MEK is a negative regulator of Stat5b in PDGF-stimulated cells

Sigrídur Valgeirsdóttir*, Aino Ruusala, Carl-Henrik Heldin

Ludwig Institute for Cancer Research, Biomedical Center, Box 595, S-751 24 Uppsala, Sweden

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Abstract In this study we show that platelet-derived growth factor (PDGF)-induced DNA binding as well as transcriptional activation of Stat5b are markedly increased by inhibition of the MAP (mitogen-activated protein) kinase kinase MEK. In addition to the previously demonstrated tyrosine phosphorylation, we show that serine and threonine phosphorylation of Stat5b is increased in response to PDGF stimulation. However, inhibition of MEK had no effect on the phosphorylation level of Stat5b or on the nuclear translocation of Stat5b. These observations indicate that MEK is a negative modulator of PDGF-induced Stat5b activation through a mechanism not involving direct phosphorylation of Stat5b.

Key words: Stat5b; Platelet-derived growth factor β-receptor; MEK; Signal transduction

1. Introduction

Platelet-derived growth factor (PDGF) is a potent growth factor for mesenchymal cells. It is made up as dimers of two related polypeptide chains, designated A and B, which are assembled as homo- or heterodimers (PDGF-AA, PDGF-BB and PDGF-AB). PDGF elicits its effect by binding to two related receptors, denoted α- and β-receptors. The binding of PDGF activates the intrinsic tyrosine kinase activity of the receptors and induces autophosphorylation of specific tyrosine residues which then allow for interactions with cytoplasmic proteins which transduce signals further into the cell (reviewed in [1]).

Signal transducers and activators of transcription (Stat) proteins were first discovered in conjunction with studies of interferon signaling. Subsequently, seven members of the Stat family have been identified, that mediate transcriptional regulation in response to a diverse group of cytokines and growth factors (reviewed in [2]). Structurally, the Stat proteins share a number of common features, including a DNA binding domain, an SH2 domain, and a putative SH3 domain. Phosphorylation of a specific tyrosine residue at the carboxy-terminal tail of the Stat proteins induces their dimerization, nuclear translocation and subsequent binding to specific promoter sequences. In cytokine receptor signaling, this tyrosine phosphorylation is mediated by members of the Janus kinase family (Jak) of tyrosine kinases [3]. In addition, tyrosine kinase receptors for PDGF, EGF and colony stimulation factor-1 (CSF-1) can also activate the Stats through tyrosine phosphorylation [4–7]. Thus, PDGF stimulation leads to tyrosine phosphorylation and DNAbinding activity of Stat1, Stat3, Stat5 and Stat6 [4,8–10]. In contrast to signaling via the cytokine receptors, PDGF- and EGF-mediated tyrosine phosphorylation of Stats seems not to require Jak kinase activity [11–13]. Whether the kinase activity of the receptor itself or another kinase is involved in tyrosine phosphorylation of Stats upon growth factor stimulation is currently not known.

In addition to the essential role of tyrosine phosphorylation for Stat activation, serine phosphorylation of Stats has also been implicated in regulation of their transcriptional activity [14–16]. Thus, serine phosphorylation has been reported to be critical for maximal transcriptional activation of Stat1α and Stat3 in response to cytokines and growth factors [15]. Although the serine kinase(s) responsible for the phosphorylation of Stats have not been characterized, a proposed phosphorylation site for the MAP (mitogen-activated protein) kinases Erk1 and Erk2, -Pro-X-Ser-Pro [17] is conserved among Stat1, Stat3, and Stat4, suggesting a role for Erk in serine phosphorylation of these proteins [15,16]. The observation that Erk2 and Stat1 interact physically after stimulation with α- or β-interferon also supports the notion that there is a functional communication between these signaling molecules when cells are activated [14]. However, Erk-independent serine phosphorylation of Stat3 has also been reported [18,19]. Stat5 is also phosphorylated on serine residues after cytokine stimulation. Whereas previous reports have indicated that Erk does not phosphorylate Stat5b, it might contribute to phosphorylation of Stat5a in response to prolactin stimulation [20–22].

The Ras/MAP kinase pathway plays an important role in transmission of signals from the activated PDGF receptor. Activation of Ras leads to the sequential activation of enzymes in the MAP kinase cascade, i.e. Raf-1, MEK and Erk. The aim of this study was to determine the role of the MAP kinase cascade in PDGF-mediated activation of Stat5.

2. Materials and methods

2.1. Cell culture

Porcine aortic endothelial (PAE) cells stably transfected with PDGF β-receptor [23] were cultured in Ham's F-12 medium (Gibco), supplemented with 10% fetal calf serum (Gibco), 100 U/ml of penicillin and 100 μg/ml of streptomycin. NIH 3T3 cells stably expressing wild-type MEK1 or catalytically inactive MEK1 (K97M), kindly provided by Natalie Ahn, Howard Hughes Medical Institute, Boulder, CO, USA [24], NIH 3T3 cells and COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL), supplemented with 10% fetal calf serum (Gibco), 100 U/ml of penicillin and 100 μg/ml streptomycin.

2.2. Antisera and reagents

The rabbit antisera against Stat5b and Stat5a were purchased from

*Corresponding author. Fax: (46) (18) 160420.

