c-Src-dependent Tyrosine Phosphorylation of IKKβ Is Involved in Tumor Necrosis Factor-α-induced Intercellular Adhesion Molecule-1 Expression

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The signaling pathway involved in tumor necrosis factor-α (TNF-α)-induced intercellular adhesion molecule-1 (ICAM-1) expression was further studied in human A549 epithelial cells. TNF-α or 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ICAM-1 promoter activity was inhibited by a protein kinase C (PKC) inhibitor (staurosporine), tyrosine kinase inhibitors (genistein and herbinycin A), or an Src-specific tyrosine kinase inhibitor (PP2). TNF-α- or TPA-induced IkB kinase (IKK) activation was also blocked by these inhibitors, which slightly reversed TNF-α-induced but completely reversed TPA-induced IkBα degradation. c-Src and Lyn, two members of the Src kinase family, were abundantly expressed in A549 cells, and their activation by TNF-α or TPA was inhibited by the same inhibitors. Furthermore, the dominant-negative c-Src (KM) mutant inhibited induction of ICAM-1 promoter activity by TNF-α or TPA. Overexpression of the constitutively active PKCs or wild-type c-Src plasmids induced ICAM-1 promoter activity, this effect being inhibited by the dominant-negative c-Src (KM) or IKKβ (KM) mutant but not by the nuclear factor-κB-inducing kinase (NIK) (KA) mutant. The c-Src (KM) mutant failed to block induction of ICAM-1 promoter activity caused by overexpression of wild-type NIK. In co-immunoprecipitation and immunoblot experiments, IKKβ was found to be associated with c-Src and to be phosphorylated on tyrosine residues after TNF-α or TPA treatment. Two tyrosine residues, Tyr188 and Tyr199, near the activation loop of IKKβ, were identified as being important for NF-κB activation. Substitution of these residues with phenylalanines abolished ICAM-1 promoter activity and c-Src-dependent phosphorylation of IKKβ induced by TNF-α or TPA. These data suggest that, in addition to activating NIK, TNF-α also activates PKC-dependent c-Src. These two pathways converge at IKKβ and go on to activate NF-κB, via serine phosphorylation and degradation of IkBα, and, finally, to initiate ICAM-1 expression.

Extravasation of leukocytes from the microvasculature at sites of inflammation or injury is a critical event in inflammation-mediated diseases, such as rheumatoid arthritis, psoriasis, bronchial asthma, atopic dermatitis, and allograft rejection (1–3). The process of leukocyte migration includes several steps (4, 5). The first of these is adhesion of the leukocyte to the endothelial cell. The initial interaction between the leukocyte and the endothelium is transient, resulting in the leukocyte rolling along the vessel wall. The rolling leukocyte then becomes activated by local factors generated by the endothelium, resulting in its arrest and firm adhesion to the vessel wall. Finally, the leukocyte squeezes between the endothelial cells and migrates to the inflammation site. These complex processes are regulated, in part, by specific adhesion molecules and their counter ligands on both circulating leukocytes and vascular endothelial cells; these include E-selectin (endothelial-leukocyte adhesion molecule-1, CD62E) and immunoglobulin superfamily members, such as intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (6, 7). In a number of inflammation and immune responses, ICAM-1 binds to two integrins belonging to the β2 subfamily, LFA-1 and Mac-1, both expressed by leukocytes and both promoting the adhesion and transendothelial migration of leukocytes (7–9). Similar processes govern the adhesion of leukocytes to lung airway epithelial cells and contribute to the damage to these cells seen in asthma (10).

Basal levels of ICAM-1 are low, but high expression can be induced in a number of cell types by a wide range of ligands, including bacterial lipopolysaccharide, phorbol esters, or inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon-γ (11–13). Induction of ICAM-1 expression requires de novo mRNA and protein synthesis (8, 14), indicating regulation at the transcriptional level. The promoter region of the human ICAM-1 gene has been cloned and sequenced and shown to contain putative recognition sequences for a variety of transcriptional factors, including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), AP-2, and the interferon-stimulated response element (15). Of these, NF-κB family proteins are essential for the enhanced ICAM-1 gene expression seen in human alveolar epithelial cells on exposure to cytokines (16, 17). The intracellular signaling pathways by which TNF-α and IL-1β cause ICAM-1 expression in A549 human alveolar epithelial cells have been explored and found to involve the sequential activation of protein kinase Cα (PKCα), protein-tyrosine kinase, nuclear factor-κB-inducing kinase (NIK), and IkB kinase β (IKKβ) (16, 17). The role of

