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Differential Role of Janus Family Kinases (JAKs) in Interferon-γ–Induced Lung Epithelial ICAM-1 Expression: Involving Protein Interactions between JAKs, Phospholipase Cγ, c-Src, and STAT1

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ABSTRACT

The signaling pathway for IFN-γ–mediated induction of ICAM-1 expression was further studied in human NCI-H292 epithelial cells. The Tyr701 phosphorylation of signal transducer and activator of transcription 1 (STAT1) induced by interferon-γ (IFN-γ) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was inhibited by the protein kinase C (PKC) inhibitor staurosporine, the tyrosine kinase inhibitor herbimycin, or the Src kinase inhibitor PP2. An association between c-Src and STAT1 was increased by IFN-γ and TPA, indicating the direct phosphorylation of STAT1 by PKC-dependent c-Src activation. Tyrosine phosphorylation of Janus kinases (JAK) 1/2 was induced by IFN-γ but not by TPA. In addition, ICAM-1 promoter activity induced by IFN-γ, but not that induced by TPA, was inhibited by the dominant-negative JAK1 and JAK2 mutants. IFN-γ–induced tyrosine phosphorylation of phospholipase C (PLC)-γ was inhibited by AG 490 (a JAK inhibitor), and the association between JAK1/2 and PLC-γ was increased after IFN-γ treatment, indicating the activation of PLC-γ via JAK1/2 phosphorylation. ICAM-1 promoter activities induced by the overexpression of wild-type JAK1- and PLC-γ2 were blocked by the PLCγ2 mutant or the dominant-negative PKCα (Lys→Arg), c-Src (Lys→Met), or STAT1 (Y701M) mutants, but not by dominant-negative STAT3 (DN) mutants. These results confirmed that IFN-γ activated PLC-γ via JAK1/2 phosphorylation to induce PKC, c-Src, STAT1 activation, and ICAM-1 expression. The association between JAK1/2 and STAT1 was increased by IFN-γ but not by TPA. It was inhibited by AG 490 but not by U73122, indicating the possible involvement of the JAK1/2-STAT1 pathway. All the results show that IFN-γ induces ICAM-1 expression by two different pathways in NCI-H292 epithelial cells. One is the JAK1/2-dependent PLC-γ pathway inducing the activations of PKCα, c-Src, and STAT1, and the other is the direct activation of STAT1 by JAK1/2.

The initial interaction between leukocytes and the endothelium seems to be transient, resulting in the leukocytes rolling along the vessel wall. These rolling leukocytes then become activated by local factors generated by the endothelium, resulting in their arrest and firm adhesion to the vessel wall. Finally, the leukocytes migrate across the endothelium. These complex processes are regulated, in part, by specific endothelial-leukocyte adhesion molecules. The intercellular adhesion molecule-1 (ICAM-1; CD54), an 80- to 114-kDa inducible surface glycoprotein belonging to the immunoglobulin superfamily, is involved in a wide range of inflammatory and immune responses (Staunton et al., 1988). During inflammation, ICAM-1 binds to two integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), belonging to the β2 subfamily, and both integrins are expressed by leukocytes and promote the adhesion and transendothelial migration of leukocytes (Staunton et al., 1988; Diamond et al., 1991). Similar processes govern leukocyte adhesion to lung airway epithelial cells and may contribute to the damage to these cells seen in asthma (Bloemen et al., 1993). ICAM-1 can be up-regulated by bacterial lipopolysaccharide, phorbol esters, and inflammatory cytokines, such as tumor necrosis factor α (TNF-

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ABBREVIATIONS: ICAM-1, intercellular adhesion molecule-1; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; JAK, Janus family kinase; STAT, signal transducers and activators of transcription; PLC, phospholipase C; PKC, protein kinase C; FCS, fetal calf serum; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; GAS, γ-activated site; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; DN, dominant negative; PI, phosphatidyl inositol; NF-κB, nuclear factor-κB; wt, wild type; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; AG 490, α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; U73122, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione.

α), interleukin-1 (IL-1), and interferon-γ (IFN-γ) (Rothlein et al., 1988; van de Stolpe et al., 1994; Read et al., 1995).

IFN-γ, a lymphocyte effector molecule produced by T cells and natural killer cells, plays an important role in macrophage activation, and it is implicated in the pathogenesis of a number of inflammatory diseases of infectious or presumed autoimmune origin (Schattner, 1994). IFN-γ has been reported to act via the JAK-STAT pathway in the regulation of gene expressions, and phospholipases are suggested to be involved in IFN-γ signaling in some cases (Sands et al., 1994). The cellular responses elicited by the interaction of many extracellular signaling molecules with their cell surface receptors are triggered by the rapid hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate. This reaction is catalyzed by phosphoinositide-specific phospholipase C (PLC) isozymes and results in the generation of two intracellular messengers, diacylglycerol and inositol 1,4,5-triphosphate, that promote the activation of protein kinase C (PKC) and the release of Ca^{2+} from intracellular stores, respectively. The PLC family comprises a diverse group of enzymes that differ in structure and tissue distribution. Ten mammalian PLC isozymes have been identified and divided into three types, PLC-β (four isozymes), PLC-γ (two isozymes), and PLC-δ (four isozymes) (Rhee and Bae, 1997). The signaling pathway for IFN-γ–induced ICAM-1 expression in NCI-H292 cells was found to involve PLC-γ2 activation via an upstream tyrosine kinase, which induced the activations of PKCα and either c-Src or Lyn, resulting in the activations of STAT1α, and GAS in the ICAM-1 promoter, followed by the initiation of ICAM-1 expression (Chang et al., 2002). To further elucidate the molecular mechanisms and the signal transduction cascades involved in IFN-γ–induced ICAM-1 expression, the associations between the phosphorylation status of JAK1/2 and PLC-γ, c-Src and STAT1α, or JAK1/2 and STAT1α were examined.

Experimental Procedures

Materials. Rabbit polyclonal antibodies specific for STAT1, JAK1, JAK2, or c-Src, and goat polyclonal antibody specific for STAT1 (Tyr701)-P were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies specific for phosphotyrosine residues (PY20) and specific for pSTAT1 (Ser727) were purchased from BD Transduction Laboratories (Lexington, KY) and Upstate Biotechnology (Lake Placid, NY), respectively. RPMI 1640 medium, fetal calf serum (FCS), penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was purchased from L.C. Services Corp. (Woburn, MA). Staurosporine was obtained from Sigma (St. Louis, MO). U73122, PP2, and herbimycin were obtained from Calbiochem (San Diego, CA). Reagents for SDS-PAGE were from Bio-Rad (Hercules, CA), and Tfx-50 and the luciferase assay kit were from Promega (Madison, WI).

Plasmids. The two ICAM-1 promotor constructs, pIC339 and pIC135, were the generous gifts from Dr. P.T. Van der Saag (Hubrecht Laboratory, Utrecht, Netherlands). The PLC-γ2 wild type and the PLC-γ2 mutant, SH2 (N), in which Arg564 is replaced by Ala, were gifts from Dr. T. Kurosaki (Kansai Medical University, Osaka, Japan). The constitutively active PKCα (Arg→Glu) and the dominant-negative mutant (Lys→Arg) were gifts from Dr. A. Altman (La Jolla Institute for Allergy and Immunology, San Diego, CA). The JAK1 wild type and dominant-negative JAK1 and JAK2 mutants were gifts from Dr. P. B. Rothman (Department of Microbiology, College of Physicians and Surgeons of Columbia University) and Dr. D. Levy (Department of Pathology, New York University, New York, NY), respectively. The STAT3 (DN) mutant was a gift from Dr. Nakajima (Department of Molecular Oncology, Osaka University, Japan).

Cell Culture. The human alveolar epithelial cell carcinoma line NCI-H292 was obtained from the American Type Culture Collection (Manassas, VA) and cultured in the RPMI 1640 medium supplemented with 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 in air. The cells were subcultured either in six-well plates for ICAM-1 promoter transfection or in 10-cm dishes for RNA extraction and cell extract preparations for coimmunoprecipitation experiments.

