Negative and Positive Regulation of MAPK Phosphatase 3 Controls Platelet-derived Growth Factor-induced Erk Activation*

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MAPK phosphatases (MKPs) are dual specificity phosphatases that dephosphorylate and thereby inactivate MAPKs. In the present study, we provide evidence that platelet-derived growth factor BB (PDGF-BB) regulates MKP3 (DUSP6), which is considered to be a phosphatase highly selective for Erk. Intriguingly, we observed that Mek is positively regulated by MKP3, whereas Erk itself is negatively regulated. In addition, we found that activation of PDGF receptor α or β leads to a rapid proteasomal degradation of MKP3 in a manner that requires Mek activation; this feed-forward mechanism was found to be essential for efficient Erk phosphorylation. We could also demonstrate that PDGF-BB stimulation induces phosphorylation of MKP3 at Ser-174 and Ser-300; phosphorylation of Ser-174 is involved in PDGF-induced MKP3 degradation, since mutation of this site stabilized MKP3. Moreover, activated Erk induces mkp3 expression, leading to restoration of MKP3 levels after 1–2 h and a concomitant dephosphorylation of Erk in cells with activated PDGFRα. Reducing the MKP3 level by small interfering RNA leads to an increased Erk activation and mitogenic response to PDGF-BB. In conclusion, MKP3 is an important regulator of PDGF-induced Erk phosphorylation acting in both a rapid positive feed-forward and a later negative feed-back loop.

Platelet-derived growth factor (PDGF) stimulates migration and proliferation of connective tissue cells and has an important role during embryonic development and wound healing (1). The biologically active form of PDGF is a disulfide-bonded dimer of A-, B-, C-, or D-polypeptide chains (2). The PDGF isoforms (PDGF-AA, -AB, -BB, -CC, or -DD) bind two structurally related tyrosine kinase receptors, denoted PDGFRα and PDGFRβ. PDGF-A, -B, and -C chains bind to PDGFRα, and PDGFR-B and -D chains bind to PDGFRβ. Ligand binding induces receptor homo- or heterodimerization and autophosphorylation. The phosphorylated tyrosine residues constitute recruitment sites for Src homology 2 domain-containing proteins, including the Grb2-Sos complex that activates Ras and the Erk MAPK pathway, the tyrosine kinase Src, the tyrosine phosphate Shp2, phosphatidylinositol 3-kinase, and phospholipase Cγ. In addition, the PDGFRβ but not PDGFRα binds RasGAP, which inactivates Ras (1, 3). These pathways mediate cell growth, survival, and migration.

The MAPK family is evolutionarily conserved, and mammalian cells possess several MAPK pathways (i.e. Erk1 and -2; p38α, -β, -δ, and -γ and JNK1, -2, and -3) (4, 5). The Erk pathway has been connected to cell proliferation and differentiation (6). In contrast, the p38 and JNK pathways have more established roles in apoptotic signaling but may under certain circumstances also contribute to cell proliferation and migration (7). Receptor tyrosine kinases often activate the Erk pathway by recruiting a complex between the adaptor protein Grb2 and the guanine exchange factor Sos to the plasma membrane, where it activates the small G-protein Ras, which then activates the three-tiered kinase module consisting of Raf, Mek, and Erk (6). A large portion of human tumors have deregulated Erk kinase activity, underscoring its importance in tumor formation (8). Unrestrained Erk kinase activity can arise from inappropriate activation of an upstream signaling component (e.g. oncogenic Ras) or loss of an inhibitory regulator, such as MAPK phosphatases.

The biological consequence of Erk activation is connected to the magnitude as well as temporal pattern of Erk phosphorylation. For example, in PC12 cells, sustained Erk activation induced by nerve growth factor leads to differentiation, whereas transient EGF-induced Erk phosphorylation results in proliferation (9). Other studies have demonstrated that sustained Erk activation is necessary for cell cycle progression (10–12). Thus, mechanisms that regulate the kinetics of Erk activation have a major biological impact, although there is significant cell type specificity regarding the outcome.

