Sequential Activation of Rac-1, SEK-1/MKK-4, and Protein Kinase Cδ Is Required for Interleukin-6-induced STAT3 Ser-727 Phosphorylation and Transactivation*

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Jan-Jacob Schuringa‡§, Lodewijk V. Dekker‡, Edo Vellenga‡§, and Wiebe Kruijer‡¶

From the ‡Department of Genetics, Biological Center, Kerklaan 30, 9751 NN Haren, The Netherlands, the §Department of Hematology, University Hospital Groningen, 9700 RB Groningen, The Netherlands, and the ¶University College London, Department of Medicine, The Rayne Institute, 5 University St., London WC1E 6JJ, United Kingdom

Activation of signal transducer and activator of transcription 3 (STAT3) by interleukin-6 (IL-6) involves phosphorylation of Tyr-705 and Ser-727, both of which are critical for STAT3 transactivation. Here, we demonstrate that IL-6 activates Rac-1 and SEK-1/MKK-4 of the stress-activated protein kinase pathway, as well as protein kinase Cδ (PKCδ), as indicated by PKCδ Thr-505 phosphorylation. However, JNK-1, the end point kinase of the stress-activated protein kinase pathway signal transduction cascade, is not activated by IL-6. PKCδ was found to be associated with SEK-1/MKK-4 in unstimulated HepG2 cells but rapidly dissociates from SEK-1/MKK-4 upon IL-6 stimulation to become associated with STAT3. Inhibition of PKCδ using rottlerin (6 μM) or by overexpression of dominant negative PKCδ demonstrates that PKCδ kinase activity is required for STAT3 Ser-727 phosphorylation and transactivation but not for STAT3 Tyr-705 phosphorylation or nuclear import. PKCδ signals downstream of Rac-1 and SEK-1/MKK-4, because enhanced STAT3 transactivation induced by overexpression of constitutive active RacV12 was strongly abrogated by rottlerin, whereas IL-6-induced SEK-1/MKK-4 Thr-223 phosphorylation was not affected under these conditions. Studying the kinetics of STAT3 and PKCδ phosphorylation in cytoplasmic and nuclear fractions revealed that STAT3 Tyr-705 phosphorylation and nuclear translocation precedes PKCδ Thr-505 and STAT3 Ser-727 phosphorylation. Furthermore, the IL-6-induced PKCδ Thr-505 and STAT3 Ser-727 phosphorylation were only observed in nuclear fractions of HepG2 cells. These results demonstrate that IL-6-induced STAT3 transactivation involves the sequential activation of Rac-1 and SEK-1/MKK-4, which leads to nuclear translocation of PKCδ by release from a SEK-1/MKK-4-containing complex. Our results further indicate that PKCδ-mediated STAT3 Ser-727 phosphorylation is mainly a nuclear event.

Signal transducers and activators of transcription (STATs) belong to a family of transcription factors that are activated in response to a variety of cytokines and growth factors (1–4). So far, seven different STATs have been identified in mammals; these STATs contain conserved DNA binding and Src homology 2 domains that include a C-terminal tyrosine phosphorylation site (4). Binding of cytokines to their corresponding receptors induces Jak kinase activity, which results in phosphorylation of STATs on a specific tyrosine residue (5–7). Tyrosine-phosphorylated STATs dimerize, translocate to the nucleus, and bind specific DNA promoter sequences (8, 9).

IL-6-induced STAT3 signaling involves the sequential activation of the gp130 receptor complex and the gp130-associated protein-tyrosine kinases Jak1, Jak2, and Tyk2 (4, 10–12). Tyrosine phosphorylation of STAT3 occurs at a single tyrosine residue (Tyr-705) that is located in conserved Src homology 2 domain, allowing homodimerization as well as heterodimerization with other STAT family members (4, 13). In addition to tyrosine phosphorylation, STAT3 is serine-phosphorylated at a single residue (Ser-727) in response to IL-6 as well as other extracellular factors, including interferon-γ and epidermal growth factor (13–17). Although the role of serine 727 phosphorylation is not yet unambiguously determined, it has been shown to strongly enhance STAT3 transcriptional activation, possibly by modulating interactions with cofactors (13, 18). The Ser-727 residue of STAT3 is located in a conserved Pro-X-Ser-Pro sequence, which is recognized by the mitogen-activated protein kinase ERK (19). ERKs belong to the family of serine/threonine kinases positioned at the end point of signal transduction cascades that are initiated at the plasma membrane by ligand-receptor interaction (20). Indeed, it has been demonstrated that epidermal growth factor-induced STAT3 Ser-727 phosphorylation involves the activation of ERKs (14). However, it has also been shown that IL-6-induced STAT3 Ser-727 phosphorylation is an ERK-independent process (14, 18, 21, 22). Lim and Cao (23) have demonstrated that stress treatment could induce STAT3 Ser-727 phosphorylation via JNK-1, whereas we demonstrated that IL-6-induced STAT3 transactivation and Ser-727 phosphorylation involves the activation of the GDP-GTP exchange factor Vav, the GTPase Rac-1, and the kinases mitogen-activated protein kinase/ERK kinase 1 and SEK-1/MKK-4 in human hematoma cells (18).