RT-PCR. Total RNA was isolated from NCI-H292 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer, then amplified for 30 cycles using two oligonucleotide primers derived from a published ICAM-1 sequence (5′-TGCGGCTGCTACCACAGTGATGAT-3′and 5′-CCATCTACAGCTTTCCGGCGCCCA-3′) and two from a β-actin sequence (5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTTGCGGGGACGATGGAGGG-3′). Each PCR cycle was carried out for 30 s at 94°C, 30 s at 65°C, and 1 min at 70°C. The PCR products were subjected to electrophoresis on a 1% agarose gel. Quantitative data were obtained using a computing densitometer and ImageQuant software (Amersham Biosciences, Piscataway, NJ).

Preparation of Cell Extracts. After pretreatment with various inhibitors for 30 min, cells were incubated with 10 ng/ml of IFN-γ or 1 μM TPA for the indicated time, then were rapidly washed with PBS, and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM NaF, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml of leupeptin, 20 μg/ml of aprotinin, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 5 mM Na-pyrophosphate, and 1% Triton X-100), as described previously (Chang et al., 2002).

Coimmunoprecipitation and Western Blot Analysis. Cell lysates containing 250 μg of protein were incubated for 1 h at 4°C with 2.5 μg of antibodies against STAT1, c-Src, JAK1, or JAK2, then protein A-Sepharose CL-4B beads (Sigma) were added and mixed for 16 h at 4°C. In this case, endogenous protein was detected. The immunoprecipitates were collected and washed three times with lysis buffer, then Laemmli buffer was added and the samples were subjected to electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at 25°C with 0.1% milk in Tris-buffered saline/Tween 20 and then incubated for 1 h at 25°C with mouse antibodies specific for phosphotyrosine residues (PY20) or PLC-γ, rabbit antibodies specific for pSTAT1 (Ser727), STAT1, c-Src, JAK1, or JAK2, or goat antibodies specific for pSTAT1 (Tyr701). Then, the membrane was incubated for 30 min at 25°C with horseradish peroxidase-labeled secondary antibody against mouse, rabbit, or goat. After each incubation, the membrane was washed extensively with Tris-buffered saline/Tween 20. The immunoreactive band was detected using enhanced chemiluminescence detection reagents (Amersham Biosciences) and visualized using Hyperfilm-ECL. Quantitative data were obtained using a computing densitometer and ImageQuant software (Amersham Biosciences).

Transient Transfection and Luciferase Activity Assay. NCI-H292 cells, 2×10^5/well, were grown in six-well plates, transfected with the human ICAM-1 promoter-firefly luciferase construct pIC339 or pIC135, using Tfx-50, as described previously (Chang et al., 2002). The following day, the cells were incubated for 5 h with either 10 ng/ml of IFN-γ or 1 μM TPA, then cell extracts were prepared and the luciferase and β-galactosidase activities were measured. The luciferase activity of each well was normalized to the β-galactosidase activity. In dominant-negative mutant experiments, cells were cotransfected with reporter/β-galactosidase (0.3 μg/ 0.1 μg) and the dominant-negative JAK1, JAK2, STAT1 (Y701M), STAT1 (S727M), or STAT3 mutants (0.6 μg) or the empty vector. In wild-type experiments, cells were cotransfected with reporter/β-galactosidase and the wild-type PLCγ2, JAK1, or Src or constitutively

active PKCα plasmids and the PLC-γ2 (SH_2(N)) mutant or the dominant-negative JAK1 (DN), JAK2 (DN), PKC-α (Lys→Arg), c-Src (K295M), or STAT1 (Y701M) mutants using SuperFect Transfection reagent (QIAGEN, Valencia, CA). Briefly, dominant-negative mutants (2.0 μg), wild-type/constitutively active plasmid or empty vector (1.5 μg), pIC135 (0.5 μg), and β-galactosidase (0.25 μg) were mixed with 1.87 μl (1:0.5) of SuperFect in 300 μl of serum-free RPMI 1640 medium. After 10 min of incubation at room temperature, 600 μl of serum-free RPMI 1640 medium was added and the mixture applied to the cells. Eight hours later, 100 μl of FCS was added to each well, resulting in cells being grown in 10% FCS. On the following day, cell extracts were prepared and the luciferase (Promega) and β-galactosidase activities were measured. The luciferase activity of each well was normalized to the β-galactosidase activity.

Results

IFN-γ Induces ICAM-1 mRNA Expression and the Effects of Various Inhibitors. It has been shown previously that the signaling pathway of IFN-γ–mediated ICAM-1 expression in NCI-H292 epithelial cells involves PLC-γ2, PKC, and c-Src (Chang et al., 2002). To determine whether the regulation occurred at the mRNA expression level, the time course of inducing ICAM-1 mRNA expression by IFN-γ and the PKC activator TPA was examined by RT-PCR. ICAM-1 mRNA levels were significantly increased after 1 h of treatment with both IFN-γ and TPA, and the levels remained high for up to 6 h of treatment, then declined (Fig. 1A). IFN-γ was more potent at initiating the transcription of ICAM-1 compared with TPA. The IFN-γ–induced increase in ICAM-1 mRNA expression could be inhibited by 30 μM U73122 (a PI-PLC inhibitor), 100 nM staurosporine (a PKC inhibitor), 1 μM herbimycin (a tyrosine kinase inhibitor), 10 μM PP2 (a Src kinase inhibitor), and 50 μM AG 490 (a JAK inhibitor) (Fig. 1B, lanes 3–7). However, the ICAM-1 mRNA

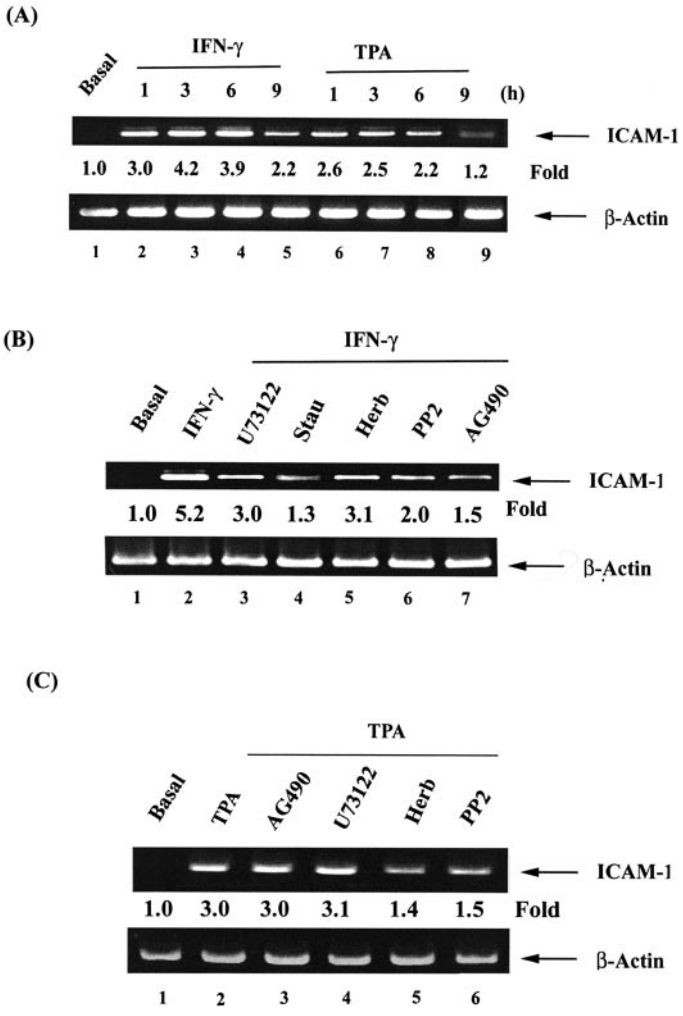

Fig. 1. Time course of ICAM-1 mRNA expression induced by IFN-γ or TPA in NCI-H292 cells and the effects of various inhibitors. A, cells were treated with 10 ng/ml of IFN-γ or 1 μM TPA for the indicated time. B, cells were pretreated for 30 min with 30 μM U73122, 100 nM staurosporine, 1 μM herbimycin, 10 μM PP2, or 50 μM AG 490 before incubation with 10 ng/ml of IFN-γ for 3 h. C, cells were pretreated with 50 μM AG 490, 30 μM U73122, 1 μM herbimycin, or 10 μM PP2 before incubation with 1 μM TPA for 3 h. Total RNA (2 μg) was used for RT-PCR.

