Protein Kinase C \( \delta \) Associates with and Phosphorylates Stat3 in an Interleukin-6-dependent Manner*

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Stat3 is activated by phosphorylation on Tyr-705, which leads to dimer formation, nuclear translocation, and regulation of gene expression. Serine phosphorylation of Stat3 by mitogen-activated protein kinase has also been observed in cells responding to epidermal growth factor and shown to affect its tyrosine phosphorylation and transcriptional activity. Serine phosphorylation of Stat3 is also induced by interleukin-6 (IL-6) stimulation, which is shown to be independent of mitogen-activated protein kinase and sensitive to the Ser/Thr kinase inhibitor H7. In this study, we investigated whether protein kinase C (PKC) is the kinase that is induced and responsible for Stat3 serine phosphorylation by IL-6 stimulation and which isoform of PKCs is likely to be involved. Here, we report that Stat3 was specifically associated with PKC \( \delta \) in vitro in an IL-6-dependent manner in several cell types. Furthermore, Stat3 was phosphorylated by PKC \( \delta \) in vivo on Ser-727, which could be inhibited either by a specific PKC \( \delta \) inhibitor or by a dominant-negative mutant of PKC \( \delta \). Finally, we showed that the phosphorylation of Stat3 by PKC \( \delta \) led to a negative regulation of Stat3 DNA binding and transcriptional activity. These results indicate that PKC \( \delta \) is likely to be the kinase that phosphorylates Stat3 in response to IL-6 stimulation and suggest a possible regulatory role of PKC \( \delta \) on Stat3 function.

Cytokines and growth factors bind to receptors on the cell surface and activate signal transduction pathways that lead to cell proliferation and differentiation. JAK-STAT is a key pathway identified in recent years that mediates cellular responses to a variety of cytokines (reviewed in Refs. 1–3). Unlike the growth factor receptors, cytokine receptors lack intrinsic tyrosine kinase activity. Ligand binding to receptors activates the receptor-associated tyrosine kinase family, such as Jak5, which phosphorylates the target tyrosine residues on the receptor (4–7). The latent cytoplasmic transcription factors, STATs, are selectively recruited to the receptors through binding of their SH2 domain to the phosphorylated tyrosine residue on the receptors and subsequently phosphorylated by Jaks on specific tyrosine residues at the COOH terminus (8, 9). STATs form homo- or heterodimers, translocate into the nucleus, bind to their recognition sequences, and regulate target gene expression (1–3). STATs were originally identified in interferon-\( \alpha \) and -\( \gamma \) signaling pathways and later found to be activated by various cytokines and growth factors (2, 10–13). Seven members have been identified so far in mammalian cells (reviewed in Ref. 14). Among them, Stat3 was identified as an acute-phase response factor activated by IL-6 in mouse liver (15) and also was cloned by low stringency screening of mouse cDNA library with a probe derived from Stat1 (16). IL-6 is a pleiotropic cytokine that controls cell growth, differentiation, and other functions in various cell types (17). Other IL-6 family members, such as IL-11, ciliary neurotrophic factor, oncostatin M, and leukemia inhibitory factor, which share the common gp130 receptor subunit, also stimulate Stat3 activation (6, 7). In response to IL-6, Stat3 is transiently associated with gp130, phosphorylated by the receptor-associated Jaks on Tyr-705, and translocated to the nucleus, where it binds to IL-6 response elements and regulates many acute-phase protein genes (6, 18, 19). Therefore, Stat3 represents an important regulator of IL-6 target gene expression. Furthermore, growth factors, such as EGF and platelet-derived growth factor (PDGF), can also activate Stat3 (13, 16). In addition to tyrosine, Stat3 is also phosphorylated on serine. Recently, Stat3 was shown to be phosphorylated on Ser-727 by extracellular signal-regulated kinase-2 (ERK2), a member of the mitogen-activated protein kinase family, in response to EGF, and this serine phosphorylation negatively affected its tyrosine phosphorylation (20–22). IL-6 also induces serine phosphorylation of Stat3. However, the involved Ser/Thr kinase has not been identified, but it has been shown to be independent of MAP kinases and sensitive to a Ser/Thr kinase inhibitor, H7 (18, 22, 23).