Recently, it has been described that PKCδ associates with and phosphorylates STAT3 on Ser-727 in an IL-6-dependent manner (24). Activation of G protein-coupled receptors, tyrosine kinase receptors, and non-tyrosine kinase receptors can activate PKCs, and it has been demonstrated that PKCδ unphosphate-buffered saline; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA response element MAP, mitogen-activated protein; SEK-1, SAPK/ERK kinase 1; M KK-4, MAP kinase kinase 4.
undergoes both tyrosine and threonine phosphorylation upon activation (25, 26). So far, the signal transduction cascade(s) involved in IL-6-induced activation of PKCδ is not well defined. Here, we further explored the role of Rac-1, SEK-1/MKK-4, and PKCδ in IL-6-induced STAT3 transactivation and Ser-727 phosphorylation. We provide evidence that PKCδ is activated by IL-6 and signals downstream of Rac-1 and SEK-1/MKK-4 in IL-6-induced STAT3 Ser-727 phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Antibodies—The human hepatoma cell line HepG2 was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Integro B.V., Zaan- dam, The Netherlands). Cells were stimulated with 25 ng/ml human IL-6 in 10% heat-inactivated fetal calf serum (Integro B.V., Zaan-dam, The Netherlands). Cells were stimulated with 25 ng/ml human IL-6 or 100 ng/ml TPA for 24 h prior to luciferase and LacZ assays. B. mammalian two hybrid assay in which HepG2 cells were transfected with the UAS-luciferase reporter containing four GAL binding sites together with expression vectors for GAL-4-JNK-1 and/or SEK-1/VP16 as indicated. Cells were stimulated with 25 ng/ml IL-6 or 100 ng/ml TPA for 24 h prior to luciferase and LacZ assays as indicated.

Transient Transfections—HepG2 cells were seeded at 3 × 10^5 cells/well in six-well plates (Costar), and 24 h later, cells were transfected with 10 μg of plasmid DNA using the calcium phosphate co-precipitation method (28). Transfection mixtures consisted of a mixture of 3 μg of pRE LUC reporter, 3 μg of pDM2-LacZ as a control to determine transfection efficiency, and 1–4 μg of expression plasmids for dominant negative or constitutive active signal transduction components, as mentioned under “Results.” When necessary, pUC18 was added to the transfection mixture to obtain a total of 10 μg of DNA. Cells were incubated with precipitate for 24 h, washed with phosphate-buffered saline (PBS), and stimulated for an additional 24 h. Cells were collected for luciferase and β-galactosidase assays as indicated.
FIG. 3. Activation and localization patterns of PKCδ and STAT3. A, HepG2 cells were transfected with the IRE-LUC reporter and pretreated with rottlerin for 30 min as indicated prior to the addition of 25 ng/ml IL-6. After 24 h, cell lysates were subjected to luciferase and LacZ assays. B, HepG2 cells were pretreated with 6 μM rottlerin as indicated, followed by IL-6 stimulation. Total cell extracts were Western blotted using antibodies against STAT3 or phosphorylated STAT3 (Ser-727 and Tyr-705). Quantifications of the designated bands are shown below the panels. C, HepG2 cells were pretreated with 6 μM rottlerin, followed by IL-6 stimulation for various time periods as indicated. Nuclear extracts were prepared and Western blotted using antibodies against STAT3. Quantifications of the designated bands are shown below the panels. D, HepG2 cells were transiently transfected with pUC18 as a control or with an expression vector encoding dominant negative PKCδ. Cells were stimulated with 25 ng/ml IL-6, and total cell extracts were Western blotted using antibodies against STAT3 and phosphorylated STAT3 (Tyr-705 and Ser-727). F, HepG2 cells were transfected with the IRE-LUC reporter, together with increasing concentrations of dominant negative PKCδ. Cells were stimulated with 25 ng/ml IL-6, and after 24 h, cell lysates were subjected to luciferase and LacZ assays.
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in 200 μl of reporter lysis buffer (PromeGA) and subjected to the assays for luciferase and β-galactosidase as previously described. The data represent two independent experiments using different batches of DNA, and in each experiment, transient transfections were performed in triplicate. Standard deviations were calculated using Sigmaplot (Jandel Corp.).

