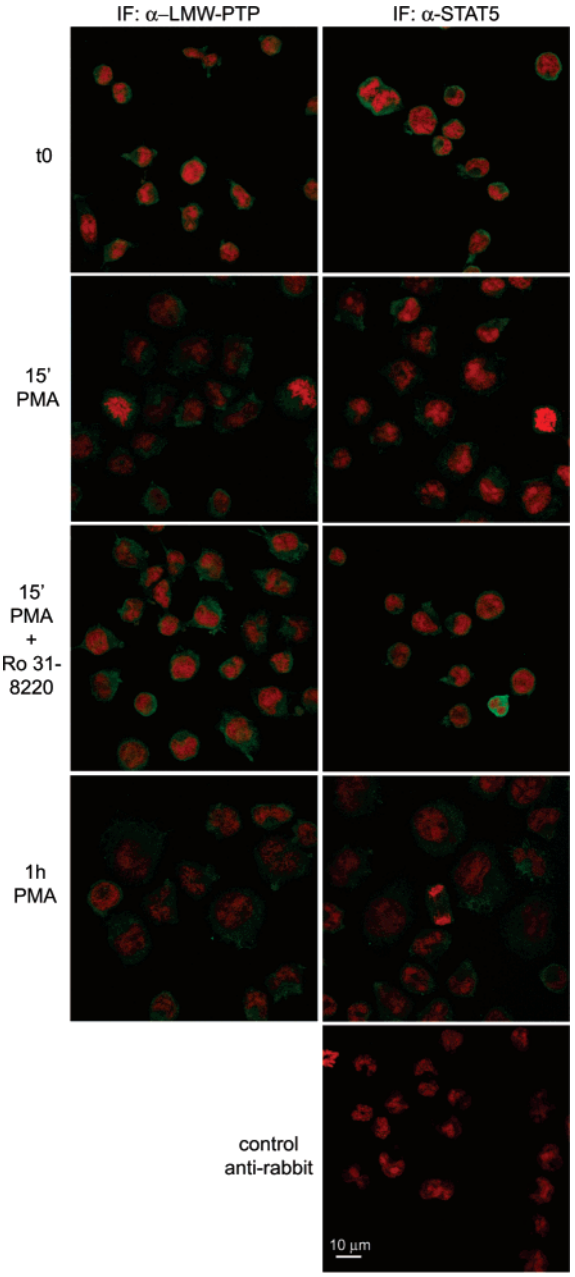

FIGURE 4: Immunofluorescence analysis of LMW-PTP and STAT5 intracellular distribution during PMA stimulation. Cells were treated with 100 nM PMA for 15 min (with or without 2 μM Ro 31-8220) or 1 h, or left untreated (t0) and were then fixed and stained with primary antibodies and Alexafluor 488 secondary antibodies. Nuclei were stained with propidium iodide. Cells in "control anti-rabbit" panel were incubated with anti-rabbit antibodies in the absence of primary antibodies.

staining with anti-LMW-PTP and anti-STAT5 antibodies, and the result shown in Figure 4 clearly demonstrates that LMW-PTP and STAT5 were diffusely distributed into the cytosol under all experimental conditions. Untreated cells, as well as cells concomitantly treated with PMA and Ro 31-8220, were cytocentrifuged onto glass cover slips before fixing and staining; they would otherwise remain in suspension, and this is why they appear somewhat spread. We can then exclude the possibility that the association of the two proteins after 15 min of PMA stimulation and their dissociation during the concomitant treatment with PKC inhibitor or after 1 h of PMA stimulation depend on a different intracellular distribution.

We have demonstrated previously that LMW-PTP interacts with the 749−757 C-terminal region of murine STAT5A (corresponding to region 750−758 of human protein) (*3*). This region contains a conserved serine/threonine residue at position 756 (757 of human protein), which seems to be highly important for the regulation of STAT5 activity. In fact, this mutation of Thr to Val causes an increase in the STAT5A transactivation potential (*11*). We then considered whether Thr757 phosphorylation was the critical regulatory event we were looking for. We first tested the ability of a Thr757/Val mutant of STAT5A to associate LMW-PTP. DAMI cells are refractory to transient transfection, so we chose NIH/3T3 cells overexpressing LMW-PTP for this purpose. The cells were transiently transfected with a plasmid encoding wild type or Thr757/Val mutant STAT5A and were then lysed and subjected to immunoprecipitation with anti-LMW-PTP or anti-STAT5 antibodies. Despite a similar