Protein kinase C (PKC) is a heterogenous family of phospholipid-dependent kinases. Activation of G protein-coupled receptors, tyrosine kinase receptors, and nonreceptor tyrosine kinases can stimulate PKC activation, which leads to long term responses of cells, such as differentiation, proliferation, and apoptosis (24, 25). PKC activity can be inhibited by the inhibitor of Ser/Thr kinases, H7 (26). At least 11 isoforms of PKCs have been identified and classified into three major categories based on differential Ca\(^{2+}\) and lipid requirements. The conventional PKCs (\( \alpha \), \( \beta \), and \( \gamma \)) require both Ca\(^{2+}\) and either diacylglycerol or phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), as cofactors. The novel PKCs (\( \epsilon \), \( \delta \), \( \eta \), and \( \theta \)) require diacylglycerol or PMA, but not Ca\(^{2+}\), whereas the atypical PKC isoforms (\( \iota \), \( \lambda \), and \( \zeta \)) are insensitive to both (reviewed in Ref. 27). PKC undergoes autophosphorylation on Ser/Thr upon activation. Initially, PKC \( \delta \) was the only isoform identified to undergo tyrosine phosphorylation in vivo in response to

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§ The abbreviations used are: Jak, Janus kinase; STAT, signal transducers and activators of transcription; IL, interleukin; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; H7, 1-(5-isouquinolinylsulfonyl)-2-methylpiperazine dihydrochloride; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PBS, phos-osphate-buffered saline; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmune precipitation; CMV, cytomegalovirus.
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several stimuli, and the kinase activity was differentially affected by its tyrosine phosphorylation, depending on the cell type (28–30). Recently, it has been shown that all PKC isoforms, including α, β, γ, ε, δ, and ζ, are tyrosine-phosphorylated and catalytically activated upon treatment of cells with H2O2 (31). In this study, we attempt to investigate whether PKC is involved in the Stat3 serine phosphorylation stimulated by IL-6. Here, we report that PKC δ specifically associates with Stat3 in several cell types in an IL-6-inducible manner. Stat3 is phosphorylated by PKC δ in vitro as well as in vivo on Ser-727, and the phosphorylation of Stat3 by PKC δ leads to an inhibition of Stat3 DNA binding and transcriptional activity.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Reagents—**HepG2 and A431 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum purchased from Life Technologies, Inc. The IL-3-dependent myeloid progenitor cell line 32D and the transfectants were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 5% WEHI-3B conditioned medium as a source of murine IL-3. Human IL-6 and EGF were purchased from Genzyme (Cambridge, MA), and PMA and phosphatidylinositol serine were from Sigma. H7 and the PKC inhibitors rottlerin and calphostin C were purchased from Biomol (Hamburg, Germany). Histone H1 and FuGENE6 transfection reagent were purchased from Roche Molecular Biochemicals.

**Antibodies, Immunoprecipitation, and Immunoblotting—**Antibodies against Stat1, Stat3, ERK2, and PKC isoforms were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY), and anti-phosphotyrosine-705-Stat3 and anti-phosphoserine-727-Stat3 were purchased from Cell Signaling Technology (Santa Cruz, CA). Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY), and anti-phosphotyrosine-705-Stat3 and anti-phosphoserine-727-Stat3 were purchased from New England Biolabs (Beverly, MA). Preparation of total cell lysates, immunoprecipitation, and immunoblotting were performed as described previously (32). In brief, cells were washed three times with cold phosphate-buffered saline (PBS) and lysed in 1 ml RIPA buffer without SDS (containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.2), 1% deoxycholic acid, 1% Triton X-100, 0.25 mM EDTA (pH 8.0), protease and phosphatase inhibitors, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, and 100 μM sodium orthovanadate). The cell lysates containing 2 mg of proteins were incubated with an anti-PKC antibody overnight at 4 °C followed by incubation with protein G plus protein A-agarose beads (Oncogene Science) for 1 h. The immunoprecipitates were washed twice with RIPA buffer and twice with cold PBS and subjected to Western blot analysis in which the immunoprecipitates were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and blotted with an anti-stat3 antibody.

**Immune Complex Protein Kinase Assay—**Cell lysates were immunoprecipitated with anti-PKC δ antibody as described above. The immunoprecipitates were washed three times with cold PBS and once with PKC kinase assay buffer (25 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.5 mM EGTA, 1 mM dithiothreitol, and 20 μg of phosphatidylinositol serine per reaction). The GST-Stat3 fusion proteins used as substrates were produced in Escherichia coli and partially purified as described previously (32). Histone H1 (10 μg) or the GST Stat3 fusion proteins (5 μg) were incubated for 10 min at 30 °C before adding cold ATP (20 μM) and 10 μCi of [γ-32P]ATP and incubated at 30 °C for various times (5 min to 10 min). The reaction mixture was boiled in Laemmli buffer, separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and exposed to x-ray film. The blots were also subjected to Amido Black staining to show the equal amount of the substrates used in each reaction.