The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; IκB, IkB kinase; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; NIK, nuclear factor-κB-inducing kinase; GST, glutathione S-transferase; IL, interleukin; PKC, protein kinase C; DME M, Dulbecco’s modified Eagle’s medium; wt, wild-type; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-O-tetradecanoylphorbol-13-acetate; DTT, dithiothreitol; TRAF, TNF receptor-associated factor; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine.
protein-tyrosine kinase has been further investigated in the present study. Using an immunocomplex kinase assay and site-directed mutagenesis, we have demonstrated that c-Src is involved in TNF-α-induced NF-κB transcriptional activation and that, in addition to serine phosphorylation of IKKβ by NIK, Tyr188 and Tyr199 phosphorylations by PKC-dependent c-Src activation also contribute to TNF-α-(132–206) (Y188F), GST-IKKβ/H9252, and IKKα/H9250 phosphorylated with TNF-α for 10 min or with TPA for 30 min, then nuclear extracts were isolated as described previously (17). Briefly, cells were washed with ice-cold phosphate-buffered saline and pelleted, then the cell pellet was resuspended in a hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 mM Na3VO4, and 1 mM Na2VO3) and incubated for 15 min on ice, then lysed by the addition of 0.5% Nonidet P-40 followed by vigorous vortexing for 10 s. The nuclei were pelleted and resuspended in extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM Na3VO4, and 1 mM Na2VO3), and the tube was vigorously shaken at 4 °C for 15 min on a shaking platform. The nuclear extract was then centrifuged, and the supernatants were aliquoted and stored at −80 °C.

Oligonucleotides corresponding to the downstream NF-κB consensus sequence (5′-AGCTTGAAATTCGCGGA-3′) in the human ICAM-1 promoter were synthesized, annealed, and end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. The nuclear extract (6–10 μg) was incubated at 30 °C for 20 min with 1 μg of [γ-32P]ATP-labeled NF-κB probe (40,000–60,000 cpm) in 10 μl of binding buffer containing 1 μg of poly(dI-dC), 15 mM HEPES, pH 7.6, 80 mM NaCl, 1 mM EGTA, 1 mM DTT, and 10% glycerol as described previously (17). DNA–nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 6% polyacrylamide gel, then the gel was vacuum-dried and subjected to autoradiography using a phosphorimager screen at −80 °C.

Site-directed Mutagenesis—Using a QuikChange™ site-directed mutagenesis kit according to the manufacturer's manual, lysine 295 in the mouse c-Src cloned in the pBluescript vector was substituted with methionine by changing the triplets from AAG to ATG. Tyrosine 199, tyrosine 188, or both sites in the human IKKα cloned in the pCMV3A vector or in the human GST-IKKα (132–206) cloned in the pGEX vector were substituted with phenylalanine by changing the triplet from TAC to TTC. The mutated primers used were as follows: primer 1 (5′-CGAGGTGGGACCTACATGAACTCTAGGACGACAAGC-3′) and primer 2 (3′-GGTCCCAACAGGTTAGTACTAGAACCTTCGCTCGT-5′) for c-Src, primer 3 (5′-CGAGGGACCTCGCTGCGG-3′) and primer 4 (3′-CCCTGCGGACCTGAAGCGGCG-5′) for IKKβ (H9253) mutation, primer 5 (5′-GGAGGACCAAGGTTACACGAGTGTCGTGG-3′) and primer 6 (3′-CCCTGCGGCTTCAAGGTCGACTGCGG-5′) for IKKα (Y9251) mutation. DNA prepared from overnight cultures of picked colonies using Miniprep (Qiagen) was subjected to restriction digestion analysis, and the nucleotide changes were confirmed by DNA sequencing. The mutated c-Src plasmid containing the point mutation was digested with EcoRI and XhoI and inserted into the pCMV3A (+) vector.

Transient Transfection and Luciferase Assay—A549 cells, grown at 50% confluency in six-well plates, were transfected with the human ICAM-1 plasmid (A549) by firefly luciferase (Luc) or 5′-Luc plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, reporter DNA (0.4 μg) and β-galactosidase DNA (0.2 μg) were mixed with 0.6 μl of Tfx-50 in 1 ml of serum-free DMEM. After 10–15 min incubation at room temperature, the mixture was applied to the cells, then, 1 h later, 1 ml of complete growth medium was added. On the following day, the medium was replaced with fresh medium. Forty-eight hours after transfection, the cells were transfected with reporter DNA (4 μg) and either the dominant-negative NIK, IKKα/H9250, or c-Src plasmid or the respective empty vector (0.4 μg). In experiments using wt plasmids, cells were co-transfected with the following mixture: 0.2 μg of reporter plasmid; 0.1 μg of β-galactosidase plasmid; 0.4 μg of the constitutively active PKCα (A/E) plasmid, wt-c-Src or NIK plasmid (or
experimental Procedures."