MAPK phosphatases (MKPs) are dual specificity phosphatases that negatively regulate the activity of MAPKs by dephosphorylating the essential threonine and tyrosine residues in the activation loop (13, 14). Treatment with growth factors, such as NGF, induces MKP expression (15–17), which can modulate both the intensity and duration of MAPK signaling. The different MKPs have distinct substrate specificities (i.e. selectively dephosphorylating Erk, p38, or JNK MAPKs), enabling the cell...
to specifically control different MAPK pathways (14). In the present study, we have explored the role of MKPs in PDGF-induced Erk activation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant human PDGF-BB was generously provided by Amgen (Thousand Oaks, CA). The inhibitor U0126 was from Calbiochem, MG132 and cycloheximide from Sigma and STI571 from Novartis Pharma AG (Basel, Switzerland). Monoclonal β-actin antibody was from Sigma (A5441), and antibodies against phosphorylated-Mek1/2 (number 9154), total Mek1/2 (number 9126), and phosphorylated Erk1/2 (number 9106) were purchased from Cell Signalling Technology (Beverly, MA). Anti-phosphotyrosine (PY99, sc-7029) and anti-ubiquitin (P4D1, sc-8017) antisera were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antiserum against MKP3 was raised by immunizing a rabbit with the synthetic peptide CPSNQNVYQVDSLQST conjugated to keyhole limpet hemocyanin. The antibodies were purified by chromatography over a column of the corresponding peptide immobilized on Sepharose beads. Purified antibodies were stored in 0.15 M NaCl, 20 mM Hepes, pH 7.4, 50% glycerol at −70 °C. A rabbit antiserum recognizing Erk was raised against a peptide corresponding to the carboxyl-terminal sequence EETARF-

**Site-directed Mutagenesis**—Point mutations were generated using the QuikChange site-directed mutagenesis system (Stratagene). In brief, two complementary oligonucleotides containing the mutant of interest were synthesized and used in the mutagenesis PCR with the wild-type MKP3 DNA as a template. The amplification mixtures were then digested with DpnI to remove template DNA. The non-digested DNA was transformed into DH5a bacteria from which the mutated DNA could be recovered. All point mutations were verified by DNA sequencing. The following oligonucleotides were used: Ser-159 → Ala, CTCGTGTAGACGACGCGCCCGCTTGGCAGT and CACTGGCAACGCAGCGCTGCTGCTACACGAG; Ser-174 → Ala, GGCTCCGGATCAGCGCTGAC-TCTTCTCGGACAATC and GATGTCGGAGGAAGACTGAC-AGGCTGTAGCCGCAGCCG; Ser-197 → Ala, CAAAACGTGCTAGCTCGGTCCAACGAC and GTCTGG-GGAAACGGGACACATCTCGGTAGGC; Ser-300 → Ala, CTTGGTGGCATTAGCGCGCCGACTCTGTTAGGC-TGTGGC and GCCACATGCTGATCTGGAGCGCTA-ATGCCAGCCAG; Cys-293 → Ala, CTGTGTTGTCTTGG-TACTGTCCTTGGCTGCCATTACCG and CTCGTTCTCGGTATAAGCGGGGACATGCTG ACGCTGCTGCTAGCCGCTATGCCAGCCAG.

**Cell Culture**—Porcine aortic endothelial (PAE) and 293T cells were cultured in Ham’s F-12 medium with 1-glutamine or Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal bovine serum and 100 units/ml penicillin, 100 µg/ml streptomycin. For serum starvation, cells were washed once and incubated in Ham’s F-12 or Dulbecco’s modified Eagle’s medium containing 1% or 0.1% fetal bovine serum, respectively.

**Transient Transfections**—Transfections were performed with Lipofectamine 2000 according to the protocol supplied by the manufacturer (Invitrogen). Cells were used for experiments 48 h after transfection.

**Immunoblotting**—Subconfluent cells were starved and incubated with inhibitors or vehicle at the indicated concentrations and thereafter stimulated with 100 ng/ml PDGF-BB for the indicated periods of time. Cells were washed with ice-cold phosphate-buffered saline and lysed (1% Triton X-100, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 20 mM Tris, pH 7.4, 1 mM Pefabloc, 1% Trasylol, 10 mM NaF, 1 mM sodium orthovanadate). Extracts were clarified by centrifugation, and protein concentration was determined by the BCA protein assay system (Pierce). Equal amounts of lysates were boiled with SDS sample buffer containing dithiothreitol and separated by SDS-PAGE. For Western blotting, samples were electrotransferred to polyvinylidene difluoride membranes (Immobilon P), which were blocked in 5% dry milk in Tris-buffered saline solution containing 0.1% Tween 20. Commercial primary antibodies were used at concentrations recommended by the suppliers, and antibodies produced in house were used at 2 µg/ml and incubated overnight in the cold. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (both from Amersham Biosciences), and proteins were visualized using ECL Western blotting detection systems from Roche Applied Science on a cooled charge-coupled device (CCD) camera (Fuji, Minami-Ashigara, Japan). For reprobing, membranes were stripped with 0.4 M NaOH for 10 min at room temperature, blocked, and incubated with the corresponding antibodies. Where quantifications of band intensities are shown, the number corresponds to the average value ± S.E.