**Separation of Nuclear and Cytoplasmic Component—**Cells were washed with PBS three times; resuspended in Buffer A containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM Nonidet P-40 with protease and phosphatase inhibitors; incubated on ice for 10 min; and homogenized in a Dounce homogenizer. The nuclei were removed by centrifugation and extracted in RIPA buffer, and the supernatant was collected as the cytoplasmic fraction.

**DNA Transfection, CAT Assay, and Mobility Shift DNA Binding Assay—**The expression plasmid of Stat3, pcR-CMV-Stat3, was obtained from Dr. J. E. Darnell (16). Expression plasmids of wild-type PKC δ and its mutant K376R were constructed by inserting the cDNA fragment (33) into the BamH I and EcoRI sites of pcR3.1. COS-1 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Transfection of the plasmids into COS-1 cells was performed by the calcium phosphate-DNA coprecipitation method. The cells were cotransfected with 4 μg of CAT-containing reporter plasmids, 5 μg of expression plasmids, and 2 μg of pCMV-β-galactosidase containing bacterial β-galactosidase gene. The total amount of DNA transfected was kept constant at 20 μg/plate with control plasmid pCMV-5. The cells were harvested at 48 h after transfection and lysed in 0.25% Triton X-100 (pH 8.0) with three freeze-thaw cycles. The lysate was spun, and the supernatant was collected and used for β-galactosidase activity and CAT assays. The pellets were resuspended in high salt buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1 mM Na3PO4, 1 mM NaVO4, 20 mM NaF, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and extracted to a crude nuclear extract for DNA mobility shift assay. The amount of cytoplasmic extract used in each CAT assay was normalized with equivalent β-galactosidase activity. Acetylated and nonacetylated forms of [14C]cholera toxin were separated by thin layer chromatography, followed by autoradiography and quantification using a Bio-Rad GS700 imaging densitometer. The mobility shift DNA binding assay was performed using hSIE as a probe under conditions described previously, except with the absence of salt in the binding buffer (32).

**RESULTS**

**IL-6 Stimulation Results in an Association of PKC δ and Stat3 in Various Cell Types—**Stat3 is phosphorylated on both tyrosine and serine by IL-6 stimulation in the human liver hepatoma cell line HepG2 (15, 23). To investigate whether PKC is the kinase involved in Ser phosphorylation of Stat3, we first examined the potential interaction between Stat3 and various isoforms of PKCs in HepG2 cells stimulated by IL-6. Lysates prepared from cells that were either left untreated or treated with IL-6 were immunoprecipitated with antibodies against individual isoforms of PKCs, including α, β, γ, ε, δ, ζ, and ζ. The immunoprecipitates were subjected to Western blot analysis using an anti-Stat3 antibody. Stat3 coimmunoprecipitated with PKC δ in an IL-6-inducible manner (Fig. 1A, top panel, lanes 1 and 2). Among other PKC isoforms, Stat3 also coimmunoprecipitated with PKC δ but was independent of IL-6 stimulation (data not shown). In contrast, Stat3 did not coimmunoprecipitate with other PKC isoforms (for example, Fig. 1A, top panel, lanes 3 and 4, show no coimmunoprecipitation of Stat3 with PKC λ, other data not shown). To test the specificity of Stat3/PKC δ interaction, a reciprocal immunoprecipitation/blotting experiment was performed. The lysates were immunoprecipitated with anti-Stat3 and blotted with anti-PKC δ antibody. Coimmunoprecipitation of PKC δ with Stat3 enhanced by IL-6 was detected (Fig. 1A, top panel, lanes 5 and 6). The amounts of immunoprecipitated PKC δ, PKC λ, and Stat3 were determined by reprobing the blots with their respective antibodies as shown in Fig. 1A, bottom panels. Other IL-6 family members, oncostatin M and leukemia inhibitory factor, which share the common gp130 receptor subunit, were also shown to stimulate the association of Stat3 with PKC δ (Fig. 1B). These results suggest cytokines of the IL-6 family stimulate a physical interaction between Stat3 and PKC δ.