Abbreviations: EGF, epidermal growth factor; Jak, Janus kinase; MBP, myelin basic protein; PAE, porcine aortic endothelial; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Stat, signal transducer and activator of transcription

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Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal anti-phosphotyrosine antibody PY20, was from Transduction Laboratories. The rabbit antiserum against Erk2 kinase (EET) was raised against a C-terminal peptide (EETARFQPGYRS). The myelin basic protein was purchased from Life Technologies-BRL. The MEK inhibitor PD98059 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA) Cells were incubated with 30–100 μM concentrations of the inhibitor at 37°C for 1 h prior to stimulation with PDGF; cells were then analyzed for Stat5 phosphorylation, nuclear translocation, DNAbinding and transcriptional activity, as described below.

2.3. Immunoblotting

Serum-starved cells were stimulated with 100 ng/ml PDGF-BB for 0, 10 or 30 min at 37°C. After washing with ice-cold phosphate buffered saline (PBS), cells were lysed in a buffer containing 1% NP-40, 10% glycerol, 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Trasylol, 100 μM Na_3VO_4. Immunoprecipitation and immunoblotting were performed as previously described [25].

2.4. Analysis of PRE binding by immunoblotting

NIH 3T3 cells or PAE cells expressing PDGF β-receptor (10^8 cells) were stimulated with 100 ng/ml PDGF-BB for 30 min at 37°C, washed and nuclear extracts prepared as described by [8]. The nuclear extracts were diluted with 1 ml of electrophoretic mobility shift assay binding buffer (12 mM HEPES pH 7.6, 8 mM KCl, 0.5 mM EDTA, 3.5% glycerol) and incubated first for 1 h with agarose-linked streptavidin (Sigma) to remove non-specific material and then overnight with immobilized oligonucleotide containing the Stat5 binding site of the bovine β-casein promoter (PRE; 5′-GA TTT CTA GGA ATT CAA-3′), obtained by incubating 40 μl of agarose-linked streptavidin with 0.2 μg of double-stranded PRE oligonucleotide biotinylated at the 5′ end of the upper strand. After washing, agarose-bound material was subjected to immunoblotting as described above.

2.5. Plasmids and cDNA transfections

The PDGF β-receptor [26] was cloned into the mammalian expression vector pcDNA3 (Invitrogen). The pXM-Stat5 plasmid [27] and the pZZ1 constructs were kindly provided by Olli Silvennoinen (University of Tampere, Finland) and Bernd Groner (Institute for Experimental Cancer Research, Freiburg, Germany). The pZZ1 construct contains the β-casein promoter from −344 to −1 in front of the luciferase gene of the pLucDSS vector [28]. Transient transfections were performed by the DEAE-dextran technique. Two μg of pXM-Stat5, β-receptor and pZZ1 cDNAs were used for transient transfections into COS-1 cells. In addition, 1.5 μg of the plasmid pCH110, encoding the β-galactosidase gene under the control of the SV40 promoter (Pharmacia Biotech), were included in each transfection as an internal control for the transfection efficiency.

2.6. Luciferase and β-galactosidase assays

COS-1 cells were left untreated or were incubated with 30 μM PD98059 for 1 h prior to stimulation with 100 ng/ml PDGF-BB for 5 h. Then β-galactosidase and luciferase activities were determined on triplicate samples using the β-galactosidase assay system (Promega) and luciferase assay system (Promega) as described by the manufacturer. Luciferase activities were corrected for the β-galactosidase values obtained in each experiment.

2.7. MAP kinase assay

Serum-starved cells were stimulated with 100 ng/ml PDGF-BB at 37°C for 0, 5, 10 or 30 min. The cells were lysed and subjected to MAP kinase assay as described previously [29], using myelin basic protein (MBP) as a substrate.

2.8. In vivo [^{32}P]orthophosphatelabeling of cells

Serum-starved cells were washed four times in phosphate-free Ham's F-12, containing 0.2% dialyzed FCS and 20 mM HEPES, pH 7.2, and then incubated for 3 h at 37°C in phosphate-free medium containing 2 mCi/ml [^{32}P]orthophosphate and 50 μM Na_3VO_4. Cells were stimulated for 10 min at 37°C with 100 ng/ml PDGF-BB, washed three times with ice-cold PBS before lysis in 1% NP-40, 10% glycerol, 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1% Trasylol, 100 μM Na_3VO_4. Insoluble material was removed by centrifugation at 14 000×*g* for 10 min at 4°C. The cleared lysates were immunoprecipitated with Stat5 antiserum and then subjected to SDS-polyacrylamide electrophoresis using 7% polyacrylamide gels. The separated proteins were transferred to a PVDF membrane and exposed on X-ray film or on a Bio-Imager screen (Fuji). The phosphorylated Stat5 was cut out and processed for trypsin cleavage and two-dimensional phosphoramino acid analysis as described previously [30].