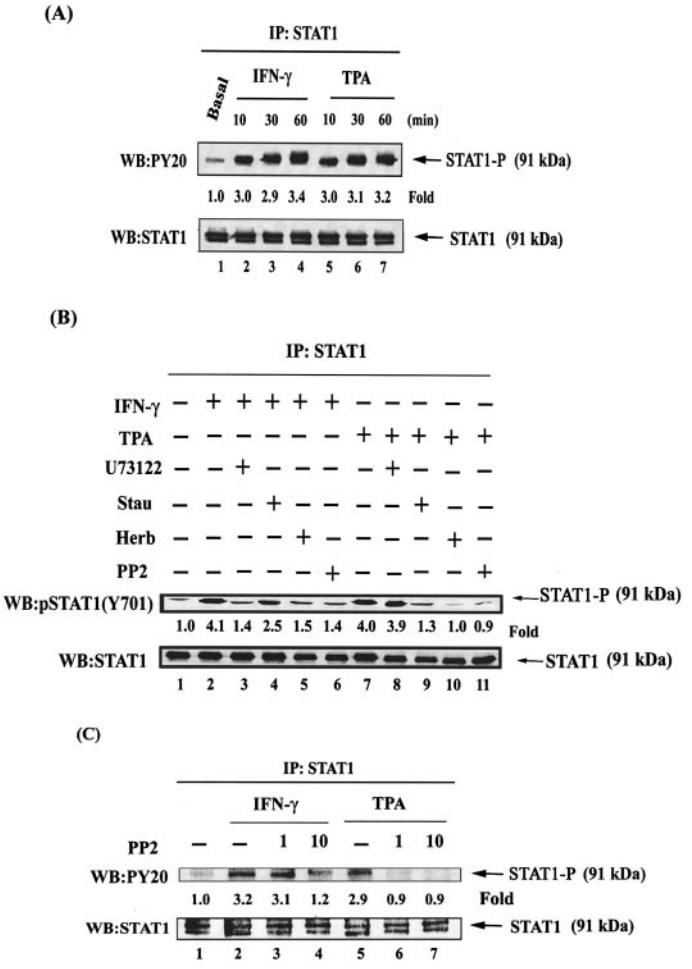

Fig. 2. Time-dependent phosphorylation of STAT1 by IFN-γ or TPA and the effects of various inhibitors. A, cells were treated with 10 ng/ml of IFN-γ or 1 μM TPA for 10, 30, or 60 min. Whole-cell lysates were immunoprecipitated with anti-STAT1 antibody, then the precipitate was subjected to SDS-PAGE and Western blotting using antibody specific for phosphotyrosine residues (PY20). B, cells were pretreated for 30 min with 30 μM U73122, 100 nM staurosporine, 1 μM herbimycin, or 10 μM PP2 before incubation with 10 ng/ml of IFN-γ for 60 min or 1 μM TPA for 30 min. Whole-cell lysates were immunoprecipitated with anti-STAT1 antibody, then the precipitate was subjected to SDS-PAGE and Western blotting with antibody specific for pSTAT1 (Tyr701). C, cells were pretreated for 30 min with 1 or 10 μM PP2 before incubation with 10 ng/ml of IFN-γ for 60 min or 1 μM TPA for 30 min. Whole-cell lysates were immunoprecipitated with anti-STAT1 antibody; then the precipitate was subjected to SDS-PAGE and Western blotting with anti-phosphotyrosine antibody (PY20). To determine the level of immunoprecipitated STAT1, the membranes were stripped and reprobed with anti-STAT1 antibody.

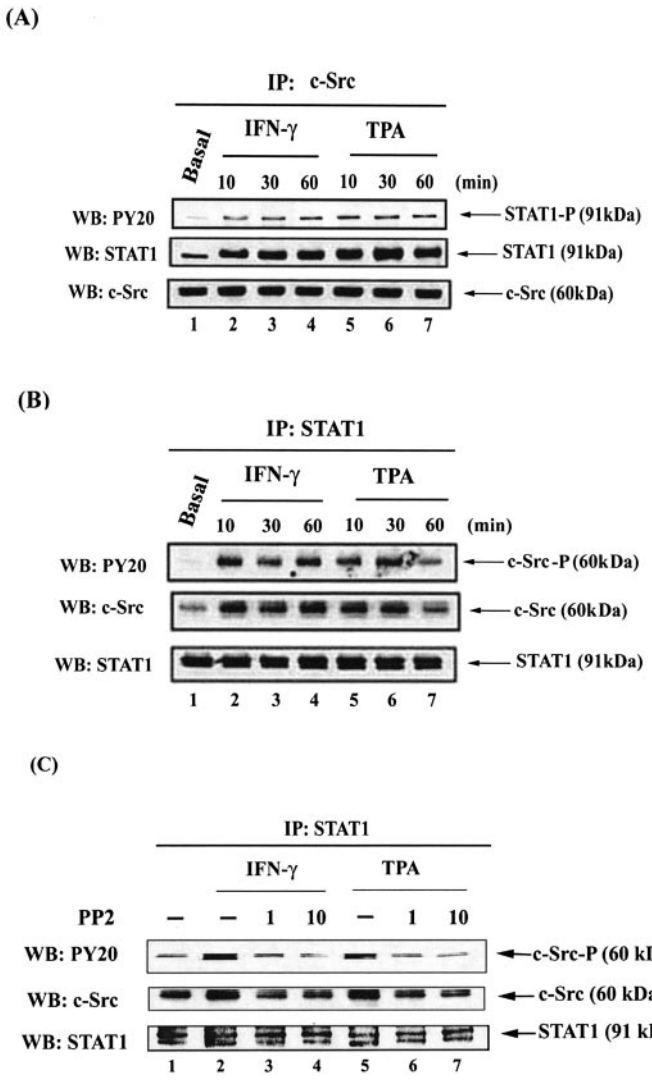

Fig. 3. Time-dependent c-Src and STAT1 interaction induced by IFN-γ or TPA. Cells were treated with 10 ng/ml of IFN-γ or 1 μM TPA for 10, 30, or 60 min. Whole-cell lysates were immunoprecipitated with anti-c-Src (A) or anti-STAT1 (B) antibody; then the precipitate was subjected to SDS-PAGE and Western blotting with anti-STAT1 or anti-phosphotyrosine (A) or anti-c-Src or anti-phosphotyrosine (B) antibody. C, cells were pretreated for 30 min with 1 or 10 μM PP2, then incubated with 10 ng/ml of IFN-γ for 60 min or 1 μM TPA for 30 min. Whole-cell lysates were immunoprecipitated with anti-STAT1 antibody; then the precipitate was subjected to SDS-PAGE and Western blotting with anti-c-Src or anti-phosphotyrosine antibody. To determine the levels of immunoprecipitated c-Src or STAT1, the membranes were stripped and reprobed with anti-c-Src (A) or anti-STAT1 (B and C) antibody, respectively.