We next performed a time course study of the Stat3/PKC δ association upon IL-6 stimulation. The association was barely detected in untreated cells, strongly increased at 15 min, decreased at 30 and 45 min, and returned to the basal level at 60 min of IL-6 stimulation (Fig. 1C, α). It was observed that the PKC δ-associated Stat3 appeared as a doublet when the SDS-PAGE was performed with a long gel in Fig. 1C. Because IL-6...
stimulates Stat3 phosphorylation on both serine and tyrosine, the doublet was further analyzed by immunoblotting with either an anti-phospho-serine antibody, 4G10, or an anti-phospho-Ser-727-Stat3 antibody (Fig. 1A, top panel), which correlated well with the strong association of Stat3 and PKCδ in HepG2 cells. On the other hand, PMA treatment, which activates PKC, did not promote an increase in either the association with the other isoforms of PKCs was not found in these cells with or without EGF or IL-6 stimulation (not shown). The PC12 cell line is a model system to study neuronal differentiation. Stat3 is not tyrosine-phosphorylated in response to nerve growth factor and EGF in this cell line (36), and no association with PKCδ was detected with either treatment. However, IL-6 stimulated both tyrosine phosphorylation of Stat3 and its association with PKCδ (data not shown). These data together suggest that the interaction of Stat3 and PKCδ induced by IL-6 is not restricted to one cell type and that the interaction is not restricted to IL-6 family of cytokines; instead, the association correlates with the tyrosine phosphorylation of Stat3, which can be induced by different ligands.

**Fig. 1.** IL-6 stimulates the association of Stat3 and PKCδ in HepG2 cells. A, HepG2 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum to approximate 80% confluence. Cells were either left untreated (–) or treated with IL-6 (50 ng/ml) for 15 min (+). Lysates were immunoprecipitated (IP) with antibodies against PKCδ, PKCλ, or Stat3. The immunoprecipitates were subjected to Western blot analysis using an anti-Stat3 or anti-PKCδ antibody (Blot) as indicated in the top panels. The blots were stripped and reprobed with the respective antibody against PKCδ, PKCλ, or Stat3 as indicated in the bottom panels. B, cells were either left untreated (–) or treated with IL-6, oncostatin M (OM) (50 ng/ml), or leukemia inhibitory factor (LIF) (50 ng/ml) for 15 min. Lysates were immunoprecipitated with anti-PKCδ antibody and blotted with anti-Stat3 (top panel) or anti-PKCδ antibody (bottom panel). C, time course of PKCδ and Stat3 interaction in response to IL-6 stimulation. HepG2 cells were either left untreated (0) or treated with IL-6 for 15, 30, 45, and 60 min. Cell lysates were immunoprecipitated with antibody against PKCδ, and the immunoprecipitates were blotted with anti-Stat3 antibody as shown in A. The blot was stripped and reprobed with anti-phosphotyrosine antibody, 4G10 (b), anti-phospho-Ser-727-Stat3 (c), or anti-PKCδ (d) antibody. Total cell lysates were also subjected to a direct Western blot analysis with either an anti-phospho-Tyr-705-Stat3 (e) or anti-Stat3 (f) antibody. The positions of Stat3 and PKCδ are indicated by arrows.

![PKCδ Associates with and Phosphorylates Stat3](image)

**Fig. 2.** Interaction of PKCδ and Stat3 in A431 cells. A431 cells were left untreated (–) or treated with EGF or IL-6 for 15 min. A, the cell lysates were immunoprecipitated with anti-PKCδ antibody and probed with anti-Stat3 antibody (top panel). The blot was stripped and reprobed with anti-PKCδ antibody (bottom panel). B, total cell lysates were subjected to a direct Western blot analysis with either anti-phospho-Tyr-705-Stat3 (top panel) or anti-Stat3 antibody (bottom panel). Stat3 and PKCδ are indicated by arrows.
that IL-6 stimulates PKC \( d \) in the kinase assays is shown in Fig. 4 (bottom panel) or anti-phospho-Tyr-705 (clear proteins with anti-Stat3 (Western blots of the cytoplasmic and nuclear proteins with anti-Stat3 (bottom panel) or anti-phospho-Tyr-705 (bottom panel) antibody.