3. Results

3.1. Increased DNA binding activity of Stat5 by inhibition of MEK

The MAP kinase pathway plays an important role in the regulation of cell growth and differentiation. One common signaling pathway leading to activation of the MAP kinases

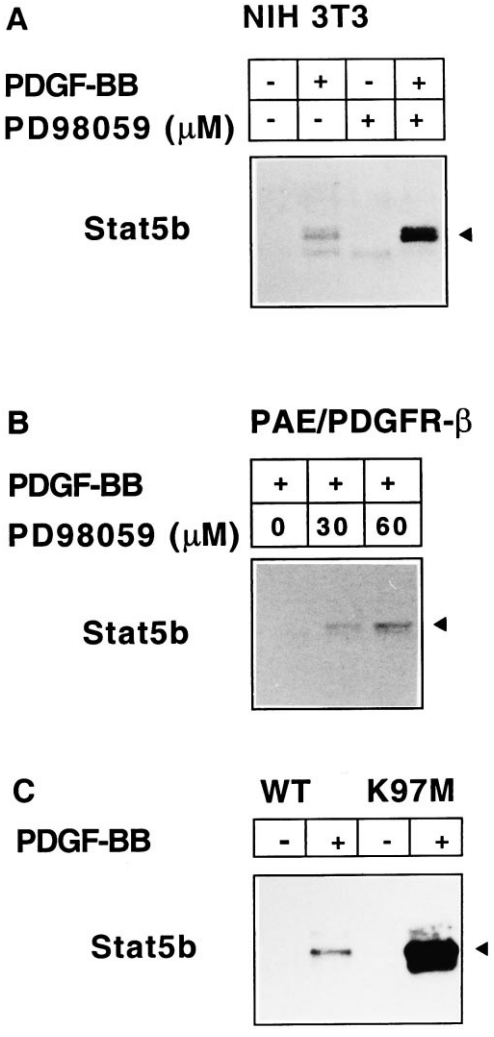

Fig. 1. Increased DNA binding activity of Stat5b by inhibition of MEK. A: NIH 3T3 cells were left untreated or were incubated with 30 μM PD98059 for 1 h prior to stimulation with PDGF-BB for 30 min at 37°C; nuclear extracts were then prepared. PREbinding complexes were isolated from the nuclear extracts using the immobilized PRE probe. DNA-bound proteins were analyzed by immunoblotting with Stat5 antiserum. B: PAE cells expressing PDGF β-receptor were incubated with 30 or 60 μM PD98059 for 1 h prior to stimulation with PDGF-BB. PRE binding activity was then analyzed as described in A. C: NIH 3T3 cells stably expressing wild-type (WT) or catalytically inactive (K97M) MEK1 were stimulated with PDGF-BB for 30 min at 37°C; nuclear extracts were then prepared and the DNA-bound proteins analyzed as described in A. The position of Stat5 is indicated on the right.

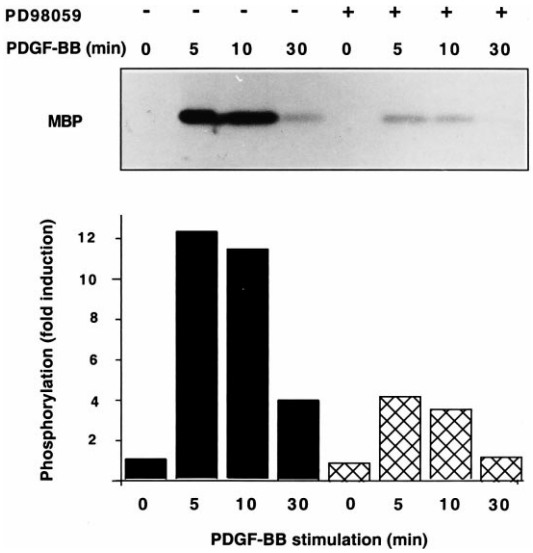

Fig. 2. Effect of PD98059 on Erk activation. NIH3T3 cells were stimulated with 100 ng/ml PDGF-BB for 30 min on ice followed by 0, 5, 10 or 30 min at 37°C, lysed and immunoprecipitated with antiserum against Erk2. The immunoprecipitated samples were subjected to in vitro kinase assay in the presence of the exogenous substrate MBP, followed by SDS-PAGE and autoradiography. The histogram in the lower part of the figure shows the relative intensities of the ^{32}P-containing MBP bands in the different lanes, quantified on a Bio-Imaging analyzer.

Erk1 and Erk2 is dependent on the upstream effectors, Ras, Raf-1 and MAP kinase kinases (MEK1 and MEK2). Since Erks have been suggested to regulate activation of Stat1 and Stat3 in cytokine signaling, we were interested in knowing whether members of the MAP kinase cascade affected PDGF-mediated activation of Stat5. We initially estimated whether the highly specific MEK inhibitor PD98059 [31,32] had any effect on the DNA binding activity of Stat5 after PDGF stimulation. For this purpose, NIH3T3 cells were incubated with 30 μM PD98059 or vehicle for 1 h at 37°C and then stimulated or not with PDGF-BB for 30 min at 37°C. Nuclear extracts were prepared and equal amount of protein incubated with biotinylated PRE probe (derived from the Stat5 binding site on the β-casein promoter), immobilized on agarose-linked streptavidin. Bound proteins were then eluted and analyzed by SDS-PAGE, followed by immunoblotting with Stat5b specific antiserum. As shown in Fig. 1A, PDGF stimulation led to binding of Stat5b to the PRE probe, in agreement with previous observations [8]. Interestingly, DNAbinding of Stat5b was markedly enhanced in cells treated with PD98059. The filter was also immunoblotted with antiserum against Stat5a, but no DNA-bound Stat5a was detected by this assay (data not shown). We also performed a similar experiment using PAE cells ectopically expressing the wild-type PDGF β-receptor. Cells were incubated with 30 or 60 μM PD98059 prior to stimulation with PDGF, and analyzed as described above. In these cells, weak DNA-binding of Stat5b was detected after stimulation with PDGF-BB, whereas pretreatment with the MEK inhibitor led to a markedly increased DNA-binding of Stat5b (Fig. 1B).