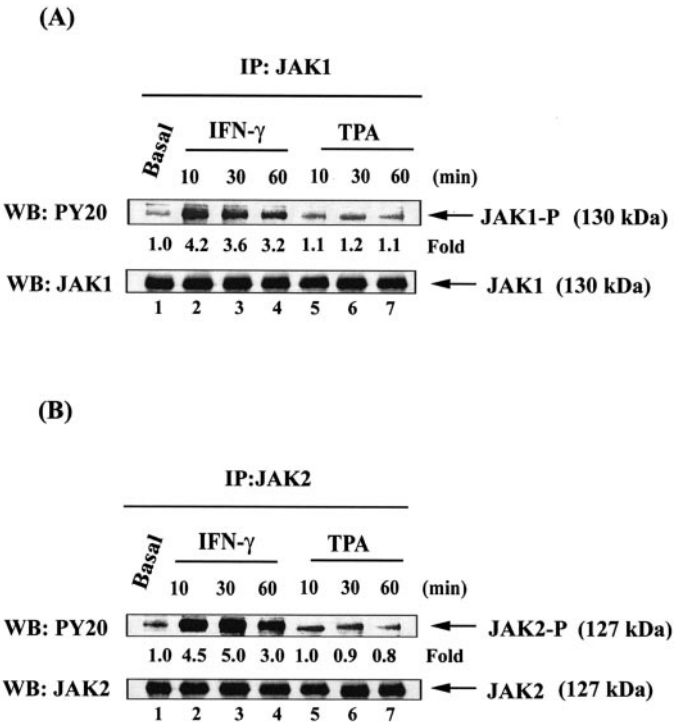

Fig. 4. Time-dependent tyrosine phosphorylation of JAK1/2 by IFN-γ but not TPA. Cells were treated with 10 ng/ml of IFN-γ or 1 μM TPA for 10, 30, or 60 min. Whole-cell lysates were immunoprecipitated with anti-JAK1 (A) or anti-JAK2 (B) antibody, then the precipitate was subjected to SDS-PAGE and Western blotting with anti-phosphotyrosine antibody. To determine the levels of immunoprecipitated JAK1 or JAK2, the membranes were stripped and reprobed with anti-JAK1 (A) or anti-JAK2 (B) antibody, respectively.

expression induced by TPA could be inhibited only by herbimycin and PP2 (Fig. 1C, lanes 5 and 6) but not by AG 490 and U73122 (Fig. 1C, lanes 3 and 4). These results suggested that PI-PLC, PKC, c-Src, and JAK are important in the mediation of ICAM-1 expression.

Stimulation with IFN-γ or TPA Increases the Tyrosine Phosphorylation of STAT1 and the Association between c-Src and STAT1. Because c-Src-dependent STAT1 activation is involved in the IFN-γ–induced ICAM-1 expression in NCI-H292 cells (Chang et al., 2002), c-Src and STAT1 coimmunoprecipitation experiments were performed to determine whether c-Src directly regulated STAT1 activity through the phosphorylation of a tyrosine residue. To determine whether c-Src bound directly to STAT1 and phosphorylated its tyrosine residue, cell lysates were immunoprecipitated with anti-STAT1 antibody, then the immunoprecipitates were separated by SDS-PAGE, transferred to membranes, and blotted with anti-phosphotyrosine antibodies. As shown in Fig, 2A, tyrosine phosphorylation of STAT1 at Y701 was seen after either IFN-γ or TPA treatment, and the effect was seen at 10 min and peaked at 60 min. However, this effect was blocked by the inhibitors of PI-PLC, PKC, tyrosine kinase, and Src kinase (Fig. 2B). On the other hand, the TPA-induced Y701 phosphorylation of STAT1 was not affected by U73122 (Fig. 2B, lane 8), but it was more sensitive to the inhibitory effect of PP2 compared with IFN-γ. PP2 (1 μM) had no inhibition on the effect of IFN-γ, whereas this concentration could completely block the effect of TPA (Fig. 2C, compare lanes 3 and 6). The results indicated that another pathway may exist in the IFN-γ–induced ICAM-1 expression, in addition to the PLCγ/PKCα/c-Src pathway.

The direct association between c-Src and STAT1 was examined further. Anti-c-Src antibody was used to precipitate c-Src from NCI-H292 cells, and the immunoprecipitated proteins were subjected to Western blotting using anti-STAT1 antibody. As shown in Fig. 3A, the amount of STAT1 coprecipitated with c-Src was increased after either IFN-γ or TPA treatment, and the associated STAT1 was tyrosine-phosphorylated (lanes 2–7). In the converse experiment, in which STAT1 was precipitated using anti-STAT1 antibody, the precipitated proteins were analyzed by Western blotting using anti-c-Src antibody. c-Src was shown to be associated with

STAT1, and the amount increased after treatment with either IFN-γ or TPA. In addition, the associated c-Src was tyrosyl-phosphorylated (Fig. 3B, lanes 2–7), and the effects were inhibited by PP2 (Fig. 3C, lanes 3–4 and 6–7). These results suggested a direct association between c-Src and STAT1, and the STAT1 was tyrosyl phosphorylated.

The Tyrosine Phosphorylation of JAK1/2 and the Association between JAK1/2 and PLC-γ Were Stimulated by IFN-γ but Not by TPA. Because IFN-γ and cytokines activate STATs via JAK kinases (Heim et al., 1995), the role of JAK1 and JAK2 in IFN-γ–induced STAT1 activation and ICAM-1 expression were examined. Tyrosine phosphorylations of JAK1 (Fig. 4A) and JAK2 (Fig. 4B) were seen after 10-min treatment with IFN-γ and declined after 60 min of treatment. However, TPA did not induce the tyrosine phosphorylation of either JAK1 or JAK2 (lanes 5–7), indicating that JAK1/2 activation occurred upstream of PKC after IFN-γ stimulation.

IFN-γ activates PLCγ via an upstream tyrosine kinase to induce the activations of PKC and c-Src, resulting in STAT1 activation, which is followed by the initiation of ICAM-1 expression in NCI-H292 cells (Chang et al., 2002; Fig. 1B). To determine whether the upstream tyrosine kinase involved in the IFN-γ–induced PLC-γ activation is JAK1 or JAK2, the effect of the PLC inhibitor U73122 and the JAK inhibitor AG 490 were tested. IFN-γ–induced phosphorylations of JAK1 and JAK2 were blocked by AG 490 but not by U73122 (Fig. 5, lanes 3–5), indicating that JAK1 and JAK2 were involved in the upstream of PLC-γ. Furthermore, IFN-γ–induced tyrosine phosphorylation of PLC-γ was inhibited by AG 490 in a dose-dependent manner (Fig. 6A, lanes 3–4). Thus, the direct association between JAK1/2 and PLC-γ was therefore examined. Anti-JAK1 and anti-JAK2 antibodies were used to precipitate JAK1 and JAK2 from NCI-H292 cells, respectively, the immunoprecipitated proteins were subjected to Western blotting using anti-PLCγ antibody. As shown in Fig. 6, B and C, an increase in the amount of PLCγ was coprecipitated with either JAK1 or JAK2 after IFN-γ stimulation and the maximal interaction was observed after 10 min of IFN-γ treatment. The results indicated that there was a transient interaction between JAK1/2 and PLCγ. Thus, IFN-γ might recruit and activate PLC-γ via JAK1/2.

The Tyrosyl Phosphorylated JAK1/2 Associated with STAT1 after IFN-γ Induction. Although previous results (Chang et al., 2002) and the above experiments demonstrated the involvement of JAK1/2/PLCγ/PKC/c-Src/STAT1 pathway in IFN-γ–induced ICAM-1 expression, the JAK1/2/STAT1 pathway had also been reported to be involved in the IFN-γ–induced activation (Heim et al., 1995). To examine the possible involvement of this pathway in the IFN-γ–induced ICAM-1 expression in NCI-H292 cells, the phosphorylation of STAT1 by JAK1/2 was examined. As shown in Fig. 7A, IFN-γ–induced tyrosine phosphorylation of STAT1 and the direct association between JAK1/2 and STAT1 was seen. In addition, the associated JAK1/2 was also tyrosyl-phosphorylated. Except for the tyrosine phosphorylation of STAT1, which was inhibited by U73122 (Fig. 7A, lane 6), all the effects were blocked by AG 490 but not by U73122 (Fig. 7A, lanes 4 and 6). After IFN-γ treatment, the increase in the amounts of JAK1 and JAK2 coimmunoprecipitated with STAT1 was parallel with that of the tyrosyl-phosphorylated STAT1 (Fig. 7, B and C, lanes 2–4). However, the amount of JAK1 coimmunoprecipitated with STAT1 did not increase after TPA treatment, despite the tyrosyl-phosphorylated STAT1 observed (Fig. 7D, lanes 2–4). These results indicated the possible existence of a JAK1/2/STAT1 pathway in the IFN-γ–induced ICAM-1 expression in NCI-H292 cells.