**[32P]Orthophosphate Labeling**—Cells were starved for 16 h, preincubated for 3 h with 4 mM/ml [32P]orthophosphate in phosphate-free Ham’s F-12 supplemented with 0.3% dialyzed fetal bovine serum, and then stimulated with PDGF-BB for 15 min. Cell lysates were subjected to immunoprecipitation with an antibody recognizing MKP3.

**Phosphopeptide Mapping, Edman Degradation, and Phosphoamino Acid Analysis**—Phosphopeptide mapping, Edman degradation, and phosphoamino acid analysis were performed essentially as described (18). In brief, tryptic peptides were recovered from the nitrocellulose membrane and separated on a Hunter thin layer electrophoresis system (HTLE-700) at 2000 V for 35 min in pH 1.9 buffer (formic acid/acetic acid/water, 4.6/15.6/8.1, v/v/v), followed by chromatography in isobutyric acid buffer (isobutyric acid/n-butyl alcohol/pyridine/acetic acid/water, 1250/38/96/58/558, v/v/v/v/v) in the second dimension. Phosphopeptides were visualized by autoradiography, extracted with 20% acetonitrile, and coupled to Seque-
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FIGURE 1. Stimulation of PDGFRα or PDGFRβ results in activation of the Erk pathway with different kinetics. PAE/PDGFRα and PAE/PDGFRβ cells were serum-starved and then stimulated with PDGF-BB (100 ng/ml) for the indicated periods of time. Erk (A), Mek (B), and PDGFR (C) levels and activation were assayed by immunoblotting (lb) of total cell lysates (TCL) for Erk or Mek and immunoprecipitated (lp) material for PDGFR with antibodies against total or phosphorylated proteins, as indicated. Quantifications shown in A and B are the averages of three independent experiments ± S.E. pTyr, phosphorytosine.

DNA Synthesis Assay—The cells plated into 24-well plates were serum-starved and then incubated for 24 h with PDGF-BB in Ham’s F-12 containing [3H]thymidine (0.1 μCi/ml). Thymidine incorporation into acid-insoluble material was measured by a scintillation counter.

Extraction of Cytoplasmic and Nuclear Fractions—293T cells were washed with PBS, collected by scraping, pelleted by centrifugation, and lysed in buffer containing 10 mM MES (pH 6.2), 10 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 5 mM dithiothreitol, and 1% Triton X-100, supplemented with protease inhibitors (1 mM Pefa Bloc, 1% Trasylol, 1 mM sodium orthovanadate). Nuclei were collected by centrifugation at 3000 × g and then lysed in buffer containing 25 mM Tris-HCl (pH 10.5), 1 mM EDTA, 0.5 mM NaCl, 5 mM β-mercaptoethanol, and 0.5% Triton X-100, supplemented with protease inhibitors. Supernatants containing nuclei proteins were collected by centrifugation at 15,000 × g for 30 min.

Real Time PCR—Total DNA-free cellular RNA was extracted from cells treated with PDGF for different periods of time with the RNeasy kit (Qiagen) and reverse-transcribed (SuperScript from Invitrogen) to create cDNA templates. The PCR was performed by the qPCR™ core kit for SYBR™ Green I (Bio-Rad) according to the manufacturer’s instructions. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control for the relative quantification of the target message. Specific primers were as follows: for mkp3, GTTTTTCCCTGAGGCCATTTC (forward) and TCACAGTGACTGAGCGGCTAAT (reverse); for glyceraldehyde-3-phosphate dehydrogenase, CCCCTTACATTGCCTGACTGACTC (forward) and GGGATTTCCATTGTGACAG (reverse).

siRNA Knockdown—Down-regulation of MKP3 was performed by using specific siRNA purchased from Dharmacon Research. For every experiment performed, nontargeting siRNA was used as a control (target sequence 5′-CGTGAGCGGAATCTTCGA-3′). Transfection of siRNA was done for 24 h with SilentFect from Bio-Rad. Levels of knockdown were tested after 40 h by measuring protein levels by immunoblotting.