Effect of Inhibitors of PKC, Tyrosine Kinase, or Src Kinase on the Induction of ICAM-1 Promoter Activity by TNF-α or TPA in A549 Cells—In our previous study (17), we found that PKC and tyrosine kinase were involved in TNF-α-induced ICAM-1 expression. Transient transfection using the ICAM-1 promoter-luciferase construct, pIC339 (-339/0) was performed to elucidate the signaling pathway mediated by these kinases. The pIC339 construct contains the downstream NF-κB site (-189/-174) responsible for mediating the induction of ICAM-1 promoter activity by TNF-α or TPA (17). As shown in Fig. 1, TNF-α led to a 2.9-fold increase in ICAM-1 promoter activity. When cells were pretreated with inhibitors of PKC (staurosporine), tyrosine kinases (genestein or herbinycin A), or Src kinases (PP2), the TNF-α-induced increase was inhibited by 69%, 84%, 65%, or 66%, respectively. TPA treatment, a direct PKC activator, resulted in a 3.5-fold increase in ICAM-1 promoter activity, and this effect was inhibited by genistein, herbinycin A, or PP2 by 74%, 60%, or 87%, respectively. None of these inhibitors alone affected the basal luciferase activity (data not shown).

Induction of IKK Activation, IkBa Degradation, and NF-κB-specific DNA-Protein Complex Formation by TNF-α and TPA, and the Inhibitory Effect of Inhibitors of PKC, Tyrosine Kinase, or Src Kinase—Because TNF-α- and TPA-induced ICAM-1 promoter activity in A549 cells is inhibited by the dominant-negative IKKβ mutant (17), endogenous IKK activity was measured by immunoprecipitation with anti-IKKβ antibody.

When cells were treated with 10 ng/ml TNF-α for 5, 10, 30, or 60 min, maximal IKK activity was seen after 5 min (Fig. 2A), whereas maximal degradation of IkB-α was seen after 10 min, IkB-α levels being restored to the resting level after 1 h of treatment (Fig. 2B). In TPA-treated cells, maximal IKK activity was seen after 30 min of treatment (Fig. 2A), whereas maximal IkB-α degradation was seen after 60 min (Fig. 2B). The TNF-α-induced IKK activation was inhibited by a PKC, tyrosine kinase, or Src kinase inhibitor by 56%, 49%, or 50%, respectively, whereas these same inhibitors suppressed TPA-induced IKK activation by 71%, 91%, or 90%, respectively (Fig. 3A). The IkBα degradation induced by TPA was reversed by PKC, tyrosine kinase, and Src kinase inhibitors, but that induced by TNF-α was only slightly affected by these inhibitors (Fig. 3B). The effect of these inhibitors on TNF-α- or TPA-induced NF-κB-specific DNA-protein binding was examined. As shown in Fig. 3C, when cells were treated with TNF-α for 10 min, increased NF-κB-specific DNA-protein binding was seen, and this effect was inhibited by PKC, tyrosine kinase, and Src kinase inhibitors by 20%, 51%, and 48%, respectively. TPA treatment for 30 min also increased NF-κB-specific DNA-protein binding, and this was more effectively suppressed by these inhibitors (75%, 74%, and 87%, respectively; Fig. 3C).
Western blotting using anti-IKK antibody, marked autophosphorylation of c-Src and Lyn was seen after 10 min and was maintained to 60 min. In 60 min, maximal c-Src and Lyn activity (enolase phosphorylation) was prepared. In experiments, whole cell lysates or nuclear extracts were prepared and subjected to Western blotting using antibodies against c-Src, Lck, Lyn, or Fyn as described under “Experimental Procedures.” In B, A549 cells were treated with 10 ng/ml TNF-α for 10, 30, or 60 min, then whole cell lysates were prepared and immunoprecipitated with anti-c-Src or anti-Lyn antibody. The kinase assay (KA) and autoradiography for phosphorylated enolase were performed on the precipitates as described under “Experimental Procedures.” Levels of immunoprecipitated c-Src and Lyn were estimated by Western blotting (WB) using anti-c-Src or anti-Lyn antibody, respectively.

seen over the same time period. TPA (1 μM) also induced c-Src and Lyn activation after 30-min treatment of A549 cells (Fig. 5). The TNF-α- and TPA-induced activation of c-Src and Lyn was inhibited by staurosporine, herbimycin A, and PP2 (Fig. 5).