The Inductions of ICAM-1 Promoter Activity by IFN-γ and TPA and the Inhibitory Effects of Dominant-Negative Mutants of JAK1, JAK2, and STAT1. IFN-γ induces JAK1/2 and STAT1 activation, and these events have downstream effects on the IFN-γ–induced ICAM-1 promoter activity (Figs. 4 and 2A; Chang et al., 2002). To further demonstrate that PKC activation occurs downstream of JAK1/2, but upstream of STAT1, transient transfection was performed with the pIC339 (−339/+1) luciferase construct containing the downstream NF-κB site and GAS of the ICAM-1 promoter. The ICAM-1 promoter activity induced by IFN-γ, but not by TPA, was attenuated by cotransfection with the dominant-negative JAK1 (DN) and JAK2 (DN) mutants (Fig. 8A). In addition, the ICAM-1 promoter activities induced by both IFN-γ and TPA were abolished by the cotransfections with the dominant-negative STAT1 (Y701M) and STAT1 (S727M), but not by STAT3 (DN) mutants (Fig. 8B). AG 490 inhibited IFN-γ- but not

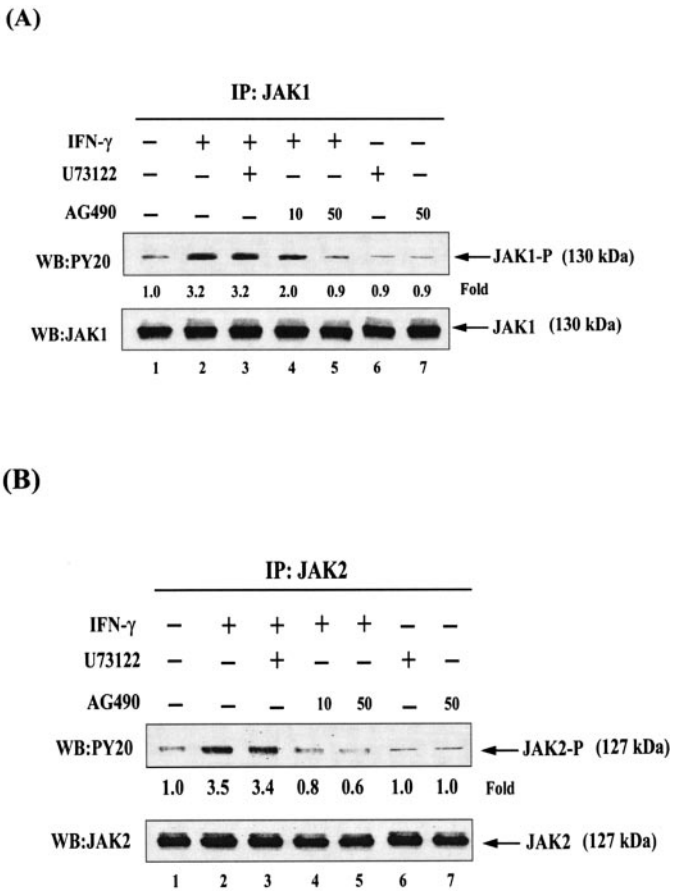

Fig. 5. Effect of various inhibitors on IFN-γ–induced tyrosine phosphorylation of JAK1/2. Cells were pretreated for 30 min with 30 μM U73122 or 10 or 50 μM AG 490 before incubation with 10 ng/ml of IFN-γ for 10 min. Whole-cell lysates were immunoprecipitated with anti-JAK1 (A) or anti-JAK2 (B) antibody, then the precipitate was subjected to SDS-PAGE and Western blotting with anti-phosphotyrosine antibody. To determine the levels of immunoprecipitated JAK1 or JAK2, the membranes were stripped and reprobed with anti-JAK1 (A) or anti-JAK2 (B) antibody, respectively.

TPA-induced ICAM-1 promoter activity, whereas PP2 blocked both (Fig. 8C). These results indicated that JAK1 and JAK2 were involved upstream and STAT1 downstream of PKC in IFN-γ–induced ICAM-1 expression.

We have shown previously that the ICAM-1 promoter activity is enhanced by the cotransfections with wild-type PLCγ2, PKCα, c-Src, JAK1, and STAT1 (Chang et al., 2002). To confirm the involvement of JAK1/2/PLCγ2/PKC/c-Src/STAT1 and JAK1/2/STAT1 pathways in the IFN-γ–induced ICAM-1 expression, the constitutively active form of PKCα (Arg→Glu), wild-type JAK1, PLCγ2, and c-Src were cotransfected with the ICAM-1-luc plasmid and resulted in the significant increases in ICAM-1 promoter activities of 4.6-, 5.3-, 5.7-, and 5.6-fold, respectively (Fig. 9). The increase in ICAM-1 promoter activity induced by these constitutive or wild-type plasmids was inhibited by the dominant-negative STAT1 (Y701M) but not by the STAT3 (DN) mutant. Furthermore, the ICAM-1 promoter activity induced by wild-type JAK1 was also inhibited by the PLCγ2 mutant and the dominant-negative c-Src (Lys→Met) mutant. However, the ICAM-1 promoter activity induced by the wild-type PLCγ2 was not affected by the dominant-negative JAK1 (DN) and JAK2 (DN) mutants, but the activity was inhibited by the dominant-negative PKCα (Lys→Arg) and c-Src (Lys→Met) mutants (Fig. 9A). This suggested that PLCγ2 lies downstream of JAK1/2. PKCα (Arg→Glu)-induced ICAM-1 promoter activity was also inhibited by the dominant c-Src (Lys→Met) mutant (Fig. 9B). When different amounts of the dominant-negative c-Src (KM) mutant DNA (1.5, 2.0, or 2.5 μg) were cotransfected with either wild-type JAK1 or PKCα (Ala→Glu), dose-dependent inhibitions on JAK1 (wt)- and PKCα (Ala→Glu)-induced ICAM-1 promoter activities were seen. Furthermore, the extent of inhibition was greater on PKCα (Ala→Glu)-induced activity than that induced by JAK1 (wt) (Fig. 9C). The JAK2 has been reported to have effects similar to those seen with JAK1 (Igarashi et al., 1994). These results confirmed the JAK1/2-STAT1 pathway as well as the JAK1/2/PLCγ2/PKC/c-Src/STAT1 pathway in the IFN-γ–induced ICAM-1 expression in NCI-H292 cells.