Statistics—Data are presented as mean ± S.D. Student’s t test was used to determine p values, and p < 0.05 was considered statistically significant.

RESULTS

PDGFRα and PDGFRβ Activate Erk with Different Kinetics—PAE cells transfected with PDGFRα or PDGFRβ (denoted PAE/PDGFRα and PAE/PDGFRβ, respectively) were used to investigate Erk activation downstream of the two PDGFR isoforms expressed at a comparable level in the same cellular background. Cells were stimulated with PDGF-BB, which binds with similar affinity to both PDGFRα and PDGFRβ, and the phosphorylation of Erk was analyzed at different points by immunoblotting using phosho-specific antibodies. We found that the kinetics of PDGF-BB-induced Erk phosphorylation was different, depending on which PDGFR isoform was expressed. In PAE/PDGFRα cells, a biphasic activation was observed with an initial peak of Erk phosphorylation after 15 min, followed by a sharp decrease and a later second increase after 4 h of stimulation (Fig. 1A, upper panels). In contrast, activation of PDGFRβ resulted in Erk phosphorylation that peaked after ~30 min of stimulation and then remained relatively constant at a lower level up to 6 h of stimulation (Fig. 1A, lower panels). Next, we analyzed the phosphorylation status of the upstream kinase Mek. As can be seen in Fig. 1B, Mek was transiently activated downstream of PDGFRα and more sustained downstream of PDGFRβ, consistent with the initial kinetics of Erk activation. For reference, the kinetics of PDGFRα and β-phosphorylation was also included in Fig. 1C.
PDGF-BB Regulates the Expression Level of MAPK Phosphatase 3—In order to investigate whether the initial rapid increase of Erk phosphorylation was accompanied by a decrease in MKP expression, we analyzed by Western blotting the protein levels of the different MKPs known to be able to dephosphorylate Erk. Although the levels of MKP1, MKP2, hVH3, and MKP4 were not influenced by PDGF-BB stimulation (supplemental Fig. 1, A and B, for PDGFRα and PDGFRβ, respectively), MKP3 underwent rapid degradation in response to PDGF-BB stimulation in both PDGFRα- and PDGFRβ-expressing cells; after 1–2 h of PDGFRα stimulation, MKP3 levels returned to initial levels, followed by a second phase of degradation (Fig. 2A, upper panel). In contrast, activation of PDGFRβ induced a slower degradation of MKP3 and a minor wave of MKP3 protein expression, followed by a second phase of MKP3 decrease (Fig. 2A, lower panel). The biphasic activation of Erk downstream of PDGFRα was inversely correlated with biphasic kinetics of MKP3 protein expression (compare Figs. 1A and 2A). This suggests that PDGFB-BB-induced changes in the intensity of Erk activity are related to the level of MKP3 protein. To further characterize the MKP3 degradation downstream of PDGFRα, we measured the protein stability in the presence of the protein synthesis inhibitor cycloheximide in the presence or absence of PDGFB-BB; the half-life of MKP3 was estimated for both the first and second waves of degradation (Fig. 2B). We found the half-life for the first wave of degradation to be ~20 min, compared with 25 min for the second wave. The high level of MKP3 expression after 1–2 h of PDGFB-BB stimulation of PDGFRα may explain why the Erk phosphorylation is low despite significant Mek phosphorylation at these time points (compare Figs. 1, A and B, and 2A). Stimulation of PDGFRβ induced a more sustained Erk phosphorylation, which is compatible with the observation that after the initial MKP3 degradation, the protein level remained low with only a minor transient increase after 2 h of PDGFB-BB stimulation (Fig. 2A, lower panel).

PDGFB-BB-induced Erk Activation Requires Proteasomal Degradation of MKP3—To investigate whether the rapid degradation of MKP3 induced by PDGFB-BB occurred through the proteasome, we analyzed the ability of PDGFB-BB to induce ubiquitination of MKP3 and the effect of treatment with the proteasomal inhibitor MG132 on MKP3 stability. Fig. 2C shows that MKP3 indeed becomes ubiquitinated in response to PDGFB-BB stimulation. Furthermore, we found that MG132 treatment completely inhibited the degradation of MKP3 both in PDGFRα- and PDGFRβ-expressing cells (Fig. 2D). Furthermore, in the presence of proteasomal inhibitor, we observed that PDGFB-BB was no longer able to induce a robust Erk activation (Fig. 2D).
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The blunted Erk phosphorylation may be attributed to stabilization of MKP3 and thus an increased dephosphorylation of Erk. It is possible that stabilization of other phosphatases also is involved. However, we were unable to detect any effects of MG132 on the levels of MKP1, MKP2, and hVH3 (Fig. 2D and supplemental Fig. 1, C and D), arguing for a relatively specific role of MKP3 in this process.