To confirm that PDGF-mediated Erk activation was inhibited in the presence of PD98059, serum-starved NIH3T3 cells were treated with 100 ng/ml PDGF for 0, 5, 10 or 30 min at 37°C. The cells were lysed, and the Erk2 protein immunoprecipitated and subjected to a kinase reaction with MBP as an exogenous substrate. Incorporation of ^{32}Pradioactivity into MBP, as analyzed by SDS-PAGE followed by autoradiography, was taken as a measure of Erk activity. PDGF stimulation of NIH3T3 cells resulted in an extensive activation of Erk2, with a 12-fold increase in phosphorylation of MBP, after 5 min of stimulation. However, in the presence of the MEK inhibitor PD98059, the Erk activation in response to PDGF stimulation was only about four-fold (Fig. 2).

In order to further investigate the potential role of MEK in inhibition of PDGF-activated Stat5, we estimated DNA binding of Stat5 in NIH 3T3 cells, stably expressing wild-type MEK1 or catalytically inactive MEK1 acting in a dominant negative manner [24]. The cells were stimulated or not with PDGF-BB for 30 min at 37°C, nuclear extracts prepared and equal amount of protein extract incubated with biotinylated PRE probe, immobilized on agarose-linked streptavidin. Bound proteins were then analyzed by SDS-PAGE, followed by immunoblotting with Stat5b. As shown in Fig. 1C, Stat5b bound the PREprobe much more efficiently in NIH 3T3 cells expressing catalytically inactive MEK1, as compared to cells expressing wild-type MEK1. Control experiments in which cellular lysates were immunoblotted with antiserum recognizing phosphorylated Erk2 revealed that Erk2 was not phosphorylated after PDGF stimulation in cells expressing dominant negative MEK1, in contrast to cells expressing wild-type MEK1 (data not shown).

Taken together, these observations indicated that MEK, or

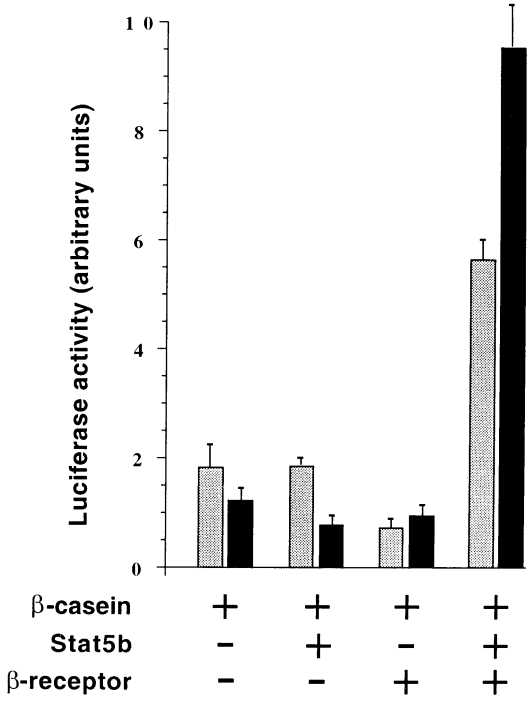

Fig. 3. Increased PDGF-induced β-casein promoter activity in the presence of MEK inhibitor. COS cells transfected with β-casein promoter luciferase construct (pZZ1), Stat5, and β-receptor (lanes 5, 6, 7 and 8) were incubated (black bars) or not (gray bars) with 30 μM PD98059 for 1 h prior to stimulation with 100 ng/ml PDGF-BB for 5 h. Cell extracts were prepared and luciferase activity determined in triplicate samples. All transfections included plasmid pCH110 and β-galactosidase assays were used as a control for transfection efficiency. Luciferase activities were normalized to the β-galactosidase activities.

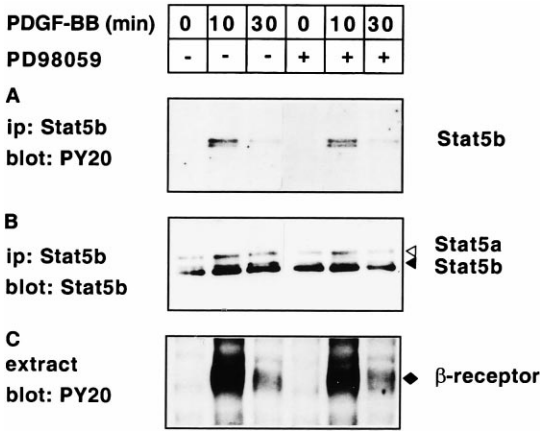

Fig. 4. Tyrosine phosphorylation of Stat5 in PDGF-stimulated cells in the absence or presence of PD98059. NIH 3T3 cells were incubated or not with 30 μM PD98059 for 1 h at 37°C and then stimulated or not with 100 ng/ml PDGF-BB for 0, 10 or 30 min at 37°C. Cells were lysed and subjected to precipitation with antiserum against Stat5b followed by SDS-PAGE and immunoblotting with PY20 (A), or antiserum against Stat5b (B). In (C), cell lysates from NIH 3T3 cells, treated or not with PD98059 and stimulated with PDGF-BB for 0, 10 or 30 min at 37°C, were separated by SDS-PAGE and immunoblotted with PY20. The positions of Stat5b (closed arrowhead), Stat5a (open arrowhead) and the PDGF β-receptor (arrow) are indicated.

its downstream effectors, are negative regulators of Stat5b DNA binding activity upon PDGF stimulation.