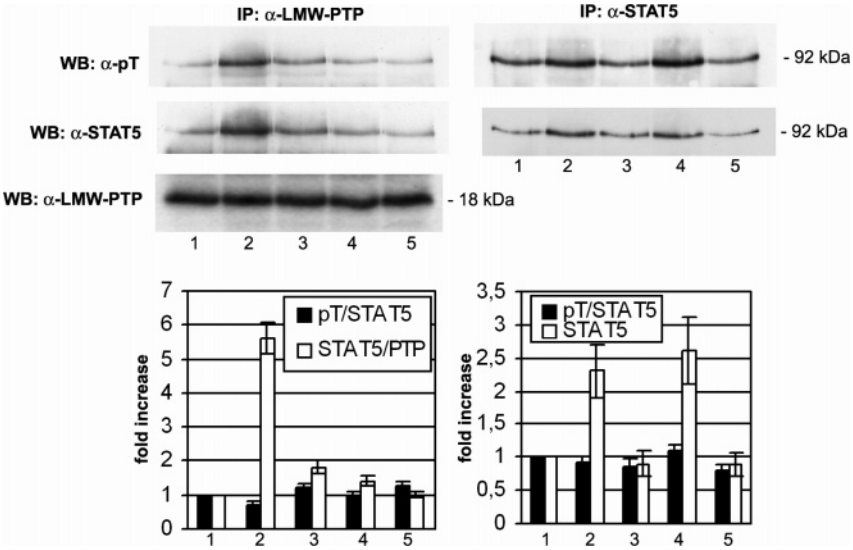

FIGURE 5: Thr757/Val mutation of STAT5 impairs its association with LMW-PTP. NIH/3T3 murine fibroblasts were transiently transfected with pXM-Thr-stat5 or pXM-Val-stat5 plasmids. Immunoprecipitates were obtained with anti-LMW-PTP or anti-STAT5 antibodies, and western blotting analysis was undertaken with anti-STAT5 and anti-LMW-PTP antibodies. 1: control cells; 2: 20 μg pXM-Thr-stat5; 3: 10 μg pXM-Thr-stat5; 4: 20 μg pXM-Val-stat5; 5: 10 μg pXM-Val-stat5. Densitometric analysis of bands was performed, and the pT/STAT5 and STAT5/PTP ratios were calculated. The intensity of transfectants was normalized with respect to controls (lane 1); means ± SEM for $n = 3$ are reported.

overexpression of both wild type and Thr757/Val STAT5, we observed an increase in STAT5 association with LMW-PTP only in cells transfected with wild type STAT5, whereas the level of association in cells transfected with Thr757/Val mutant was similar to that of controls (Figure 5). The residual coimmunoprecipitation of STAT5 with LMW-PTP that may be observed in NIH/3T3 cells overexpressing Thr757/Val STAT5 represents wild type STAT5 naturally expressed by these cells; in fact the intensity of such association is comparable to that of control cells. This result demonstrates that Thr757, whose mutation determines an increase in STAT5 activity (*11*), is essential for STAT5 interaction with LMW-PTP.

We then studied the threonine phosphorylation of wild type and Thr757/Val STAT5 under these experimental conditions. Immunorecognition with anti-phosphothreonine antibodies confirmed the expected threonine phosphorylation of LMW-PTP-associated wild type STAT5 (Figure 5, left panels). Analysis of total STAT5 revealed that wild type and Thr757/Val mutant were threonine phosphorylated to a similar extent (Figure 5, right panels). This suggests that, under such conditions, Thr757 was not phosphorylated; otherwise the threonine phosphorylation level of wild type STAT5 should be significantly higher than that of Thr757/Val mutant, resulting from the sum of the phosphorylation of Thr757 and of another unknown threonine residue that is also present in Thr757/Val mutant. We then considered whether STAT5 interaction with LMW-PTP depends on two distinct prerequisites: (a) the presence of Thr757; (b) the phosphorylation of a different threonine residue.

To settle the contribution of Thr757 phosphorylation in the regulation of STAT5 and LMW-PTP interaction, we set up the following experiment. We hypothesized that, in view of the essential role of the 750−758 region of STAT5A for its interaction with LMW-PTP, a peptide corresponding to this region competes with the whole protein for its association with the phosphatase. If Thr757 phosphorylation was needed for STAT5 interaction with LMW-PTP, then a peptide