In order to determine whether activation of the Erk pathway is important for the degradation of MKP3, we treated cells with the low molecular weight inhibitor U0126, which inhibits Mek and consequently Erk activation, and investigated the effect on PDGF-BB-induced MKP3 degradation. Treatment with U0126 resulted in a significant reduction in the rapid degradation of MKP3 after PDGF-BB stimulation, both in PDGFRα- and PDGFRβ-expressing cells (Fig. 2E; compare bands after 30 min of PDGF-BB stimulation in the absence or presence of U0126). Furthermore, we observed that the increased levels of MKP3 normally seen after 2 h of PDGF-BB stimulation did not occur in the presence of U0126, and in fact we found a decrease in MKP3 level (Fig. 2E). In Fig. 4B, we show that the mkp3 gene is activated by PDGF-BB in a manner requiring Erk activity. Thus, the decrease in MKP3 level after 2 h of PDGF-BB stimulation in the presence of U0126 may be caused by general protein turnover in combination with inhibited expression of the mkp3 gene. Another possibility is that U0126 has been partially metabolized, thereby allowing for weak PDGF-BB-induced MKP3 degradation in combination with partially inhibited mkp3 gene expression. Normally, MKP3 appears as multiple bands in immunoblotting. However, after U0126 treatment, the slowest migrating band disappeared, suggesting that it can represent a phosphorylated species of MKP3 (Fig. 2E). In support of this notion, PDGF-BB induced phosphorylation of MKP3 in a Mek-dependent manner, as determined by metabolic [32P]orthophosphate labeling of cells treated with PDGF-BB in the absence or presence of U0126 as indicated (Fig. 3A).

To identify the phosphorylation sites in MKP3 following PDGF-BB stimulation, we performed two-dimensional phosphopeptide mapping of the tryptic MKP3 digest (Fig. 3B). Two phosphopeptides (denoted peptide 1 and peptide 2) were recovered and subjected to Edman degradation and phosphoamino acid analysis (Fig. 3C). Matching the cycle in which radioactivity was released during Edman degradation and the result from the phosphoamino acid analysis with the sequences of all tryptic fragments of MKP3 revealed Ser-174 and Ser-300 as novel PDGF-BB-induced phosphorylation sites in MKP3. In the case of Ser-174 (peptide 1), only one MKP3-derived tryptic peptide was consistent with the cycle where radioactivity was released, whereas both Ser-300 and Ser-328 could match the pattern obtained for peptide 2. However, through mutational analysis we could exclude Ser-328 (data not shown). To elucidate whether the PDGF-induced phosphorylation was impor-
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In order to investigate the mechanism(s) behind the induction of MKP3 1–2 h after PDGF-BB stimulation, we performed quantitative real time PCR analysis of

m kp3 mRNA expression. PDGF-BB stimulation was found to cause about a 3-fold increase of m kp3 mRNA in PDGFRα-expressing cells and about a 2-fold induction in cells expressing PDGFRβ; in both cases, mRNA induction occurred after 1 h of PDGF stimulation (Fig. 4A).

In order to explore whether activation of Erk is important for induction of the m kp3 gene, we analyzed the effect of treatment of cells with U0126. After pretreatment with U0126, we could not observe the increase in MKP3 protein levels usually detected after 2 h of PDGF-BB stimulation of PDGFRα and to a lesser extent PDGFRβ (Fig. 2E). Instead, we found a gradual decrease in MKP3 protein levels, probably due to general protein turnover. In addition, siRNA-mediated silencing of Erk also abolished the increase in MKP3 protein levels after 2 h of PDGF-BB treatment (data not shown). This prompted us to investigate whether Erk pathway activation was necessary for the PDGF-BB-induced transcriptional activation of the m kp3 gene. Therefore, we stimulated cells with PDGF-BB for various periods of time in the absence or presence of U0126 and then prepared total RNA. Using quantitative real time PCR we could indeed demonstrate a requirement for Erk activation in the induction of m kp3 gene expression (Fig. 4B). However, we observed an escape from the U0126-mediated inhibition in m kp3 expression after 2 h of PDGF-BB treatment. Possible explanations for this include metabolism of the U0126 compound after prolonged incubation or activation of the m kp3 gene through an alternative mechanism that does not require the Erk pathway. Thus, the Erk pathway is involved both in a positive feed-forward mechanism to cause degradation of MKP3 and in a negative feed-back loop by inducing the expression of MKP3 at later time points.