**Cellular Localization of PKC \( d \)/Stat3 Association**—To elucidate the possible role of PKC \( d \)/Stat3 interaction, we determined the cellular localization of these two proteins as well as their association. Extracts of cytoplasm and nucleus were prepared from HepG2 cells with or without IL-6 treatment, followed by immunoprecipitation and immunoblotting analysis. The results showed that PKC \( d \) was distributed almost exclusively in cytoplasm in both untreated and IL-6-treated cells (Fig. 3A, bottom panel). In contrast, Stat3 was localized in cytoplasm in untreated cells (Fig. 3B, top panel, lanes 1 and 3). Upon IL-6 stimulation, about one-third of the Stat3 proteins were translocated into the nucleus, and two-thirds of them remained in the cytoplasm (Fig. 3B, top panel, compare lanes 2 and 4). No association of Stat3 and PKC \( d \) was detected in cytoplasmic and nuclear fractions in untreated cells (Fig. 3A, top panel, lanes 1 and 3), whereas IL-6 induced the interaction that occurred mainly (more than 90%) in the cytoplasmic fraction (lane 2). The associated Stat3 in cytoplasm was tyrosine-phosphorylated, as verified by reprobing the blot with the anti-Tyr-705-Stat3 antibody (Fig. 3A, middle panel, lane 2). In agreement with these results, we observed that although tyrosine-phosphorylated Stat3 was accumulated in the nucleus after IL-6 stimulation (Fig. 3B, bottom panel, lane 4), certain amounts of Stat3 that remained in the cytoplasm were also tyrosine-phosphorylated (lane 2). These data suggest that PKC \( d \) may associate with the tyrosine-phosphorylated Stat3 localized in the cytoplasm. Such association may further facilitates the phosphorylation of Stat3 by PKC \( d \) (see below). This observation is in agreement with the previous reports that the tyrosine phosphorylation appears prior to serine in cells response to EGF and IL-6 (18, 34) and that the most serine phosphorylation of Stat1 occurs in the cytoplasm (37). The possible role of such association may involve regulation of Stat3 nuclear translocation.

**PKC \( d \) Phosphorylates Stat3 in Vitro**—We performed an immune complex protein kinase assay to determine whether PKC \( d \) can phosphorylate Stat3. HepG2 cells were left untreated or treated with IL-6 for 15 min (+). The lysates were immunoprecipitated with anti-PKC \( d \) antibody, and the PKC \( d \) activity was measured by immune complex protein kinase assay using either histone H1 (A) or GST-Stat3 (B) as a substrate as described under “Experimental Procedures.” The incubation time in kinase reaction was 5 min (lanes 1 and 2) or 10 min (lanes 3 and 4). Amido Black staining of the blots indicating the amounts of H1 or GST-Stat3 fusion protein used in the in vitro kinase assays is shown in the bottom panels. C, amount of immunoprecipitated PKC \( d \) used in the kinase assay was detected by probing the blot in B with anti-PKC \( d \) antibody.

**PKC \( d \) Phosphorylates Stat3 on Ser-727 in Vivo**—We further analyzed Stat3 phosphorylation stimulated by IL-6 in vivo. HepG2 cells were labeled with \[^{32}P\]orthophosphate, immunoprecipitated with Stat3 antibody, and analyzed by SDS-PAGE. As shown in Fig. 5A, Stat3 was weakly phosphorylated in untreated cells, and the phosphorylation was increased in IL-6-treated cells. The labeled Stat3 proteins were digested with trypsin and further analyzed by two-dimensional phosphopeptide mapping. Two weakly phosphorylated peptides (labeled a and b) were observed in untreated cells, which were enhanced in the IL-6-treated cells with an additional spot (Fig. 5B, c). Notably, the pattern of the peptide mapping shown in Fig. 5B (right panel) was very similar to that observed in COS-1 cells transfected with Stat3 and stimulated by EGF (34). In those cells, spots a and b were identified as phosphoserine-containing peptides phosphorylated by ERK2, whereas spot c was the Tyr-705-containing peptide (34). It has been known that Ser-727 is the major phosphorylation site of ERK2 and spots a and b were Ser-727-containing peptides (21, 22, 34). We therefore examined the activation of ERK2 by IL-6 in HepG2 cells by

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2 Neeraj Jain and Xinmin Cao, unpublished data.
PKC isoforms at high concentrations (30–100 μM) phosphorylated ERK2 in these cells (controls, pervanadate, and PMA induced strong tyrosine phosphorylation, a specific inhibitor of PKC. Go6976 inhibits PKC was observed only in cells overexpressing the wild-type PKCδ.

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Fig. 5. Two-dimensional phosphopeptide mapping of Stat3 phosphorylation in IL-6-stimulated HepG2 cells. A, cells were labeled with [32P]orthophosphate and either left untreated (−) or treated with IL-6 for 15 min. Lysates were immunoprecipitated with Stat3 antibody. The immunoprecipitates were separated by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and autoradiographed. B, the Stat3 bands in A were excised, digested with trypsin, and subjected to two-dimensional phosphopeptide mapping as described under “Experimental Procedures.” The major phosphopeptides are labeled a, b, and c.