Western Blotting and Immunoprecipitations—A total of 1 × 10^6 cells were lysed on ice in lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM dithiothreitol, 1 mM NaVO₃ (ortho), 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, and 0.4 mM phenylmethylsulfonyl fluoride). Prior to SDS-polyacrylamide gel electrophoresis and immunoprecipitations, protein concentrations were determined (Bio-Rad), and equal amounts were used in the experiments. Whole-cell extracts were boiled for 5 min in the presence of Laemmli sample buffer prior to separation on 12.5% SDS-polyacrylamide gels. The proteins were transferred to a nitrocellulose filter (Millipore) in Tris-glycine buffer at 100 Volts for 1.5 h using an electrot blotter (Amersham Pharmacia Biotech). Membranes were blocked with PBS buffer containing 5% nonfat milk prior to incubation with antibodies. Binding of each antibody was detected by chemiluminescence using ECL according to the manufacturer’s recommendations (Amersham Pharmacia Biotech). For immunoprecipitations, whole cell lysates were incubated with anti-SEK-1/MKK-4 or anti-PKCδ antibodies, precipitated with protein A-Sepharose beads (Amersham Pharmacia Biotech), and washed twice with lysis buffer. The precipitates were boiled for 5 min in Laemmli sample buffer and subjected to 12.5% SDS-polyacrylamide gel electrophoresis. Blots were scanned and quantified using Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

Fluorescence Microscopy—HepG2 cells were grown overnight on glass coverslips and pretreated with 6 μM rottlerin where indicated, followed by stimulation with IL-6 for 15 min. Cells were fixed in 4% paraformaldehyde in PBS for 30 min and permeabilized in 0.1% Triton X-100 in PBS for 5 min. Cells were stained by incubation with paraformaldehyde in PBS for 30 min and permeabilized in 0.1%

microscopy demonstrated that IL-6, unlike the stress inducers UV, Anisomycin, and TPA, did not induce JNK-1 nuclear translocation (data not shown). Taken together, these results indicate that the end point kinase JNK-1 of the stress-activated protein kinase pathway signal transduction cascade is not involved in IL-6-induced STAT3 Ser-727 phosphorylation and transactivation.

PKCδ Kinase Activity and Its Role in STAT3 Ser-727 Phosphorylation—Because JNK-1 is not involved in IL-6-induced STAT3 transactivation and PKCδ physically interacts with STAT3 in an IL-6-dependent manner, the role of PKCδ on STAT3 Tyr-705 and Ser-727 phosphorylation, nuclear import, and STAT3 transactivation was studied. Pretreatment of HepG2 cells with the PKCδ inhibitor rottlerin, which effectively inhibits PKCδ activity but does not affect the activity of other PKC isoforms at concentrations ranging from 0 to 6 μM (31, 32), strongly reduced STAT3 transactivation in a dose-dependent manner (Fig. 3A). Rottlerin did not inhibit luciferase activity because the dose-dependent inhibition of rottlerin on IL-6-induced STAT3 transactivation was still observed in experiments on a shorter time-scale in which cells were stimulated with IL-6 for 6 h instead of 24 h (data not shown). The reduced STAT3 transactivation in the presence of rottlerin was coupled to a reduced IL-6-induced STAT3 Ser-727 phosphorylation, whereas the IL-6-induced STAT3 Tyr-705 phosphorylation was unaffected (Fig. 3B). Inhibition of PKCδ activity only affects IL-6-induced STAT3 Ser-727 phosphorylation and not STAT3 nuclear import, as determined by Western blotting of nuclear fractions (Fig. 3C) and intracellular localization studies using fluorescence microscopy (Fig. 3D). To specifically determine the role of PKCδ in IL-6-induced STAT3 Ser-727 phosphorylation and transactivation, the regulatory domain of PKCδ (amino acids 1–298) was overexpressed; this domain functions as a dominant-negative as determined by in vitro kinase assays using purified PKCδ and GAP-43 as a substrate. In in vitro kinase assays, PKCδ-induced phosphorylation of GAP-43 was strongly reduced by the addition of the purified regulatory domain of PKCδ, indicating that this dominant-negative PKCδ inhibits the phosphorylation potential of wild-type PKCδ (data not shown). Accordingly, overexpression of dominant-negative PKCδ reduced IL-6-induced STAT3 Ser-727 phosphorylation, whereas the IL-6-induced STAT3 Tyr-705 phosphorylation remained unaffected (Fig. 3E). In addition, overexpression of dominant-negative PKCδ reduced STAT3 transactivation, although at high concentrations of dominant-negative PKCδ, inhibition of STAT3 transactivation was less effective (Fig. 3F).