MKP3 Promotes Persistent Mek Activation but Negatively Regulates PDGF-BB-induced Erk Activation and Proliferation—To further elucidate the functional role of MKP3, we downregulated its expression using siRNA and analyzed the effects on PDGF-induced Mek and Erk phosphorylation as well as the mitogenic response. We found that down-regulation of MKP3 by ~70% enhanced the intensity of Erk phosphorylation in response to PDGF-BB, consistent with the role of MKP3 as a negative Erk regulator, but had no major effect on the kinetics
of Erk phosphorylation (Fig. 5A). We observed that siRNA-mediated down-regulation of MKP3 reduced Mek phosphorylation in response to PDGF-BB stimulation of the PDGFRα (Fig. 5B, upper panels). In concurrence, MKP3 overexpression led to enhanced Mek but reduced Erk activation (Fig. 5B, middle panels). In addition, stabilization of MKP3 through proteasomal inhibition led to a prolonged Mek activation (Fig. 5B, lower panels). This may reflect loss of a negative feed-back control of Mek by Erk (20). Hence, MKP3 had opposing effects on Mek and Erk phosphorylation, but the dominant function was to act as a negative regulator of PDGF-mediated Erk activation.

In cells with enhanced Erk activation after silencing of MKP3, we observed a significant increase in the mitogenic response to PDGF-BB in both PAE/PDGFRα and PAE/PDGFRβ cells, in accordance with a critical role of Erk in mediating proliferative signals (Fig. 6).

**DISCUSSION**

In the present study, we have investigated the mechanisms by which PDGF-BB activates the Erk MAPK pathway. We provide...
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In addition, a possibility that remains to be elucidated is that the catalytic activity of the MKPs may be regulated by PDGF stimulation although their expression level remains constant.

We observed that PDGF-BB induced a slower migration of MKP3 in SDS-PAGE, and we were able to show that PDGF-BB induced phosphorylation of MKP3 in a Mek-dependent manner. Moreover, this modification was required for degradation of MKP3, which we speculate may occur through a phosphorylation-dependent recruitment of a ubiquitin ligase. In accordance with this hypothesis, it has been reported that Erk-mediated phosphorylation of MKP1 (Ser-296 and Ser-323) leads to recruitment of the ubiquitin-ligase SCFe6p2 (22). However, manipulation of SCFe6p2 levels did not influence the PDGF-BB-induced MKP3 degradation in our model system (data not shown). In contrast, phosphorylation of MKP1 (Ser-359 and Ser-364) by Erk has been associated with increased MKP1 stability (25). Thus, regulation of MKP1 protein stability is complex, and it appears that different phosphorylation sites may differentially control MKP1 degradation. It has been demonstrated that serum stimulation of fibroblasts leads to MKP3 degradation through Erk- and mTor-mediated phosphorylation of Ser-159 and Ser-197 (19, 26). In our model system, we were able to identify the novel phosphorylation site Ser-174 as important in regulating PDGF-induced MKP3 protein degradation. When we expressed MKP3 with Ser-174 mutated to Ala (in combination with mutation of the active site Cys-293 to Ala in order to avoid complete Erk dephosphorylation) together with PDGFRα in 293T cells, we found that the mutant MKP3 form was stabilized compared with MKP3 only containing the C293A mutation. In fact, also in the absence of PDGF-BB stimulation, we observed a stabilization of the Ser-174 (and Ser-159) mutant forms of MKP3, implying that there is a constitutive rate of MKP3 degradation, which may be accelerated by PDGF-BB treatment. Consistently, also in the absence of PDGF-BB stimulation we observed MKP3 migrating as multiple bands suggesting basal phosphorylation when expressed in 293T cells. In addition, endogenous MKP3 was stabilized by MG132 both in the absence and presence of PDGF-BB stimulation, suggesting a constitutive proteasomal turnover. Although we were not able to detect PDGF-BB-induced phosphorylation of Ser-159 and Ser-197, which are phosphorylated after serum stimulation (19), we could confirm a role of Ser-159 in MKP3 stability by site-directed mutagenesis. This observation may be explained by phosphorylation of Ser-159 at low stoichiometry induced by the low amount of serum present in our starvation medium. An alternative explanation could be that mutation of Ser-159 could influence interaction(s) with protein(s) necessary for MKP3 degradation. We were not able to detect an effect of mutating Ser-197 to Ala on MKP3 degradation. A possible explanation for the lack of effect of the S197A mutation on MKP3 stability in our experiments, although such an effect has been previously reported (19, 26), may be that Ser-197 interacts in a phosphorylation-dependent manner with a scaffolding protein not expressed in our model system.