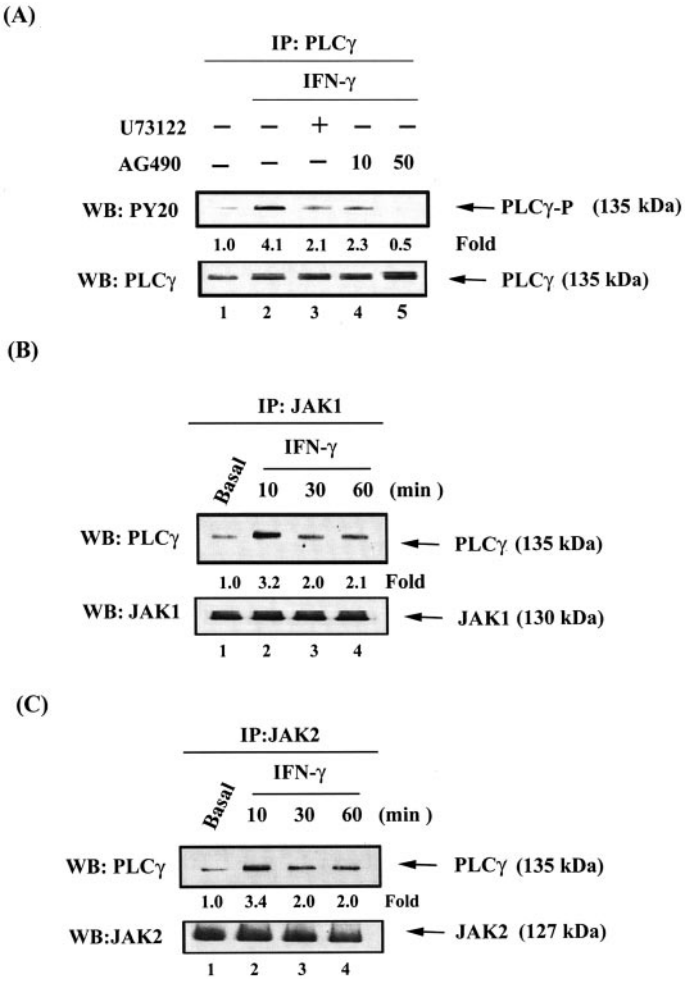

Fig. 6. JAK-dependent tyrosine phosphorylation of PLCγ and time-dependent JAK1/2 and PLCγ interaction by IFN-γ. A, cells were pretreated for 30 min with 30 μM U73122, 10 or 50 μM AG 490 before incubation with 10 ng/ml of IFN-γ for 10 min. Whole-cell lysates were immunoprecipitated with anti-PLCγ antibody, then the precipitate was subjected to SDS-PAGE and Western blotting with anti-phosphotyrosine antibody. B and C, cells were treated with 10 ng/ml of IFN-γ for 10, 30, or 60 min. Whole-cell lysates were immunoprecipitated with anti-JAK1 (B) or anti-JAK2 (C) antibody, then the precipitate was subjected to SDS-PAGE and Western blotting with anti-PLCγ antibody. To determine the levels of immunoprecipitated PLCγ, JAK1, or JAK2, the membranes were stripped and reprobed with antibodies against PLCγ (A), JAK1 (B), or JAK2 (C), respectively.

Discussion

The PKC-dependent c-Src activation pathway is involved in the IFN-γ–induced STAT1α and GAS activation, resulting in the ICAM-1 expression in NCI-H292 alveolar epithelial cells and causing monocyte adherence to these cells (Chang et al., 2002). Because the involvement of PKC/c-Src/STAT1α pathway had been demonstrated, the tyrosine phosphorylation of STAT1 by c-Src was examined. Several lines of evidence showed that this occurs. First, in anti-STAT1 immunoprecipitates, STAT1 was found to be tyrosine-phosphorylated at Tyr701 after either IFN-γ or TPA stimulation. Second, the Src kinase inhibitor PP2 inhibited this effect. Third, using either immunoprecipitation with anti-c-Src antibody followed by Western blotting with anti-STAT1 antibody or immunoprecipitation with anti-STAT1 antibody followed by Western blotting with anti-c-Src antibody, a direct association between c-Src and STAT1 was increased after IFN-γ or TPA treatment. Fourth, the STAT1 was phosphorylated at Tyr701 in anti-c-Src immunoprecipitates, and c-Src was tyrosyl phosphorylated in anti-STAT1 immunoprecipitates as well (see Fig. 3). Many cytokines and growth factors are also reported to activate STATs via members of the cytoplasmic Src tyrosine kinase family (Bowman et al., 2000). A role for Src kinase in STAT activation was first suggested by studies aimed at investigating the molecular mechanisms associated with the Src-mediated transformation of fibroblasts and hematopoietic cell lines. STAT3 activation is required for the v-Src-mediated transformation of NIH3T3 cells (Bromberg et al., 1998; Turkson et al., 1998), and a direct association of v-Src and STAT3 has been observed in the 32Dcl3 cells (Reddy et al., 2000). In addition to v-Src, c-Src plays a role in IL-3-, epidermal growth factor-, and platelet-derived growth factor–induced STAT3 and STAT1 activation (Cirri et al., 1997; Chaturvedi et al., 1998; Olayioye et al., 1999). In A431 cells, epidermal growth factor induces the activations of STAT1/3 and c-Src kinase, and a direct association between the two components has been demonstrated (Olayioye et al., 1999). In NIH3T3 cells, both

the tyrosine phosphorylation and the DNA binding activity of STAT1 and STAT3 are up-regulated in the cells overexpressing c-Src, and the coimmunoprecipitation of STAT1 and c-Src had also been observed (Cirri et al., 1997). The involvement of PKC-dependent c-Src activation in the expression of inducible inflammatory genes may be a common signaling pathway, because both the TNF-α- and IL-1β–induced ICAM-1 and cyclooxygenase-2 expression in alveolar epithelial cells also involves this pathway (Chen et al., 2000a,b, 2001; Huang et al., 2003a,b). A similar PKC-dependent c-Src activation pathway has been found in the A7r5 vascular smooth muscle cells, in which TPA induces the Rho-dependent actin reorganization (Brandt et al., 2002). How the PKC activates the c-Src remains to be investigated, but it is known to induce rapid threonine phosphorylation of Btk in platelets, and the association between PKC and Btk occurs via the Pleckstrin homology domain of Btk (Crosby and Pool, 2002).

STATs are latent cytoplasmic transcription factors that transduce the signal from the cell membrane to the nucleus upon phosphorylation/activation. JAK is one of the protein tyrosine kinases that can induce the phosphorylations of STATs. The intracellular signaling of IFN-γ has been shown to act through the JAK/STAT pathway (Heim et al., 1995). The IFN-γ–induced Tyr701 phosphorylation of STAT1 is mediated by JAK (Kohlhuber et al., 1997; Bromberg and Darnell, 2000), and this single phosphotyrosine residue is required for the activations of genes induced by IFN-γ (Shuai et al., 1993). Several lines of evidence have shown the involvement of JAK1 and JAK2 in IFN-γ–induced ICAM-1 expression in the NCI-H292 cells. First, the IFN-γ–induced ICAM-1 mRNA expression was attenuated by the JAK inhibitor, AG 490. Second, the IFN-γ–induced ICAM-1 promoter activity was blocked by the dominant-negative JAK1 and JAK2 mutants. Third, in either the anti-JAK1 or the anti-JAK2 immunoprecipitates, both JAK1 and JAK2 were tyrosine phosphorylated after IFN-γ treatment. These results indicated that JAK1/2 activation is indeed involved in the IFN-γ–induced ICAM-1 expression. Five other findings showed that, in addition to the involvement of the PLCγ2/PKCα/c-Src/STAT1 pathway, which was already known (Chang et al., 2002) and confirmed in the present study, the JAK1/2/STAT1 pathway is also involved in the IFN-γ–mediated induction of ICAM-1 expression. First, the tyrosine phosphorylation of STAT1 by IFN-γ was inhibited by the JAK inhibitor AG 490