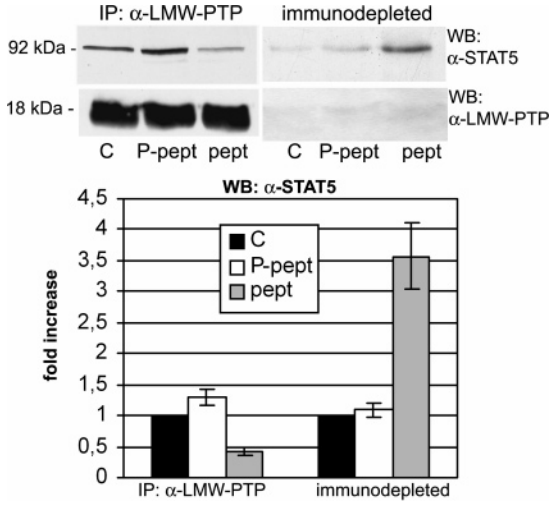

FIGURE 6: Competition between STAT5 and its 750−758 peptide for interaction with LMW-PTP. Lysates from PMA-stimulated DAMI cells were divided into aliquots that were supplemented with equal quantities of BSA (control, C), 750−758 peptide (pept), or the corresponding Thr757 phosphorylated peptide (P-pept). Immunoprecipitates were obtained with anti-LMW-PTP antibodies and were analyzed, together with the immunodepleted lysates, by western blotting. The graph shows the increase in bands intensity, normalized by the control value, for anti-STAT5 western blotting (quoting means ± SEM for $n = 3$).

phosphorylated on this residue would compete more efficiently. DAMI cells were stimulated for 15 min with 100 nM PMA to promote the maximal association of STAT5 with LMW-PTP; they were then lysed and a peptide was added corresponding to the 750−758 region of STAT5A, phosphorylated or not on Thr757. Immunoprecipitation with anti-LMW-PTP antibodies was then performed. The results in Figure 6show that whereas the peptide was effective in competing with STAT5 for its association with LMW-PTP, reducing it to one-third of controls, this was not the case for the phosphopeptide. Correspondingly, a considerable amount of STAT5 (three times that in controls) remained in the

immunodepleted lysate after coimmunoprecipitation in the presence of the peptide, whereas no significant differences from control were observed in the presence of the phosphopeptide. LMW-PTP was equally and totally immunoprecipitated from cell lysates in all cases.

This experiment, while confirming the importance of the 750−758 region of STAT5A in its interaction with LMW-PTP, demonstrates clearly that Thr757 should not be phosphorylated, in order to allow for this interaction.

DISCUSSION

STAT5 is believed to play an important role in myeloid differentiation and also in the maintenance of lymphoid precursors, as demonstrated by the complex hemeatopoietic phenotype displayed by STAT5A/B (−/−) double knockout mice (*19*). Accurate temporal regulation of STAT5 activity during hemeatopoietic development is a basic prerequisite for generating the necessary equilibrium between proliferation and terminal differentiation.

We describe in this paper the participation of LMW-PTP in the temporal regulation of STAT5 activation at the start of PMA-induced megakaryocyte differentiation. We show that STAT5 undergoes a delayed increase in tyrosine phosphorylation, which is the essential event in its activation pathway, with a peak at around 1 h of PMA stimulation (Figure 1). In view of the interaction of LMW-PTP with STAT5 in DAMI proliferating cells, we investigated their relationship during the first hour of PMA stimulation. We discovered a new correlation between STAT5 threonine phosphorylation, its association with LMW-PTP, and the maintenance of weak STAT5 tyrosine phosphorylation (Figure 2). We further demonstrated that the early threonine phosphorylation of STAT5 depends on the activation of PKC; in fact, it was prevented by concomitant treatment with a specific PKC inhibitor (Figure 3A,B). PKCα, -ϵ, and -θ, were previously identified as isoforms that may be involved in PMA-induced DAMI differentiation, and their translocation to the membrane was observed after 15 min of PMA stimulation (*20*). Accordingly, we determined that the decrease in STAT5 threonine phosphorylation following 1 h of PMA stimulation is associated with a decrease in PKCα protein levels, probably due to its down-regulation (*17*) (Figure 3C).

Of the interactions that have so far been demonstrated between LMW-PTP and its substrates, this one has unique features. First, it does not involve the LMW-PTP active site and STAT5 phosphotyrosine residue; in fact it is not prevented by competitive inhibitors such as phosphate and vanadate (*3*) but actually involves a small region of STAT5 (749−757 of murine STAT5A) which does not contain the phosphotyrosine residue (*3*). Second, this interaction involves a cytosolic substrate: the other LMW-PTP substrates identified so far are all located either in the plasma membrane (PDGFr, bFGFr, M-CSFr, insulin receptor, EphA2), (*10*, *21*, *22*, *23*, *24*) or in specialized contact regions between the membrane and cytoskeleton (FAK, Rho-GAP, β-catenin) (*18*, *25*, *26*) or are associated to membrane receptors (Grb2) (*27*) and become accessible to LMW-PTP after its translocation from the cytosol to the particulate fraction. In any case, LMW-PTP in nonadherent cells is essentially cytosolic (Figure 4), so that other mechanisms are expected to regulate its activity on specific substrates.