Evidence that MKP3, a cytoplasmic Erk-selective phosphatase (21), has an important role in the regulation of Erk phosphorylation in response to PDGF-BB stimulation. In an early phase after PDGF-BB stimulation, MKP3 is ubiquitinated and targeted for proteasomal degradation in a Mek-dependent manner, requiring phosphorylation of Ser-174 or Ser-159 in MKP3. The MKP3 degradation is essential for activation of Erk. At a later phase, mkp3 is induced and MKP3 is synthesized, leading to an efficient dephosphorylation and deactivation of Erk. Interestingly, both the degradation and subsequent synthesis of MKP3 are dependent on activation of the Erk pathway, indicating that they represent positive feed-forward and negative feedback mechanisms, respectively (Fig. 7). In fact, it was reported by Lin and Yang (22) that Erk activation induced proteasomal degradation of MKP3, which at a later time point dephosphorylates Erk (negative feed-back mechanism).

Stimulation of cells expressing PDGFRα or PDGFRβ led to a transient increase in gene expression of the dual specificity phosphatases mkp1, mkp2, mkp3, and hvh3, but this did not translate into changes in protein levels for MKP1, MKP2, and hVH3. This finding may be explained by the presence of a translational block that possible could be released by a signal not present in our cellular model. In contrast, the increase of mkp3 mRNA induced by PDGFRα translated into efficient MKP3 protein synthesis, whereas PDGFRβ-mediated mkp3 gene expression resulted in only a minor increase in MKP3 protein levels. This control of protein translation, which differs downstream of PDGFRα and PDGFRβ, provides an additional level of regulation. The phosphatases MKP1, MKP2, MKP4, MKP6, and hVH3 are able to dephosphorylate Erk (14, 23, 24). Since the protein levels of these MKPs are not affected by PDGF-BB treatment, they may provide PDGF-independent phosphatase activity that inactivates Erk in the absence of a continued activating signal.

FIGURE 7. Schematic representation of MKP3-mediated regulation of Erk signaling. Initially, PDGF-BB causes Mek-dependent degradation of MKP3, which allows a rapid increase in Erk phosphorylation (positive feed-forward mechanism). At the same time, PDGF-BB induces Erk-dependent synthesis of MKP3, which at a later time point dephosphorylates Erk (negative feed-back mechanism).
In addition, Erk activation is necessary for PDGF-mediated mkp3 gene expression, which ultimately halts Erk signaling after prolonged ligand stimulation. In concurrence, it was recently demonstrated that Erk via Ets factors is required for mkp3 gene expression in response to FGF (27). Moreover, also serum-induced MKP1 and MKP2 protein expression is sensitive to inhibition of the Erk pathway (28).

Our data suggest that MKP3 acts as a positive regulator of Mek. The reduced Mek phosphorylation observed after MKP3 down-regulation and, conversely, enhanced phosphorylation when MKP3 was overexpressed can be due to changes in Erk activity; Erk may, through direct phosphorylation of Mek, Raf, or Sos, negatively regulate Mek activation (20). Thus, by changing the level of MKP3, the ability of Erk to establish negative feed-back loops could be affected.

We observed that the difference in MKP3 protein expression downstream of PDGFα and PDGFβ was correlated with a difference in temporal pattern of Erk phosphorylation. Since the kinetics of Erk phosphorylation is of importance for the end effect on cell growth versus differentiation (9), the difference in kinetics could contribute to the subtle differences in signaling via the two PDGF receptors. siRNA-mediated down-regulation of MKP3 resulted in a marked increase in Erk phosphorylation in response to PDGF-BB, despite less persistent Mek activation. Consistent with reports suggesting that a sustained and robust Erk activation is necessary to induce cell proliferation (10–12), we observed an increased mitogenic response to PDGF-BB when MKP3 was down-regulated. Our data indicate that MKP3 serves to dampen the PDGF-induced Erk activation and proliferative signaling. In summary, Erk exploits MKP3 to regulate its own activity in two signal transduction phases: first in the initial amplification of PDGF-BB-induced Erk activation through phosphorylation-induced MKP3 degradation and later, after prolonged PDGF-BB stimulation, in the reduction in Erk activity via resynthesis of MKP3.