Checking its tyrosine phosphorylation. Consistent with previous reports (22, 23), the results showed that IL-6 did not induce ERK2 phosphorylation (Fig. 5C, top panel, lane 2), whereas the controls, pervanadate, and PMA induced strong tyrosine phosphorylation of ERK2 in these cells (lanes 4 and 5). These results suggest that Stat3 is phosphorylated on Ser-727 in HepG2 cells stimulated by IL-6 and that ERK2 is not involved in this phosphorylation.

To show that PKC δ is the possible Ser/Thr kinase that phosphorylates Stat3 in IL-6-stimulated cells, we examined the Stat3 phosphorylation by PKC δ in vivo. The 32D cell line is an IL-3-dependent myeloid progenitor line with low expression levels of PKC δ, η, and α (38). 32D cells overexpressing wild-type PKC δ or the kinase-defective ATP-binding mutant (PKC-δK376R) (33) were used for this purpose. These cells were not responsive to IL-6 and therefore were treated with PMA to activate PKC. As shown in Fig. 6A, phosphorylation of Stat3 was observed only in cells overexpressing the wild-type PKC δ, which was further increased after treatment of PMA. On the other hand, no phosphorylation of Stat3 was detected in the parental 32D cells or the cells overexpressing the mutant PKC δ (PKC-δK376R) with or without treatment of PMA. The labeled Stat3 protein in wild-type PKC δ-expressing cells was further analyzed by two-dimensional phosphopeptide mapping. The pattern of the peptide mapping was similar to that of phospho-Stat3 from COS-1 cells overexpressing ERK2 (34), as well as from HepG2 cells stimulated by IL-6 except lack of spot c (the Tyr-705-containing peptide). Similar to HepG2 cells, ERKs were not activated by PMA stimulation in 32D cell lines (data not shown). These data suggest that PKC δ can phosphorylate Stat3 on Ser-727 even at micromolar concentrations (39, 40). HepG2 cells were pretreated with these inhibitors followed by IL-6 stimulation, and the phosphorylation of Stat3 on Ser-727 was evaluated. The results showed that rottlerin inhibited IL-6-stimulated serine phosphorylation of Stat3 in a dose-dependent manner (Fig. 7A, top panel). In contrast, the inhibitor Go6976 did not inhibit serine phosphorylation of Stat3 (Fig. 7B, top panel). The amount of Stat3 in both experiments are shown in the bottom panels. In another set of experiment, HepG2 cells were transfected with wild-type (PKC δ WT) or the dominant-
negative mutant of PKC δ (PKC δ−). The cells were left untreated or treated with IL-6, and Ser-727 phosphorylation was tested. As shown in Fig. 7C, top panel, a basal level of Ser-727 phosphorylation was detected in the untreated cells that were transfected with either control plasmid or the wild-type PKC δ (lanes 1 and 2), which decreased in cells transfected with PKC δ− (lane 3). On the other hand, treatment with IL-6 increased Ser-727 phosphorylation in the cells transfected with control plasmid (lane 4), which was further increased with transfection of the wild-type PKC δ (lane 5) and inhibited with PKC δ− (lane 6). The expression of Stat3 and PKCs is shown in Fig. 7C, middle and bottom panels, respectively. It has been noticed that the expression of the wild-type PKC δ was higher than that of the mutant, and the reason for this is yet unknown. Nevertheless, these data suggest that PKC δ, but not the other PKC isomer, is likely to be the kinase that phosphorylates Stat3 on Ser-727 by IL-6 stimulation.