The importance of STAT5 threonine phosphorylation in modulating the interaction with LMW-PTP during 1 h of PMA stimulation is strengthened by the observation that, although cells undergo obvious morphological transformation and become adherent and increasingly spread, LMW-PTP and STAT5 remain essentially cytosolic (Figure 4). We verified that LMW-PTP redistribution to the cytoskeleton is a much later event (after 6 h of PMA stimulation, data not shown) so that it is not involved in its association with STAT5 at the start of megakaryocyte differentiation.

A low base level of STAT5 association with LMW-PTP is present both in DAMI and in NIH/3T3 cells in the absence of specific stimuli (ref *3* and Figures 2, 5). In these cases too, coimmunoprecipitated STAT5 was threonine phosphorylated, and since such experiments were always conducted in the presence of FCS, this could be responsible for the low base level of PKC activity. We observed that serum starvation greatly reduced the basal association of STAT5 with LMW-PTP (data not shown). We cannot exclude the possibility that other FCS-stimulated threonine kinases are effective in such situations in maintaining a low base level of STAT5 threonine phosphorylation and consequently of its association to LMW-PTP.

P.M. Gowri et al. (*11*) showed that conversion of Thr757 to Val enhanced the STAT5A transactivation potential. They concluded that the effect is not due to the absence of interaction with a regulatory phosphotyrosine protein phosphatase, because the rates of decay of tyrosine phosphorylation and of DNA association of wild type and Thr757/Val mutant STAT5 in the nucleus are similar. They did not consider the possible participation of a cytosolic tyrosine phosphatase in the control of STAT5 activity, however. In fact, their results clearly show that the level of tyrosine phosphorylation and of DNA association of Thr757/Val STAT5 is higher than that of wild type STAT5 in the nucleus, though their decay kinetics are similar. We believe that these data are consistent with the participation of a cytosolic tyrosine phosphatase, controlling the level of STAT5 tyrosine phosphorylation prior to its nuclear translocation, and whose interaction with STAT5 may be impaired by Thr757/Val mutation. It follows from these facts that Thr757 is essential for STAT5 interaction with LMW-PTP, since its mutation to valine prevents STAT5 coimmunoprecipitation with LMW-PTP (Figure 5).

The question naturally arises of whether the phosphorylation of Thr757 is necessary for the interaction of the two proteins. Our results show the opposite: the peptide corresponding to the 750−758 region, once threonine phosphorylated, was no longer able to compete with STAT5 for its interaction with LMW-PTP. Furthermore, a prediction of STAT5A phosphorylation sites executed on the NetPhos 2.0 Server (*28*) and the NetPhosK 1.0 Server (*29*) assigned a very low score for Thr757 phosphorylation relative to other threonine residues. We conclude that, despite the necessity of Thr757 for interaction of STAT5 with LMW-PTP, PKC determines the phosphorylation of other residues outside the 749−757 region during the association of the two proteins and probably determines conformational changes which increase their mutual affinity. This proposal is consistent with data obtained previously with a Thr757/Asp mutant of STAT5 (*11*), which, though mimicking Thr757 phosphory-

lation, did not exhibit different transcriptional activity from wild type STAT5.

A new model for STATs regulation has recently been proposed (*30*). In this model, tyrosine-unphosphorylated STATs shuttle continuously between cytosol and the nucleus by means of a mechanism that does not require metabolic energy or transport factors (*31*, *32*). Following cytokine stimulation, STATs undergo phosphotyrosine-dependent dimerization with a switch to carrier-dependent nuclear import (*33*). Dimerized STATs cannot exit the nucleus, so that nuclear dephosphorylation, which is controlled by the DNA off-rate, is essential for STATs recycling to the cytosol (*34*).

According to this model, cytoplasmic tyrosine dephosphorylation of STAT5 would prevent its dimerization, maintaining it in the "continuously shuttling" state in which STAT5, although entering the nucleus, does not interact with DNA. Also, nuclear phosphatases would regulate the shutdown of the STAT5 transcriptional signal, dissolving dimers and allowing STAT5 to exit from the nucleus. We propose that the cytosolic phosphatase LMW-PTP regulates the onset of STAT5 activity by associating with it and maintaining it in a tyrosine dephosphorylated state until a stimulus promotes their dissociation.

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