Furthermore, the kinetics of nuclear translocation and activation of PKCδ and STAT3 were studied. IL-6-induced PKCδ Thr-505 phosphorylation was transient, reaching maximal levels between 5 and 30 min, and was only detected in nuclear and not cytoplasmic fractions of HepG2 cells. IL-6-induced STAT3 Ser-727 phosphorylation was also only observed in the nuclear fractions, reaching maximal levels between 10 and 30 min. In contrast, STAT3 Tyr-705 phosphorylation and STAT3 nuclear import occurred with faster kinetics, which were first detected at 2 min after IL-6 stimulation (Fig. 4).

PKCδ Signals Downstream of Rac-1 and SEK—Because PKCδ is activated by IL-6 and dynamically associated with both SEK-1/MKK-4 and STAT3 in an IL-6-dependent manner, we questioned whether PKCδ signals downstream of the signal
transduction cascade that includes Rac-1 and SEK-1/MKK-4 in IL-6-induced STAT3 Ser-727 phosphorylation and transactivation. First, constitutive active RacV12 was overexpressed in HepG2 cells and STAT3 Ser-727 phosphorylation was studied. In control pUC transfected cells, IL-6 induced Ser-727 phosphorylation within 15 min, whereas no basal STAT3 Ser-727 phosphorylation was observed (Fig. 5A). Overexpression of RacV12 strongly enhanced both basal and IL-6-induced STAT3 Ser-727 phosphorylation, whereas dominant negative RacN17 had the opposite effect (Fig. 5A). STAT3 Tyr-705 phosphorylation (Fig. 5A) and STAT3 nuclear import (data not shown) were not affected by RacV12 overexpression. Overexpression of constitutive active RacV12 also enhanced both basal and IL-6-induced SEK/MKK-4 Thr-223 phosphorylation, whereas overexpression of dominant negative RacN17 abrogated the IL-6-induced activation of SEK-1/MKK-4 (Fig. 5A), indicating that Rac-1 signals upstream from SEK/MKK-4. Furthermore, the IRE-LUC reporter was transiently transfected together with expression vectors encoding dominant negative RacN17 or constitutive active RacV12 mutants, and PKCδ activity was blocked with rottlerin. As depicted in Fig. 5B, rottlerin augmented the inhibitory effects of RacN17, whereas the enhancing effects of RacV12 were completely inhibited. Similarly, rottlerin augmented the inhibitory effects of overexpressed
dominant negative SEK(A-L) on IL-6-induced STAT3 transactivation (Fig. 5C). Because rottlerin did not inhibit IL-6-induced SEK-1 Thr-223 phosphorylation (Fig. 5D), these results strongly suggest that PKCδ signals downstream of Rac-1 and SEK-1/MKK-4 in the IL-6-induced STAT3 transactivation.

Discussion

In addition to STAT3 tyrosine phosphorylation, STAT3 is phosphorylated on a specific Ser-727 residue in response to growth factors and cytokines (13–18, 33). In this study, we have investigated the involvement of PKCδ and members of the stress-activated protein kinase cascade in IL-6-induced STAT3 Ser-727 phosphorylation and transactivation. Our results demonstrate that in unstimulated HepG2 cells, PKCδ strongly associates with SEK-1/MKK-4 and is released from this complex in an IL-6-dependent manner, which subsequently leads to STAT3 association and Ser-727 phosphorylation independent of JNK-1. Based on these findings, we conclude that PKCδ might be considered as the end point kinase of the IL-6-activated Rac-1, SEK-1/MKK-4 signal transduction cascade and that activated PKCδ is responsible for STAT3 Ser-727 phosphorylation and transactivation through an increased interaction with STAT3.