3.2. Increased Stat5 transcriptional activity in the presence of MEK inhibitor

We next examined the effect of MEK inhibition on the transcriptional activity of Stat5. PDGF β-receptor, Stat5 and the Stat5-responsive β-casein promoter-luciferase construct were coexpressed in COS cells and the cells stimulated with 100 ng/ml PDGF-BB for 5 h in the presence or absence of 30 μM PD98059. The transcriptional activation of the β-casein promoter-luciferase reporter gene upon stimulation with PDGF-BB was then measured. As shown in Fig. 3, PDGF-stimulated luciferase activity was almost twice as high in cells treated with PD98059. Thus, the transcriptional activity of Stat5 appears to be negatively modulated by MEK in PDGF-stimulated cells.

3.3. Tyrosine phosphorylation of Stat5b and the PDGF β-receptor is not affected by treatment with MEK inhibitor

Tyrosine phosphorylation of Stats is critical for their dimerization, nuclear translocation and subsequent transcriptional activity. Therefore, we examined whether tyrosine phosphorylation of Stat5 was modulated by inhibition of MEK. Serum-starved NIH 3T3 cells were incubated or not with PD98059 prior to stimulation with PDGF for 0, 10 or 30 min at 37°C. Cells were lysed and immunoprecipitated with Stat5b antiserum and the immunoprecipitates analyzed by SDS-PAGE followed by immunoblotting with phosphotyrosine antibodies (PY20). As shown in Fig. 4A, tyrosine phosphorylation of Stat5b was virtually unaffected by the presence of 30 μM PD98059. The amount of Stat5 was similar in all lanes as revealed by immunoblotting of the same filter with Stat5b antiserum (Fig. 4B). As shown in Fig. 4B, this antiserum crossreacted with Stat5a, however, Stat5a was not recognized by phosphotyrosine antibodies by this approach. In order to confirm the specificity of PD98059, we investigated whether phosphorylation of PDGF β-receptor was affected by the inhibitor. Therefore cell lysate from NIH3T3 cells stimulated with PDGF were separated by SDS-PAGE, followed by immunoblotting with phosphotyrosine antibodies. As shown in Fig. 4C, tyrosine phosphorylation of the PDGF β-receptor was not affected by pretreatment with the MEK inhibitor, in agreement with previous reports [32]. By a similar approach we also found that tyrosine phosphorylation of PLC-γ was not affected by PD98059 (data not shown). These data indicate that the increased DNAbinding activity of Stat5b upon MEK inhibition is not due to a change in tyrosine phosphorylation of Stat5b.

3.4. Serine/threonine phosphorylation of Stat5 is not reduced by MEK inhibition

To further analyze the phosphorylation level of Stat5b in the presence or absence of PD98059, serum-starved NIH 3T3 cells, treated or not with PD98059, were labeled for 3 h with [^{32}P]orthophosphate and then stimulated or not with PDGF for 10 min at 37°C. After cells lysis, Stat5b was immunoprecipitated from the lysate and analyzed by SDS-PAGE. The phosphorylated Stat5b was visualized by autoradiography, transferred to a PVDF membrane, and analyzed by phosphoramino acid analysis. In PDGF-stimulated cells, there was about two-fold induction of serine/threonine as well as tyrosine phosphorylation of Stat5b; treatment with the MEK inhibitor did not lead to a decrease in PDGF-stimulated or background phosphorylation of Stat5b; actually, slight increases were noticed (Fig. 5). The observation that incubation

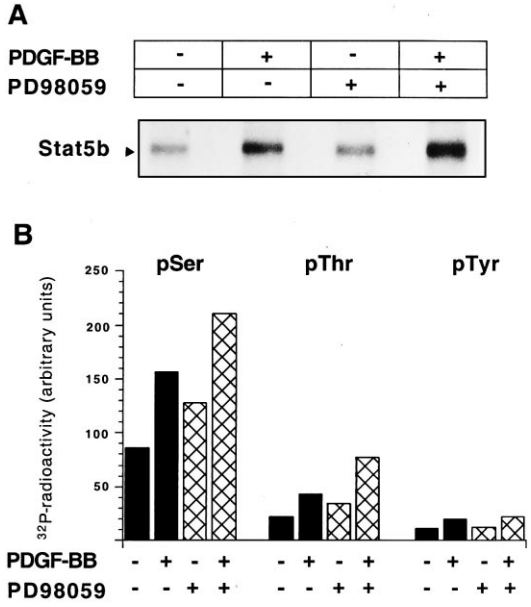

Fig. 5. Phosphorylation of Stat5 in the presence or absence of MEK inhibitor. A: Serum-starved NIH 3T3 cells were incubated with 30 μM PD98059 or vehicle for 1 h and then labeled in vivo with 2 mCi/ml [^{32}P]orthophosphate. Cells were left unstimulated or stimulated with 100 ng/ml PDGF for 10 min at 37°C, lysed and immunoprecipitated with antiserum against Stat5b. The immunoprecipitates were analyzed by SDS-PAGE and electrotransferred to a PVDF membrane and analyzed by autoradiography. B: A histogram showing the relative intensities of the [^{32}P]orthophosphate-containing phosphoserine (pSer), phosphothreonine (pThr), phosphotyrosine (pTyr), in the absence (black bars) or presence (hatched bars) of 30 μM PD98059, quantified on a Bio-Imaging analyzer.