Induction of IKKβ Activation by Overexpression of PKCa or c-Src, and the Inhibitory Effect of Dominant-negative Mutants of c-Src or IKKβ—The TNF-α- or TPA-induced activation of c-Src and Lyn was inhibited by PKC, tyrosine kinase, or Src kinase inhibitors. This indicated that PKC-dependent c-Src and Lyn activation was required to induce IKK and NF-κB activation in A549 cells. To further examine the involvement of c-Src, a dominant-negative mutant was generated by site-directed mutagenesis of mouse c-Src lysine 295 to methionine. Overexpression of c-Src (KM) attenuated the TNF-α-induced promoter activity induced by overexpression of NIK wt (Fig. 7), These results indicate the involvement of both the PKC/c-Src/IKKβ and c-Src pathways in TNF-α-induced ICAM-1 expression in A549 cells.

Expression in nuclear extracts was determined by electrophoretic mobility shift assay as described under “Experimental Procedures.”

pressed in brain, in Jurkat and HL-60 cells, and in the human alveolar epithelial cell lines NCI-H292 and A549. Lck was abundantly expressed in brain and Jurkat cells, but only weakly expressed in NCI-H292 and A549 cells. Lyn was abundantly expressed in brain and in Jurkat, HL-60, NCI-H292, and A549 cells, whereas Fyn was only expressed in brain and in Jurkat and HL-60 cells. c-Src and Lyn in A549 cells were therefore isolated by immunoprecipitation using anti-c-Src or anti-Lyn antibody, and their kinase activity was measured using enolase as substrate. As shown in Fig. 4B, when A549 cells were treated with 10 ng/ml TNF-α for 10, 30, or 60 min, maximal c-Src and Lyn activity (enolase phosphorylation) was seen after 10 min and was maintained to 60 min. In addition, marked autophosphorylation of c-Src and Lyn was

FIG. 3. Effect of various inhibitors on TNF-α- or TPA-induced IKK activation, IκBα degradation, and NF-κB-specific DNA-protein complex formation in epithelial cells. A549 cells were pretreated for 30 min with 300 nM staurosporine, 1 μM herbimycin A, or 10 μM PP2 before incubation with 10 ng/ml TNF-α for 10 min or 1 μM TPA for 30 min, then whole cell lysates or nuclear extracts were prepared. In A, whole cell lysates were immunoprecipitated with anti-IKKβ antibody, and the kinase assay (KA) and autoradiography for phosphorylated GST-IκBα-(1–100) were performed on the precipitates as described under “Experimental Procedures.” Levels of immunoprecipitated IKKβ were estimated by Western blotting (WB) using anti-IKKβ antibody. In B, cytosolic levels of IκBα were measured by Western blotting using anti-IκBα antibody as described under “Experimental Procedures.” In C, the NF-κB-specific DNA-protein activity in nuclear extracts was determined by electrophoretic mobility shift assay as described under “Experimental Procedures.”

FIG. 4. Src family expression and time-dependent activation of c-Src or Lyn by TNF-α in A549 cells. In A, Jurkat, HL-60, NCI-H292, or A549 cells and brain lysates were prepared and subjected to Western blotting using antibodies against c-Src, Lck, Lyn, or Fyn as described under “Experimental Procedures.” In B, A549 cells were treated with 10 ng/ml TNF-α for 10, 30, or 60 min, then whole cell lysates were prepared and immunoprecipitated with anti-c-Src or anti-Lyn antibody. The kinase assay (KA) and autoradiography for phosphorylated enolase were performed on the precipitates as described under “Experimental Procedures.” Levels of immunoprecipitated c-Src or Lyn were estimated by Western blotting (WB) using anti-c-Src or anti-Lyn antibody, respectively.
PKC-dependent c-Src Activation in IKKβ Activation

Effect of Various Inhibitors on TNF-α- or TPA-induced c-Src or Lyn Activation in Epithelial Cells. A549 cells were pre-treated with 300 nM staurosporine, 1 μM herbimycin A, or 10 μM PP2 for 30 min before incubation with 10 ng/ml TNF-α for 10 min or 1 μM TPA for 30 min. Whole cell lysates were prepared and immunoprecipitated with anti-c-Src or anti-Lyn antibody, and the kinase assay (KA) and autoradiography for phosphorylated enolase were performed on the precipitate as described under “Experimental Procedures.” Levels of immunoprecipitated c-Src or Lyn were estimated by Western blotting (WB) using anti-c-Src or anti-Lyn antibody, respectively.