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Cytokines and growth factors stimulate Stat3 DNA binding and its transcriptional activity to its target genes, and the tyrosine phosphorylation of Stat3 is a prerequisite for these activities. On the other hand, the Ser-727 phosphorylation alone does not stimulate Stat3 DNA binding (1–3). We investigated the effect of PKC δ on Stat3 DNA binding and its transcriptional activity. COS-1 cells express very low level of Stat3 (21). In these cells, DNA binding and the transcriptional activity of Stat3 can be strongly stimulated by EGF and weakly stimulated by IL-6. The expression plasmids of Stat3, PKC δ, and its dominant-negative mutant, PKC δ−, were transfected either alone or together in the presence of a reporter plasmid containing three copies of SIE, a high affinity binding site for Stat3, followed by a CAT gene. The cells were either left untreated or treated with EGF or IL-6, and the DNA binding and the CAT activities were analyzed. It has been reported (16) that EGF induced the activation of Stat3 and Stat1 to form three complexes with SIE: SIF-A (Stat3 homodimer), SIF-B (Stat1/3 heterodimer), and SIF-C (Stat1 homodimer). DNA binding activity of Stat3 was not stimulated in the untreated cells either transfected with Stat3 and PKC δ alone or cotransfected together (Fig. 8A, lanes 3–7). However, EGF stimulated the endogenous Stat1 to form Stat1 homodimer in cells transfected with control plasmid (lane 8). Stat3 homodimer was strongly stimulated in cells transfected with Stat3 after EGF treatment (lane 9), which was inhibited by cotransfection of the wild-type PKC δ (lane 12). On the other hand, cotransfection of the mutant PKC δ with Stat3 did not affect EGF-induced Stat3 DNA binding activity (lane 13). PKC δ also reduced endogenous Stat1 activity weakly (lane 10). Consistently, similar results of CAT assays were observed. CAT activity in the Stat3-transfected cells was stimulated by EGF (about 3-fold), compared with that in the untreated cells (Fig. 8B). This activity, however, was inhibited almost 50% by cotransfection with the wild-type PKC δ, but not by the mutant PKC δ (PKC δ−). Although the induction of the Stat3 activity by IL-6 was not as strong as by EGF in COS-1 cells, a similar inhibition by PKC δ of Stat3 DNA binding and transcriptional activity stimulated by IL-6 was also detected (data not shown). Notably, a basal transcriptional activity of Stat3 was observed in the untreated cells that were also inhibited by wild-type PKC δ. These results indicate that PKC δ negatively regulates Stat3 activities in COS-1 cells.

**DISCUSSION**

In this study, we investigated the possible role of PKC on phosphorylation and regulation of STAT proteins. We detected that only PKC δ interacts with Stat3 in an IL-6-dependent manner in several cell types, whereas other PKC isoforms either associate with Stat3 constitutively, such as PKC β and ε in HepG2 cells, or do not associate with Stat3, such as in the A431 cells. Stat1 has also been reported to be activated by IL-6 stimulation (6). We therefore examined the interaction of PKC δ and Stat1 in HepG2, PC12, and A431 cells stimulated by IL-6, interferon-γ, or EGF. No association was detected in these cells (data not shown). These results suggest that the PKC δ specifically interacts with Stat3. In addition to cytokines of IL-6 family, EGF also stimulates the association of PKC δ and Stat3 in A431 cells. Because EGF also stimulates the Stat3 tyrosine phosphorylation in A431 cells and IL-6, oncostatin M, and leukemia inhibitory factor do so in HepG2 cells, it is likely that the tyrosine phosphorylation of Stat3 is important for the association. The correlation of the time course between the association and the tyrosine phosphorylation of Stat3 supports this
hypothesis. On the other hand, serine phosphorylation may not
be required for this association because we have observed that
PKC\textsubscript{d} can associate with tyrosine-phosphorylated Stat3 pro-
teins that are not serine-phosphorylated (Fig. 1C, a–c). More-
over, in contrast to IL-6, which stimulated Stat3 tyrosine phos-
phorylation and the association with PKC\textsubscript{d} in PC12 cells,
nerve growth factor and EGF stimulated strong serine phos-
phorylation of Stat3 without triggering the tyrosine phospho-
rylation, and no association of Stat3 with PKC\textsubscript{d} was detected
(data not shown). Furthermore, the association induced by IL-6
was inhibited by staurosporine, a tyrosine kinase inhibitor, but
not by H7 (data not shown). This result also suggests that Stat3
association with PKC\textsubscript{d} is independent of PKC\textsubscript{d} activation
because H7 inhibits PKC\textsubscript{d} activity. Indeed, PKC\textsubscript{d} is strongly
activated by PMA, but no association was observed in PMA-
treated HepG2 and A431 cells (data not shown). These data
together suggest that the tyrosine phosphorylation of Stat3,
but not the serine phosphorylation of Stat3 or the activation of
PKC\textsubscript{d}, is likely to be important for Stat3 association with
PKC\textsubscript{d}.