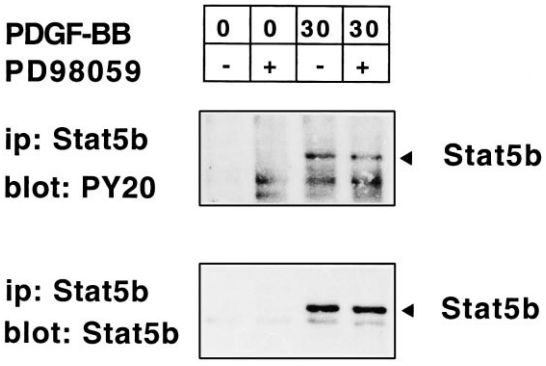

Fig. 6. Nuclear translocation of Stat5b is not affected by MEK inhibitor. NIH 3T3 cels were incubated or not with 30 μM PD98059 for 1 h at 37°C, and then stimulated or not with 100 ng/ml PDGF-BB for 30 min on ice, followed by 10 or 30 min at 37°C. Nuclear extracts were prepared and Stat5b immunoprecipitated from the lysate. The precipitate was analyzed by SDS-PAGE and transfered to filter, followed by immunoblotting with PY20 and Stat5b antiserum. The position of Stat5b (closed arrowhead) is indicated on the right.

with the MEK inhibitor did not result in a decreased serine/threonine phosphorylation of Stat5b, suggests that MEK or Erk do not phosphorylate Stat5b directly after PDGF stimulation.

3.5. *Nuclear translocation of Stat5 is not affected by treatment with MEK inhibitor*

Tyrosine phosphorylation of Stat5 leads to homo- or heterodimerization of Stat5 subunits, followed by translocation to the nucleus. We therefore estimated whether the translocation of Stat5 was affected by the MEK inhibitor. For that purpose, cytoplasmic and nuclear extracts were prepared and equal amounts of nuclear extracts subjected to immunoprecipitation with Stat5b antiserum, followed by SDS-PAGE analysis and immunoblotting. As shown in Fig. 6A, a similar amount of tyrosine phosphorylated Stat5b was detected in the nuclear extract after PDGF-stimulation in cells treated or not with MEK inhibitor. Immunoblotting with Stat5b antiserum revealed that also the amount of Stat5b in the nucleus was similar in cells treated or not with MEK inhibitor (Fig. 6B). Thus, the induced DNA binding was not due to changes in nuclear translocation of Stat5b. In contrast to the case for Stat5b, tyrosine phosphorylated Stat5a was not detected in the nucleus after PDGF stimulation (data not shown).

4. Discussion

In this study we have determined the effect of MEK inhibition on Stat5 activation in PDGF-stimulated cells. We show that treatment of cells with the MEK inhibitor PD98059 leads to an increased DNA binding and transcriptional activity of Stat5b. Moreover, DNA binding of Stat5b upon PDGF stimulation was markedly increased in cells expressing catalytically inactive MEK1 as compared to cells expressing wild-type MEK1. These data suggest that MEK or its downstream effectors Erks are negative regulators of Stat5b after PDGF stimulation. Interestingly, it has been shown that the DNA binding activity of Stat5 and the transcription of β-casein gene in response to lactogenic hormones are impaired in mammary epithelial cells transformed by the Ha-*ras* and the v-*raf* oncogenes [33]. These observations are in line with our results showing increased activation of Stat5b by inhibition of MEK.

The molecular mechanism involved in the modulation of PDGF-stimulation of Stat5b by MEK, remains to be elucidated. Stat5 has been shown to be phosphorylated on serine residues upon cytokine stimulation; Ser-730 was recently shown to be the major phosphorylation site in Stat5b, and Ser-725 and Ser-780 have been suggested as phosphorylation sites in Stat5a [21,22]. Whereas serine phosphorylation of Stat5b was not essential for its transcriptional activity in response to stimulation by growth hormone and prolactin [22,34], it appeared to be important in response to IL-2 stimulation [20]. In our study, phosphoramino acid analysis of Stat5b immunoprecipitated from [^{32}P]orthophosphate-labeled cells revealed that phosphorylation of Stat5b on serine and threonine residues increased upon stimulation with PDGF. However, PDGF-induced serine/threonine phosphorylation of Stat5b was not reduced in the presence of PD98059, suggesting that Erk does not phosphorylate Stat5b. Serine phosphorylation of Stat5b has previously been proposed to be responsible for its electrophoretic mobility shift upon stimulation [20,34]. As shown in Figs. 4B and 6B, the electrophoretic mobility shift of Stat5b was not affected by the MEK inhibitor, which further supports the notion that serine phosphorylation of Stat5b was not impaired. Our results are in agreement with previous studies showing that Erk is not required for transcriptional activation of Stat5 in response to IL-2 and prolactin stimulation [20,35,36].

Since serine/threonine phosphorylation of Stat5b was not affected by PD98059, the effect of MEK on Stat5b activation appears to be indirect. Increased activation of Stat5 was noticed after 1 h of incubation with the MEK inhibitor, suggesting that the induction of gene transcription was not required. Furthermore, translocation of Stat5b into the nucleus was not affected upon MEK inhibition, suggesting that MEK modulates Stat5b activity in the nucleus, e.g. by affecting interactions with other transcription factor(s). Stats have been shown to act together with other classes of transcription factors, e.g. the glucocorticoid receptor together with which Stat5b stimulates, in a synergistic manner, the β-casein promoter [37,38].