Effect of Various Dominant-negative Mutants on TNF-α- or TPA-induced ICAM-1 promoter activity in A549 cells. A549 cells were co-transfected with pIC339 and the dominant-negative c-Src (K295M), NIK (KA), or IKKβ (KM) mutant, or the respective empty vector, then treated for 6 h with 10 ng/ml TNF-α or 1 μM TPA. Luciferase activity was then measured as described under “Experimental Procedures,” and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01 compared with TNF-α or TPA alone.

Effect of PP2—Because c-Src-dependent IKK activation was shown to be involved, co-immunoprecipitation of c-Src and IKKβ was performed to examine whether c-Src directly regulates IKK activity through phosphorylation of tyrosine residues. When cells were treated with TNF-α for 5, 10, or 15 min, IKKβ was tyrosine-phosphorylated in a time-dependent manner, the maximal effect being seen at 10 min; a similar effect was seen after 30-min treatment with TPA (Fig. 8A). Both effects were inhibited by PP2 (Fig. 8A). To demonstrate that c-Src associated with IKKβ and phosphorylated its tyrosine residues, cell lysates were immunoprecipitated with anti-IKKβ antibodies, then the immunoprecipitates were separated by SDS-PAGE, transferred to membranes, and blotted with anti-phosphotyrosine antibodies. As shown in Fig. 9B, tyrosine phosphorylation of IKKβ was seen after TNF-α or TPA treatment, the effect being maximal at 10 or 30 min, respectively, and inhibited by PP2. When cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with anti-IKKβ or anti-c-Src antibody, both IKKβ and c-Src were shown to be tyrosine-phosphorylated after TNF-α or TPA treatment, and these effects were again inhibited by PP2 (Fig. 8C). These results indicate that c-Src can associate with IKKβ and phosphorylate its tyrosine residues after TNF-α or TPA stimulation. The association between c-Src and IKKβ was further examined. Anti-IKKβ antibody was used to precipitate IKK from A549 cells, then the immunoprecipitated proteins were subjected to Western blotting using anti-c-Src antibody. As shown in Fig. 9A, an increased amount of c-Src co-precipitated with IKKβ after TNF-α or TPA stimulation. In the converse experiment in which c-Src was precipitated using anti-c-Src antibody, IKKβ was shown to be associated with c-Src in a time-dependent manner after TNF-α or TPA treatment (Fig. 9B). These results show that there is an association between c-Src and IKKβ and that IKKβ tyrosine residues are phosphorylated.

Inhibitory Effect of the Dominant-negative Mutants IKKβ (Y188F), IKKβ (Y199F), or IKKβ (FF) on TNF-α- and TPA-induced ICAM-1 Promoter Activity and on the PKCα- and c-Src-induced, but not the NIK-induced, Increase in NF-κB Activity—The above experiments demonstrate that c-Src directly interacts with IKKβ and phosphorylates its tyrosine residues after TNF-α or TPA stimulation. When the amino sequences of subdomains VII and VIII in the kinase domain of PKCα, AKT1, and IKKα were aligned, the tyrosine residues were found to be conserved (Fig. 9C). Hypothesizing that Tyr188 and/or Tyr199 of IKKβ were the targets for c-Src phosphorylation after TNF-α or TPA stimulation, we used site-directed mutagenesis to gen-
PKC-dependent c-Src Activation in IKKβ Activation

Fig. 8. Tyrosine phosphorylation of IKKβ induced by TNF-α or TPA and the inhibitory effect of PP2. Control cells or cells pre-treated for 30 min with 10 μM PP2 were stimulated with TNF-α for 5, 10, or 15 min or with TPA for 10 or 30 min. In A, crude lysates were prepared. In B and C, equal amounts (1 mg) of cell lysate were immunoprecipitated (IP) with anti-IKKβ (A) or anti-phosphotyrosine (PY) (B) antibodies. Crude lysates and immunoprecipitated proteins were separated by SDS-PAGE on a 10% gel and immunoblotted (WB) with anti-phosphotyrosine (PY) (A and B), anti-IKKβ (C), or anti-c-Src (C) antibodies or reprobed with anti-IKKβ (A and B) antibody.