Although an H7-sensitive Ser/Thr kinase was suggested to
be involved in Stat3 serine phosphorylation stimulated by IL-6
(22, 23), the kinase was not identified. In this study, we have
shown that the PKC\textsubscript{d} activity was stimulated by IL-6 (Fig. 4).
Furthermore, the Stat3 serine phosphorylation was also stim-
ulated by IL-6, which was inhibited by a specific inhibitor of
PKC\textsubscript{d}, rottlerin, but not by an inhibitor of PKC\textsubscript{a, b, and g},
Go6976 (Fig. 7). These results suggest that PKC\textsubscript{d} is the kinase
inducible by IL-6 and phosphorylates Stat3 on serine in HepG2
cells. However, the other PKC isoforms, such as the atypical
PKC isoforms, which are not inhibited by Go6976, and the
calmodulin-kinase III, which is also inhibited by rottlerin, may
also be involved in serine phosphorylation of Stat3 in IL-6
stimulated cells. To exclude these possibilities, we further

FIG. 8. PKC\textsubscript{d} negatively regulates Stat3 activity. COS-1 cells were co-
transfected with vector plasmid (−), Stat3 (pRo/CMV-Stat3), wild-type PKC\textsubscript{d} (PKC\textsubscript{d}-WT), and/or a dominant-negative PKC\textsubscript{d} (PKC\textsubscript{d}\textsuperscript{Δ}) expression plasmid, together
with the reporter plasmid pSIE-CAT and pCMV-β-galactosidase. Cells were left un-
treated or treated with EGF for 15 min for DNA binding assays and 6 h for CAT as-
says. Cell lysates and nuclear extracts were prepared as described under “Exper-
imental Procedures.” A, mobility shift DNA binding assay using hSIE as a
probe. FP (lane 1) indicates free probe. B, CAT assays were performed and normal-
ized to β-galactosidase activity as an internal control. The CAT activity ob-
tained from two independent transfection experiments was quantified using Bio-
Rad GS700 imaging densitometer, and the S.D. values are denoted by error bars
in the bottom panel. The top panel shows a representative result of the CAT assays.
showed that the Stat3 Ser-727 phosphorylation induced by IL-6 was enhanced by transfection of the wild-type PKC δ but inhibited by the dominant-negative mutant of PKC δ (Fig. 7C). These data demonstrated that PKC δ is the major kinase that phosphorylates Stat3 on Ser-727 by IL-6 stimulation. On the other hand, we also observed that the Stat3 was completely serine-phosphorylated within 15 min of EGF stimulation in A431 cells (indicated by the slow migrating band in Fig. 2B, lane 2), whereas only about 50% of Stat3 was serine-phosphorylated with IL-6 stimulation in both A431 (Fig. 2B) and HepG2 (Fig. 1C, a–c, lane 2) cells. This indicates that ERKs stimulated by EGF phosphorylate Stat3 more strongly than PKC δ stimulated by IL-6. However, the serine phosphorylation patterns of Stat3 by ERK2 and PKC δ analyzed by two-dimensional phosphopeptide mapping were shown to be similar (Fig. 6B and Ref. 33). These data indicate that Ser-727 is the major phosphorylation site, which could be important for regulating its activity. It is unknown, however, whether the intensity of the serine phosphorylation of Stat3 by different kinases plays a role in fine tuning Stat3 activity.

Although the role of the Stat3 serine phosphorylation and the involved kinases in regulation of Stat3 activity remains unclear, negative effects on Stat3 activity have been suggested in several reports. For instance, Chung et al. (22) reported that phosphorylation on Ser-727 of Stat3 negatively modulated its transcriptional activity. We also found that activation of ERK2 by its upstream kinase, MEK1, abrogated Stat3 transcriptional activity stimulated by EGF through inhibition of its tyrosine phosphorylation and DNA binding activity (34). These data were in agreement with the report showing that activation of MAP kinases by MEK1 or by PMA inhibits Stat3 activity stimulated by IL-6 (41). Our results here clearly showed that PKC δ inhibits Stat3 DNA binding and its transcriptional activities (Fig. 8). The results are also consistent with a previous report that PKC δ inhibited Stat1 tyrosine phosphorylation and DNA binding stimulated by tyrosine kinase Bmx (42). The mechanism of the repression by PKC δ and MAP kinase is unknown and could be a complicated issue. A few possibilities may be considered. First, the repression may be due to a direct phosphorylation by ERK2 or PKC δ on Ser-727 that blocks the subsequent phosphorylation on Tyr-705. In PC12 cells, we observed that pretreatment of the cells with nerve growth factor, subsequent phosphorylation on Tyr-705. In PC12 cells, we observed that pretreatment of the cells with nerve growth factor, subsequent phosphorylation on Tyr-705.

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