The results presented in the present communication illustrates one mechanism for modulation of Stat activation. Several other such mechanisms have recently been proposed, including dephosphorylation of the regulatory C-terminal tyrosine residue of Stats which results in loss of dimerization and inactivation of DNA binding [39], proteolytic degradation, negative regulation by cytokine-inducible proteins of the SOCS/JAB/SSI family [40–42], and through inhibition by the PIAS (protein inhibitor of activated Stats) family of proteins, which act as negative regulators of Stat DNA binding activity [43,44].

Stat5a and Stat5b are closely related proteins, encoded by separate genes. In our study we saw that both Stat5b (Fig. 4) and Stat5a (data not shown) were tyrosine phosphorylated in response to PDGF stimulation. However, only tyrosine phosphorylated Stat5b, and not Stat5a, was detected in the nucleus upon PDGF-stimulation of NIH 3T3 cells and only Stat5b was detected in complex with PRE probe in the DNA-binding assays. Studies on Stat5 knockout mice have shown that Stat5a and Stat5b have separate physiological functions. Thus, Stat5a has been shown to be important for mammary gland development and lactogenesis [45], whereas Stat5b is

involved in sexual dimorphism of body growth rates and liver gene expression [46]. The effects of disruption of the Stat5b gene on body fat deposition were marked with significantly less adipose tissue both in Stat5b$^{-/-}$ females and males compared with wild-type mice. Furthermore, the expression of Stat5b has been shown to be tightly correlated with the adipocyte phenotype of 3T3-L1 cells, with a markedly elevated expression in adipocytes as compared with their fibroblast precursors [47]. Previous studies have indicated that PDGF, together with insulin, affects adipose differentiation of 3T3-L1 preadipocytes [48,49]. Therefore, it is possible that PDGF-stimulated Stat5b has a role in this differentiation process. It is noteworthy that previous studies have indicated that MAP kinase activity affects adipocytic differentiation of 3T3-L1 cells [50,51]; it is an interesting possibility, which remains to be elucidated, that the modulation of Stat5b activation by MEK might play a role in differentiation of preadipocytes.

PDGF activates Stat5b by promoting its phosphorylation on tyrosine residues [8]. At the same time PDGF induces activation of the MAP kinase cascade, which leads to inhibition of Stat5b activity as suggested by the data shown in the present study. This is thus yet another example that stimulatory and inhibitory pathways for various responses, are initiated in parallel at the activated PDGF receptor (reviewed in [1]). We noticed that treatment with PD98059 markedly reduced PDGF-mediated DNA synthesis (data not shown) in agreement with previous observations [52]. Thus, the DNA binding activity of Stat5b increased simultaneously as the PDGF-induced mitogenicity decreased; however, it remains to be determined whether Stat5b directly modulates PDGF-induced mitogenicity. An important issue for future studies is to clarify the mechanism of induction of Stat5b activity by PDGF and to determine the functional role of Stat5b in PDGF signaling.