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REFERENCES

1. Heldin, C. H., and Westermark, B. (1999) Physiol. Rev. 79, 1283–1316
2. Fredriksson, L., Li, H., and Eriksson, U. (2004) Cytokine Growth Factor Rev. 15, 197–204
3. Pawson, T. (1995) Nature 373, 573–580
4. Chang, L. and Karin, M. (2001) Nature 410, 37–40
5. Johnson, G. L., and Lapadat, R. (2002) Science 298, 1911–1912
6. Yoon, S., and Seger, R. (2006) Growth Factors 24, 21–44
7. Dent, P., Yacoub, A., Fisher, P. B., Hagan, M. P., and Grant, S. (2003) Oncogene 22, 5885–5896
8. Hoshino, R., Chatani, Y., Yamori, T., Tsuruo, T., Oka, H., Yoshida, O., Shimada, Y., Ari-i, S., Wada, H., Fujimoto, J., and Kohno, M. (1999) Oncogene 18, 813–822
9. Marshall, C. J. (1995) Cell 80, 179–185
10. Cook, S. J., and McCormick, F. (1996) Biochem. J. 320, 237–245
11. Meloche, S., Seuwen, K., Pages, G., and Pouyssegur, J. (1992) Mol. Endocrinol. 6, 845–854
12. Yamamoto, T., Ebisuya, M., Ashida, F., Okamoto, K., Yonehara, S., and Nishida, E. (2006) Curr. Biol. 16, 1171–1182
13. Camps, M., Nichols, A., and Arkinstall, S. (2000) FASEB J. 14, 6–16
14. Farooq, A., and Zhou, M. M. (2004) Cell. Signal. 16, 769–779
15. Camps, M., Chabert, C., Muda, M., Boschert, U., Gillieron, C., and Arkinstall, S. (1998) FEBS Lett. 425, 271–276
16. Hirsch, D. D., and Stork, P. J. (1997) J. Biol. Chem. 272, 4568–4575
17. Misra-Press, A., Rim, C. S., Yao, H., Roberson, M. S., and Stork, P. J. (1995) J. Biol. Chem. 270, 14587–14596
18. Blume-Jensen, P., Wernstedt, C., Heldin, C. H., and Ronnstrand, L. (1995) J. Biol. Chem. 270, 14192–14200
19. Marchetti, S., Gimond, C., Chambard, J. C., Touboul, T., Roux, D., Pouyssegur, J., and Pages, G. (2005) Mol. Cell. Biol. 25, 854–864
20. Shaul, Y. D., and Seger, R. (2007) Biochim. Biophys. Acta 1773, 1213–1226
21. Muda, M., Boschert, U., Dickinson, R., Martinou, J. C., Martinou, I., Camps, M., Schlegel, W., and Arkinstall, S. (1996) J. Biol. Chem. 271, 4319–4326
22. Lin, Y. W., and Yang, J. L. (2006) J. Biol. Chem. 281, 915–926
23. Mandl, M., Slack, D. N., and Keyse, S. M. (2005) Mol. Cell. Biol. 25, 1830–1845
24. Marti, F., Krause, A., Post, N. H., Lyddane, C., Dupont, B., Sadelain, M., and King, P. D. (2001) J. Immunol. 166, 197–206
25. Brondello, J. M., Pouyssegur, J., and McKenzie, F. R. (1999) Science 286, 2514–2517
26. Bermudez, O., Marchetti, S., Pages, G., and Gimond, C. (2008) Oncogene 27, 3685–3691
27. Ekerot, M., Stavridis, M. P., Delavaine, L., Mitchell, M. P., Staples, C., Owens, D. M., Keenan, I. D., Dickinson, R. J., Storey, K. G., and Keyse, S. M. (2008) Biochem. J. 412, 287–298
28. Brondello, J. M., Brunet, A., Pouyssegur, J., and McKenzie, F. R. (1997) J. Biol. Chem. 272, 1368–1376