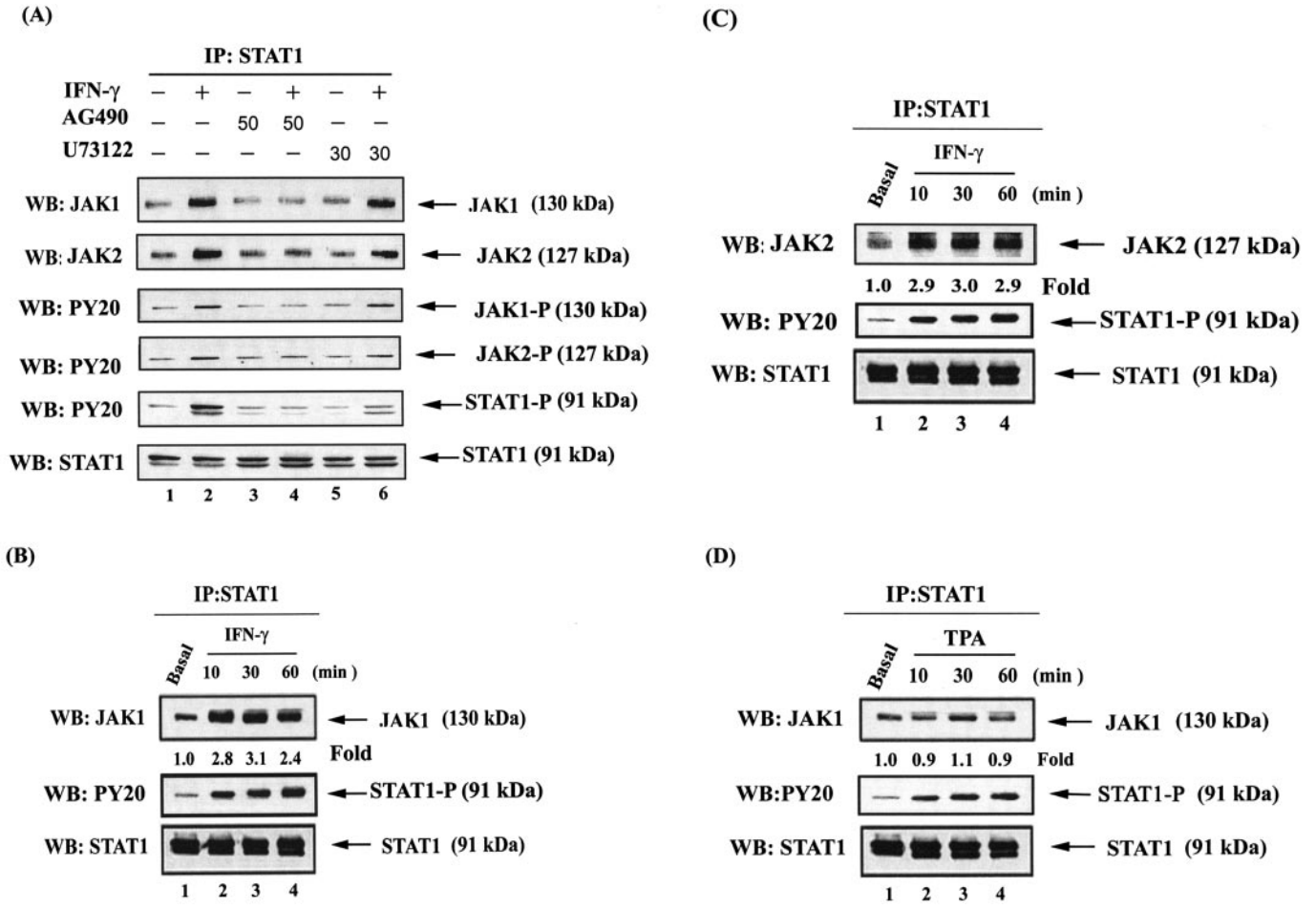

Fig. 7. Induction of the JAK-dependent tyrosine phosphorylation of STAT1 and the JAK1/2-STAT1 interaction by IFN-γ. A, cells were pretreated with 50 μM AG 490 or 30 μM U73122 for 30 min before incubation with 10 ng/ml of IFN-γ for 60 min. Whole-cell lysates were immunoprecipitated with anti-STAT antibody, then the precipitate was subjected to SDS-PAGE and Western blotting with anti-JAK1, anti-JAK2 or anti-phosphotyrosine antibody. B and C, after IFN-γ treatment, whole-cell lysates were immunoprecipitated with anti-STAT1 antibody, then the precipitate was subjected to SDS-PAGE and Western blotting with anti-JAK1, anti-JAK2 or anti-phosphotyrosine antibody. D, after TPA treatment, whole-cell lysates were immunoprecipitated with anti-STAT1 antibody, then the precipitate was subjected to SDS-PAGE and Western blotting with anti-JAK1 or anti-phosphotyrosine antibody. To determine the levels of immunoprecipitated STAT1, the membranes were stripped and reprobed with anti-STAT1 antibody.

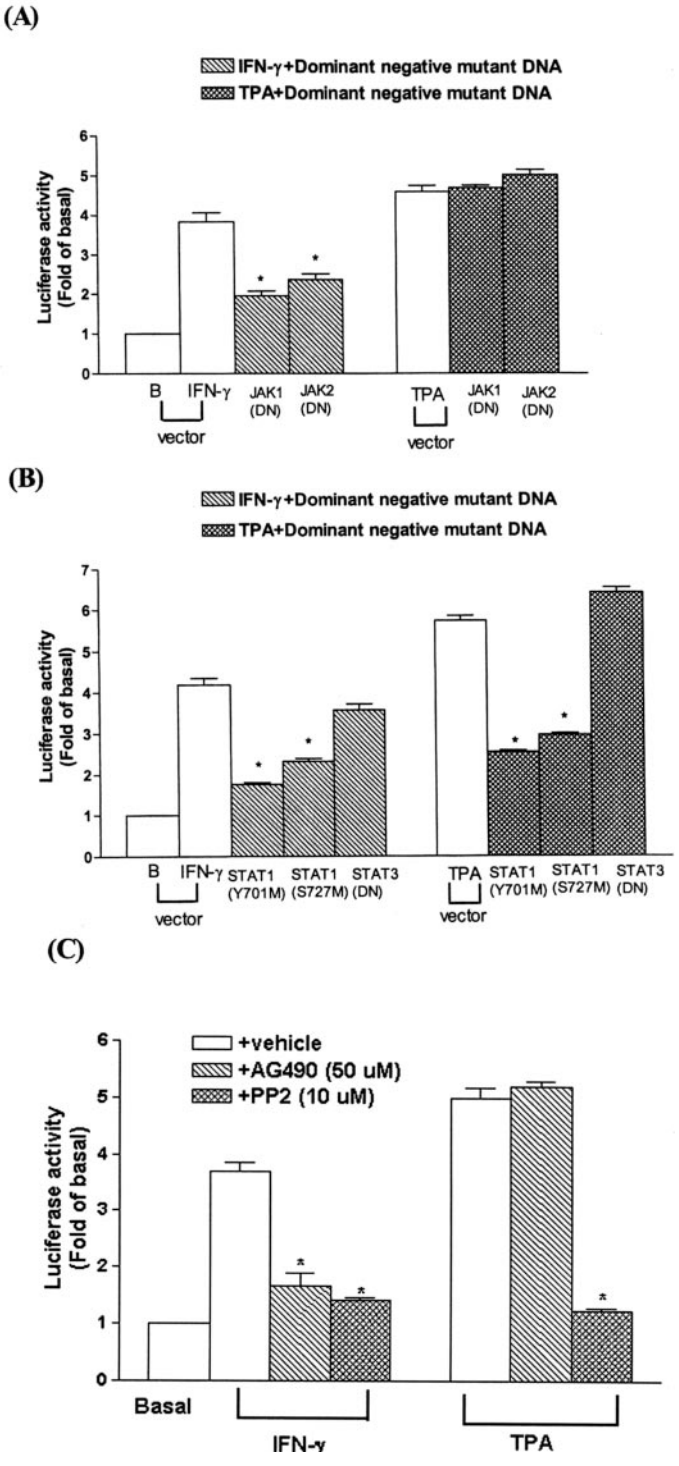

Fig. 8. Effect of dominant-negative mutants on IFN-γ- or TPA-induced ICAM-1 promoter activity. Cells were cotransfected with pIC339 and the dominant-negative JAK1 or JAK2 (A) or STAT1 (Y701M), STAT1 (S727M), or STAT3 (B) mutant, or empty vector. C, cells were cotransfected with pIC135 and the indicated dominant-negative mutant. Luciferase activity was assayed. The results were normalized to the β-galactosidase activity and are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. *, $p < 0.05$ compared with IFN-γ or TPA alone.