erate the dominant-negative tyrosine mutants, IKKβ (Y188F), IKKβ (Y199F), and IKKβ (Y188F, Y199F). Overexpression of these mutants attenuated the TNF-α- or TPA-induced ICAM-1 promoter activity, the double mutant having a greater inhibitory effect than either of the single mutants (Fig. 10A). The dominant-negative IKKβ (KM) mutant, with Lys44 mutated to methionine, had a similar inhibitory effect to that of IKKβ (Y188F) or IKKβ (Y199F) on TNF-α- and TPA-induced ICAM-1 promoter activity, whereas IKKα (AA), with Ser177 and Ser181 mutated to alanine, was as effective as IKKβ (Y188F) or IKKβ (Y199F) in inhibiting TNF-α-induced ICAM-1 promoter activity but had no effect on TPA-induced ICAM-1 promoter activity (Fig. 10A).

To further confirm the involvement of tyrosine phosphorylation in the PKCα/c-Src/IKKβ pathway and serine phosphorylation in the NIK/IKKβ pathway, the dominant-negative IKKβ mutants with either a tyrosine or serine mutation were co-transfected with PKCα (A/E), wt c-Src, or wt NIK to examine their inhibitory effects on the constitutively active or wt plasmid-induced NF-κB activity. As shown in Fig. 10B, PKCα (A/E) or wt c-Src-induced NF-κB activity was inhibited by all three tyrosine mutants but not by the double-serine mutant, whereas the converse was true for NIK-induced NF-κB activity. Because Tyr188 and Tyr199 in IKKβ were found to be critical for the PKCα/c-Src/IKKβ pathway to elicit NF-κB activation, leading to induction of TNF-α- or TPA-stimulated ICAM-1 promoter activity (Fig. 10), endogenous c-Src phosphorylation of Tyr188 and Tyr199 in IKKβ was further examined. c-Src was immunoprecipitated using anti-c-Src antibody, and its ability to phosphorylate IKKβ was measured using GST-IKKβ-(132–206) as an in vitro substrate. When cells were treated with TNF-α or TPA, IKKβ was phosphorylated by c-Src in a time-dependent manner. The maximal effect was seen at 10-min treatment with TNF-α or 30-min treatment with TPA (Fig. 11B), and both effects were inhibited by PP2 (Fig. 11B). The c-Src-dependent IKKβ phosphorylation was specific for Tyr188/ Tyr199, because it was not seen when either or both tyrosine residues were substituted with phenylalanines (Fig. 11C).

**DISCUSSION**

The PKC-dependent tyrosine kinase activation pathway is involved in TNF-α-induced NF-κB activation and ICAM-1 expression in A549 alveolar epithelial cells and in causing monocytes to adhere to these cells (17). The role and molecular identity of the tyrosine kinase involved have been further characterized in the present study. TNF-α- and TPA-induced ICAM-1 promoter activity were both inhibited by PKC, tyrosine kinase, and Src kinase inhibitors, indicating the possible involvement of the Src tyrosine kinase family downstream of PKC activation in the induction of ICAM-1 expression. IKKβ, but not IKKα, is involved in the TNF-α- and TPA-induced ICAM-1 promoter activity (17), and TNF-α- or TPA-induced stimulation of IKK activity and parallel degradation of IκBα was seen in the present study. The TNF-α- and TPA-induced IKK activity and NF-κB-specific DNA-protein binding were attenuated by PKC, tyrosine kinase, and Src kinase inhibitors, indicating that the Src tyrosine kinase family is involved down-
stream of PKC in the induction of IKKβ activation leading to NF-κB activation and ICAM-1 expression in A549 cells. Western blot analysis showed that c-Src and Lyn were abundantly expressed in A549 cells and that TNF-α and TPA induced activation of these two Src tyrosine kinases. The c-Src and Lyn activation induced by either stimulus was also inhibited by PKC, tyrosine kinase, and Src kinase inhibitors. Taken together, these results demonstrate that the tyrosine kinase involved downstream of PKC is c-Src or Lyn. The involvement of PKC/c-Src/Iκκβ activation in TNF-α-induced ICAM-1 expression was confirmed by the finding that the dominant-negative c-Src (KM) mutant attenuated the TNF-α- and TPA-induced ICAM-1 promoter activity.