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References

[1] Heldin, C.-H., Östman, A. and Rönnstrand, L. (1998) Biochim. Biophys. Acta 1378, F79–F113.
[2] Hoey, T. and Schindler, U. (1998) Curr. Opin. Genet. Dev. 8, 582–587.
[3] Horvath, C.M. and Darnell Jr., J.E. (1997) Curr. Opin. Cell Biol. 9, 233–239.
[4] Silvennoinen, O., Schindler, C., Schlessinger, J. and Levy, D.E. (1993) Science 261, 1736–1739.
[5] Ruff-Jamison, S., Chen, K. and Cohen, S. (1993) Science 261, 1733–1736.
[6] Ruff-Jamison, S., Chen, K. and Cohen, S. (1995) Proc. Natl. Acad. Sci. USA 92, 4215–4218.
[7] Novak, U., Harpur, A.G., Paradiso, L., Kanagasundaram, V., Jaworowski, A., Wilks, A.F. and Hamilton, J.A. (1995) Blood 86, 2948–2956.
[8] Valgeirsdóttir, S., Paukku, K., Silvennoinen, O., Heldin, C.-H. and Claesson-Welsh, L. (1998) Oncogene 16, 505–515.
[9] Raz, R., Durbin, J.E. and Levy, D.E. (1994) J. Biol. Chem. 269, 24391–24395.
[10] Patel, B.K.R., Wang, L.-M., Lee, C.-C., Taylor, W.G., Pierce, J.H. and LaRochelle, W.J. (1996) J. Biol. Chem. 271, 22175–22182.
[11] Leaman, D.W., Pisharody, S., Flickinger, T.W., Commane, M.A., Schlessinger, J., Kerr, I.M., Levy, D.E. and Stark, G.R. (1996) Mol. Cell. Biol. 16, 369–375.
[12] Vignais, M.-L., Sadowski, H.B., Watling, D., Rogers, N.C. and Gilman, M. (1996) Mol. Cell. Biol. 16, 1759–1769.
[13] Quelle, F.W., Thierfelder, W., Witthuhn, B.A., Tang, B., Cohen, S. and Ihle, J.N. (1995) J. Biol. Chem. 270, 20775–20780.
[14] David, M., Petricoin III, E., Benjamin, C., Pine, R., Weber, M.J. and Larner, A.C. (1995) Science 269, 1721.
[15] Wen, Z., Zhong, Z., Darnell, J. and James, E. (1995) Cell 82, 241–250.
[16] Zhang, X., Blenis, J., Li, H.-C., Schindler, C. and Chen-Kiang, S. (1995) Science 267, 1990.
[17] Gonzalez, F.A., Raden, D.L. and Davis, R.J. (1991) J. Biol. Chem. 266, 22159–22163.
[18] Chung, J., Uchida, E., Grammer, T.C. and Blenis, J. (1997) Mol. Cell. Biol. 17, 6508–6516.
[19] Ceresa, B.P., Horvath, C.M. and Pessin, J.E. (1997) Endocrinology 138, 4131–4137.
[20] Beadling, C., Ng, J., Babbage, J.W. and Cantrell, D.A. (1996) EMBO J. 15, 1902–1913.
[21] Pircher, T.J., Flores-Morales, A., Mui, A.L.-F., Saltiel, A.R., Norstedt, G., Gustafsson, J.-Å. and Haldosén, L.-A. (1997) Mol. Cell. Endocrinol. 133, 169–176.
[22] Yamashita, H., Xu, J., Erwin, R.A., Farrar, W.L., Kirken, R.A. and Rui, H. (1998) J. Biol. Chem. 273, 30218–30224.
[23] Mori, S., Rönnstrand, L., Yokote, K., Engström, Å., Courtneidge, S.A., Claesson-Welsh, L. and Heldin, C.-H. (1993) EMBO J. 12, 2257–2264.
[24] Mansour, S.J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande Woude, G.F. and Ahn, N.G. (1994) Science 265, 966–970.
[25] Valgeirsdóttir, S., Eriksson, A., Nistér, M., Heldin, C.-H., Westermark, B. and Claesson-Welsh, L. (1995) J. Biol. Chem. 270, 10161–10170.
[26] Claesson-Welsh, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C. and Heldin, C.-H. (1988) Mol. Cell. Biol. 8, 3476–3486.
[27] Wakao, H., Gouilleux, F. and Groner, B. (1994) EMBO J. 13, 2182–2191.
[28] Gouilleux, F., Wakao, H., Mundt, M. and Groner, B. (1994) EMBO J. 13, 4361–4369.
[29] Reuter, C.W.M., Catling, A.D. and Weber, M.J. (1995) Methods Enzymol. 255, 245–256.
[30] Blume-Jensen, P., Wernstedt, C., Heldin, C.-H. and Rönnstrand, L. (1995) J. Biol. Chem. 270, 14192–14200.
[31] Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. and Saltiel, A.R. (1995) J. Biol. Chem. 270, 27489–27494.
[32] Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J. and Saltiel, A.R. (1995) Proc. Natl. Acad. Sci. USA 92, 7686–7689.
[33] Happ, B., Hynes, N.E. and Groner, B. (1993) Cell Growth Differ. 4, 9–15.
[34] Ram, P.A., Park, S.-H., Choi, H.K. and Waxman, D.J. (1996) J. Biol. Chem. 271, 5929–5940.
[35] Kirken, R.A. et al. (1997) J. Biol. Chem. 272, 15459–15465.
[36] Wartmann, M., Cella, N., Hofer, P., Groner, B., Liu, X., Hennighausen, L. and Hynes, N.E. (1996) J. Biol. Chem. 271, 31863–31868.
[37] Lechner, J., Welte, T., Tomasi, J.K., Bruno, P., Cairns, C., Gustafsson, J.-Å. and Doppler, W. (1997) J. Biol. Chem. 272, 20954–20960.
[38] Stöcklin, E., Wissler, M., Gouilleux, F. and Groner, B. (1996) Nature 383, 726.
[39] Haspel, R.L., Salditt-Georgieff, M. and Darnell Jr., J.E. (1996) EMBO J. 15, 6262–6268.
[40] Starr, R. et al. (1997) Nature 387, 917–921.
[41] Endo, T.A. et al. (1997) Nature 387, 921–924.
[42] Naka, T. et al. (1997) Nature 387, 924–928.
[43] Chung, C.D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P. and Shuai, K. (1997) Science 278, 1803.
[44] Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D. and Shuai, K. (1998) Proc. Natl. Acad. Sci. USA 95, 10626–10631.

[45] Liu, X., Robinson, G.W., Wagner, K.-U., Garrett, L., Wynshaw-Boris, A. and Hennighausen, L. (1997) Genes Dev. 11, 179–186.
[46] Udy, G.B., Towers, R.P., Snell, R.G., Wilkins, R.J., Park, S.-H., Ram, P.A., Waxman, D.J. and Davey, H.W. (1997) Proc. Natl. Acad. Sci. USA 94, 7239–7244.
[47] Stephens, J.M., Morrison, R.F. and Pilch, P.F. (1996) J. Biol. Chem. 271, 10441–10444.
[48] Bachmeier, M. and Löffler, G. (1995) Eur. J. Cell Biol. 68, 323–329.
[49] Bachmeier, M. and Löffler, G. (1997) Eur. J. Biochem. 243, 128–133.
[50] de Mora, J.F., Porras, A., Ahn, N. and Santos, E. (1997) Mol. Cell. Biol. 17, 6068–6075.
[51] Sale, E.M., Atkinson, P.G.P. and Sale, G.J. (1995) EMBO J. 14, 674–684.
[52] Weber, J.D., Raben, D.M., Phillips, P.J. and Baldassare, J.J. (1997) Biochem. J. 326, 61–68.