In HepG2 cells, IL-6 induces both SEK-1/MKK-4 Thr-223 and PKCδ 84 h-505 phosphorylation. PKCδ threonine 505 is located in the activation loop, and it has been demonstrated that phosphorylation at this site is required for its maximal catalytic activity in eukaryotic cells (34). Previously, it has been demonstrated that IL-6 induces PKCδ kinase activity in HepG2 cells (24), and here we identify the residue threonine 505 as a regulatory PKCδ phosphorylation site in response to IL-6 stimulation. Blocking PKCδ kinase activity with rottlerin does not affect the activity of SEK-1/MKK-4, suggesting that IL-6 first activates and phosphorylates SEK-1/MKK-4, followed by the phosphorylation and release of PKCδ. Because IL-6 induces Thr-505 phosphorylation of PKCδ, it is plausible that SEK-1/MKK-4 kinase activity is required for this phosphorylation event, and experiments are currently under way to support this hypothesis.

Further support of the idea that PKCδ signals downstream of SEK-1/MKK-4 in IL-6-induced STAT3 transactivation is derived from overexpression of constitutive active and dominant negative variants of SEK-1/MKK-4 and its upstream regulator Rac-1. Overexpression of constitutive active RacV12 strongly enhanced both SEK-1/MKK-4 Thr-223 and STAT3 Ser-727 phosphorylation, as well as STAT3 transactivation. Inhibition of PKCδ activity with rottlerin completely abolished the RacV12 enhanced STAT3 transactivation, but did not affect the IL-6-induced SEK-1/MKK-4 Thr-223 phosphorylation. These data imply that IL-6-induced SEK-1/MKK-4 kinase activity is required for this phosphorylation event, and experiments are currently under way to support this hypothesis.

The association of PKCδ and SEK-1/MKK-4 in IL-6 signal transduction is somewhat remarkable, as JNK-1 is generally regarded as the end point kinase of the stress-activated signal transduction cascade. However, in HepG2 cells, neither JNK-1 activation nor JNK-1 association with SEK-1/MKK-4 could be demonstrated in response to IL-6. These results strengthen the view that in unstimulated HepG2 cells, JNK-1 and PKCδ are anchored in different signal transduction protein complexes through physical association with SEK-1/MKK-4. These complexes...
plexes are activated in a strictly ligand-dependent manner, which may further involve segregation of the activated components into different cellular compartments. Further studies are required to clarify these issues.

PKCδ activity is essential for IL-6-induced STAT3 signaling based on the following observations: (i) the PKCδ specific inhibitor rottlerin inhibits IL-6-induced STAT3 Ser-727 phosphorylation, and (ii) rottlerin strongly reduces IL-6-induced STAT3 transactivation. Although rottlerin also inhibits calmodulin-kinase III activity, we confirmed the role of PKCδ in STAT3 Ser-727 phosphorylation by overexpression of a dominant negative mutant of PKCδ, which reduced STAT3 Ser-727 phosphorylation as well as STAT3 transactivation, whereas IL-6-induced STAT3 Tyr-705 phosphorylation remained unaffected. The role of serine 727 phosphorylation in STAT3 transactivation has not yet been unambiguously determined, but the present study demonstrates a direct correlation between STAT3 Ser-727 phosphorylation and transactivation. Treatment with rottlerin severely reduced the IL-6-induced STAT3 Ser-727 phosphorylation, as well as STAT3 transactivation, whereas STAT3 Tyr-705 phosphorylation or nuclear import was not affected. In addition, we and others demonstrated that overexpression of either STAT3ζ, which lacks the C-terminal transactivation domain that includes residue Ser-727, or a STAT3 S727A mutant severely impairs the IL-6-induced STAT3 transactivation, confirming the role of Ser-727 phosphorylation on STAT3 transactivation (13, 18). In contrast to these findings, it has also been reported that overexpression of constitutive active mutants of PKCδ strongly enhanced STAT3 Ser-727 phosphorylation but severely impair IL-6-induced STAT3 transactivation DNA binding and its transcriptional activities (24). Also, STAT3 Tyr-705 phosphorylation induced by the tyrosine kinase Bmx has been shown to be inhibited by overexpression of PKCδ (35). It is conceivable that these observations are due to secondary effects such as the activation of tyrosine phosphatases or the up-regulation of negative feedback proteins such as suppressors of cytokine signaling, rather than a specific in-

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