In nonstimulated cells, NF-κB dimers are present as cytoplasmic latent complexes due to the binding of specific inhibitors, the IκBs, that mask their nuclear localization signal. Following stimulation by pro-inflammatory cytokines, the IκBs are rapidly phosphorylated at two conserved N-terminal serine residues, and this post-translational modification is rapidly followed by their polyubiquitination and proteasomal degradation (18, 19). This leads to the unmasking of the nuclear localization signal in NF-κB dimers, followed by their translocation to the nucleus, binding to specific DNA sites (κB sites), and targeting of gene activation. The protein kinase that phosphorylates IκBs in response to pro-inflammatory stimuli has been identified biochemically and molecularly (20–24). Named IκK, it exists as a complex, termed the IKK signalsome, which is composed of at least three subunits, IKKα (IKK1), IKKβ (IKK2), and IKKγ (25). IKKα and IKKβ are very similar protein kinases that act as the catalytic subunits of the complex (20–24). In mammalian cells, IKKα and IKKβ form a stable heterodimer that is tightly associated with IKKγ, a regulatory subunit (26). The IKKs bind NIK (22, 23), a member of the mitogen-activated protein kinase kinase kinase family, that interacts with the TRAF6-associated IL-1 receptor complex or TRAF2-associated TNF receptor complex, thereby linking IκB degradation and NF-κB activation to IL-1β or TNF-α stimulation.
PKC-dependent c-Src Activation in IKKβ Activation

The activities of both IKKα and IKKβ are reported to be regulated by NIK (28). Our results show that the TNF-α-induced increase in ICAM-1 promoter activity was inhibited by the dominant-negative NIK (KA) and IKKβ (KM) mutants (Fig. 6) (17). The dominant-negative IKKβ (KM) mutant attenuated wt NIK-induced ICAM-1 promoter activity, indicating the involvement of the NIK/IKK pathway in TNF-α-induced ICAM-1 expression. To elucidate the relationship between the PKC/c-Src/IKKβ and NIK/IKKβ pathways in TNF-α-induced ICAM-1 expression, overexpression of a constitutively active PKCα plasmid and the wt c-Src, NIK, and IKKβ plasmids was used. These plasmids all induced increase in ICAM-1 promoter activity, and their effects were blocked by the dominant-negative IKKβ (KM) mutant. The effect of the constitutively active PKCα (A/E) was blocked by the dominant-negative c-Src (KM) mutant, but not by the NIK (KA) mutant. The effect of the wt c-Src plasmid on ICAM-1 promoter activity was not affected by the dominant-negative NIK (KA) mutant, and the wt NIK plasmid was not affected by the dominant-negative c-Src (KM) mutant (Fig. 7B). These results show that the PKC/c-Src/IKKβ and NIK/IKKβ pathways function in parallel in the TNF-α-mediated induction of ICAM-1 expression in A549 cells. The existence of these two pathways explains why inhibitors of PKC, tyrosine kinases, or Src kinase could reverse TPA- but not TNF-α-induced ICα-Bβ degradation, because TNF-α could still act via the NIK/IKKβ pathway in the presence of these inhibitors.

c-Src is involved in NF-κB activation in bone marrow macrophages, U937 cells, and B cells (29–31). In bone marrow macrophages, TNF-α induces activation of c-Src, which associates with IκB-α and phosphorylates Tyr12 of IκB-α, leading to NF-κB activation and IL-6 release (29). In contrast to the rapid degradation of serine-phosphorylated IκB-α (32), tyrosine-phosphorylated IκB-α is not subject to rapid proteolysis (29, 33). In the present study of TNF-α-induced ICAM-1 expression, the downstream target of c-Src was IKKβ and rapid degradation of IκB-α was seen (Fig. 2B). Involvement of a tyrosine kinase upstream of IKK activation has also been reported in CD23 signaling in U937 cells (30) and in B cell antigen receptor stimulation (31). A similar PKC-dependent c-Src activation pathway has been found in human osteoblasts, in which GGF-2 increases N-cadherin expression, in A7r5 vascular smooth muscle cells, in which TPA induces Rho-dependent actin reorganization, and in SH-SY5Y neuroblastoma cells, in which TPA induces Cas-Crk complex formation (34–36). Furthermore, the PKC/c-Src/IKKβ pathway, here shown to be involved in induction of ICAM-1 expression, might be a common signaling pathway for inducible gene expression, because TNF-α, IL-1β, or interferon-γ-induced COX-2 or ICAM-1 expression in human alveolar epithelial cells also involves PKC-dependent activation of c-Src or Lyn (16, 37, 38).2

Because involvement of the PKC/c-Src/IKKβ pathway had been demonstrated, tyrosine phosphorylation of IKKβ by c-Src was further examined. Several lines of evidence show that this occurred. First, in both crude cell lysates and immunoprecipitates formed using anti-IKKβ antibody, IKKβ was found to be tyrosine-phosphorylated after TNF-α or TPA stimulation. Second, in immunoprecipitates formed using anti-phosphotyrosine antibody, both IKKβ and c-Src were tyrosine-phosphorylated after treatment with TNF-α or TPA. Third, all these effects were inhibited by PP2. Fourth, using either immunoprecipitation with anti-IKKβ antibody followed by blotting with anti-c-Src antibody or immunoprecipitation with anti-c-Src antibody followed by blotting with anti-IKKβ antibody, an association between c-Src and IKKβ was demonstrated and shown to be increased after TNF-α or TPA treatment. Fifth, an in vitro kinase assay demonstrated that c-Src directly phosphorylated IKKβ at Tyr188 and Tyr199. IKKβ is a Thr/Ser kinase and phosphorylation of Ser177 and Ser181 in the kinase domain is necessary for its activation, because substitution of these two residues with alanines reduces IKKβ activity and leads to reduced Rel A nuclear translocation and NF-κB-dependent gene expression (21, 39). MEKK1 and NIK are reported to phosphorylate these two serine residues (40). The present experiments further demonstrated Tyr188 and Tyr199 phosphorylation by c-Src via a PKC-dependent activation pathway. This tyrosine phosphorylation of IKKβ was essential for TNF-α-induced ICAM-1 expression in A549 cells, because the dominant-negative mutants, IKKβ (Y188F) and IKKβ (Y199F), or IKKβ (FF), abrogated the effects of both TNF-α and TPA. Tyrosine phosphorylation of Thr/Ser kinases, such as PKCs and Akt, has also been reported to be important for their activation (41, 42). Akt activation by extracellular stimuli is a multistep process involving translocation and phosphorylation. Two phosphorylation sites, Thr308 and Ser473, have been shown to be critical for growth factor-induced activation of Akt (43–45). In addition to the phosphorylation of these two sites, tyrosine phosphorylation plays an important role in regulation of Akt activity. Both the EGF-induced tyrosine phosphorylation and kinase activity of Akt are blocked by PP2, and Src phosphorylates Tyr312 and Tyr326 of Akt both in vivo and in vitro (41). It is noteworthy that these tyrosine residues are conserved in about 50% of Ser/Thr kinases and that phosphorylation of the corresponding residues, Tyr312 and Tyr326, in PKCδ is also critical for PKCδ activation in response to H2O2 (42). Phosphorylation of the two conserved tyrosine residues in the kinase domains of Ser/Thr kinases may therefore be a general mechanism by which Akt, PKCδ, and IKKβ are regulated (41, 42, and present study) (Fig.

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2 W.-C. Huang, J.-J. Chen, and C.-C. Chen, unpublished data.
The Src tyrosine kinase family therefore directly regulates IKKβ activity via phosphorylation at Tyr^{188} and Tyr^{199}, rather than solely by NIK-mediated phosphorylation at Ser^{177} and Ser^{181}, as previously suggested (27). Three findings further support the notion that the PKC/ε-Src/IKKβ pathway induces tyrosine phosphorylation, whereas the NIK/IKKβ pathway induces serine phosphorylation. First, NF-κB activity induced by PKCα (εB) or wt-c-Src was inhibited by the tyrosine mutants, IKKβ(Y188F), IKKβ(Y199F), or IKKβ(FF), but not by IKKβ(AA), in which Ser^{177} and Ser^{181} are mutated. Second, wt NIK-induced NF-κB activity was inhibited by IKKβ(AA) but not by IKKβ(Y188F). IKKβ(Y199F), or IKKβ(FF) (Fig. 10B). Third,TPA-induced ICAM-1 promoter activity was not affected by IKKβ(AA) (Fig. 10A). Our data demonstrate, for the first time, that, in addition to phosphorylation of Ser^{177} and Ser^{181}, Tyr^{188} and Tyr^{199} phosphorylation of IKKβ is required for its full activation and biological functions.

In summary, the signaling pathways involved in TNF-α-induced ICAM-1 expression in A549 cells have been further explored. In addition to activating the NIK/IKKβ pathway, TNF-α activates the PKC-dependent c-Src pathway. These two pathways converge at IKKβ, and are, respectively, responsible for phosphorylation of Ser^{177}/Ser^{181} and Tyr^{188}/Tyr^{199} of IKKβ, then go on to activate NF-κB, via serine phosphorylation and degradation of IkB-α, then, finally, initiate of ICAM-1 expression. A schematic diagram showing the involvement of these two pathways in TNF-α-induced ICAM-1 expression in A549 epithelial cells is shown in Fig. 12.