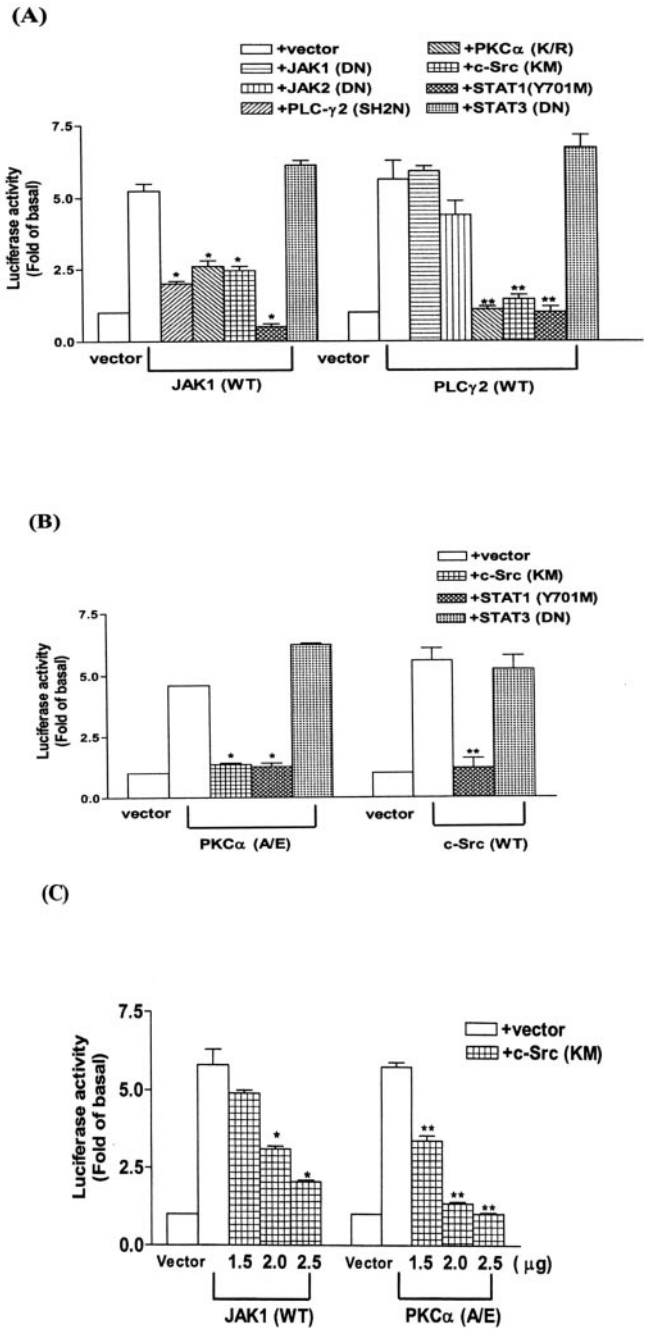

Fig. 9. Effects of various dominant-negative mutants on wild-type plasmid-stimulated ICAM-1 promoter activity. KM, Lys→Met; K/R, Lys→Arg; A/E, Arg→Glu. A, NCI-H292 cells were cotransfected with 1.5 μg of wild-type JAK1 (wt) or PLCγ2 (wt) and 2.0 μg of the dominant-negative JAK1 (DN), JAK2 (DN), PLCγ2 (SH2(N)), PKCα (Lys→Arg), c-Src (Lys→Met), STAT1 (Y701M), or STAT3 (DN) mutant, or the empty vector. B, NCI-H292 cells were cotransfected with 1.5 μg of constitutively active PKCα (Arg→Glu) or wild-type c-Src (wt) and 2.0 μg of the dominant-negative mutant, c-Src (Lys→Met), STAT1 (Y701M), or STAT3 (DN), or the empty vector. C, NCI-H292 cells were cotransfected with 1.5 μg of wild-type JAK1 (wt) or constitutively active PKCα (Arg→Glu) and different amount (1.5, 2.0, or 2.5 μg) of dominant-negative mutant c-Src (Lys→Met) DNA or the empty vector. Luciferase activity was assayed. The results were normalized using the β-galactosidase activity and are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate. A, *, $p < 0.05$ compared with JAK1 (wt); **, $p < 0.05$ compared with PLC-γ2 (wt). B, *, $p < 0.05$ compared with PKCα (Arg→Glu); **, $p < 0.05$ compared with c-Src (wt). C, *, $p < 0.05$ compared with JAK1 (wt); **, $p < 0.05$ compared with PKCα (Arg→Glu).

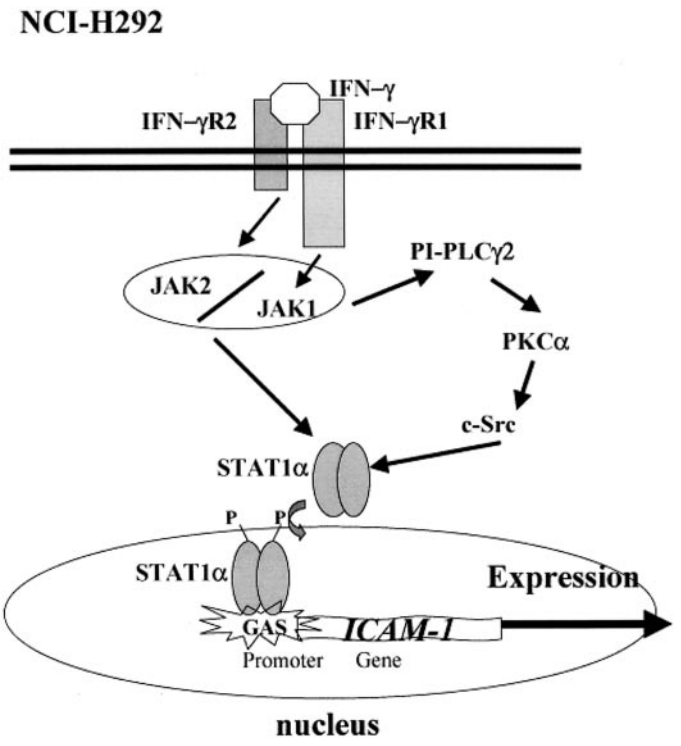

Fig. 10. Schematic representation of the signaling pathways involved in IFN-γ–induced ICAM-1 expression in NCI-H292 epithelial cells. IFN-γ binds to IFN-γR and activates JAK1/2, which either acts via PI-PLCγ2 to induce PKCα and c-Src activation, leading to tyrosine phosphorylation of STAT1α, or acts directly via tyrosine phosphorylation of STAT1α. These two pathways converge at STAT1α, resulting in activation of STAT1α and GAS in the ICAM-1 promoter, followed by initiation of ICAM-1 expression.

(Fig. 7A). Second, the direct association between JAK1, JAK2, and STAT1 were increased after the IFN-γ treatment using immunoprecipitation with anti-STAT1 antibody followed by blotting with either anti-JAK1 or anti-JAK2 antibody (Fig. 7, B and C), and this effect was inhibited by AG 490 but not U73122 (Fig. 7A). Third, the IFN-γ–induced ICAM-1 expression and the tyrosine phosphorylation of STAT1 were less sensitive to the inhibitory effect of PP2 than those induced by TPA, which directly activated PKC (Fig. 5C in Chang et al., 2002; Fig. 2C). Fourth, the association between JAK1 and STAT1 did not increase after TPA treatment despite the observation of tyrosine phosphorylation of STAT1 (Fig. 7D), which is caused by the activation of PKC/c-Src pathway. Fifth, JAK1 and JAK2 were shown to act directly on both the STAT1 and the PLCγ/PKC/c-Src/STAT pathway (see below). Because PKC could only act through the c-Src/STAT pathway, it explained why the wild-type JAK1-induced ICAM-1 promoter activity was less sensitive to the inhibitory effect of the dominant-negative c-Src (Lys→Met) mutant than that induced by the constitutively active PKCα (Ala→Glu) (Fig. 9C).

The relationship between the PLCγ2/PKCα/c-Src/STAT1 and JAK1/2/STAT1 pathways in IFN-γ–induced ICAM-1 expression was elucidated by the overexpression of either wild-type JAK1 or PLCγ2. These two plasmids both induced an increase in ICAM-1 promoter activity, and their effects were blocked by the dominant-negative mutants PKCα (Lys→Arg), c-Src (Lys→Met), and STAT1 (Y701M), but not by the STAT3 mutant. The effect of wild-type JAK1 was also blocked by the PLC-γ2 (SH2(N)) mutant, but that induced by the wild-type PLC-γ2 was not affected by the dominant-negative JAK1 and JAK2 mutants (Fig. 9A). Furthermore, the dominant-negative JAK1 and JAK2 mutants did not affect the induction of ICAM-1 promoter activity by TPA (Fig. 8A). These results indicated that JAK1 and JAK2 also acted upstream of PLC-γ2. PLC-γ is an SH2 domain-containing protein that uses this module to link the phosphotyrosine-containing sequences in a receptor protein or the cytoplasmic protein tyrosine kinase to PI hydrolysis (Schlessinger, 1994). Several nonreceptor tyrosine kinases are involved in PLC-γ activation. For example, PLC-γ1 is tyrosine phosphorylated after recruitment to the phosphorylated Syk through its SH2 domains in the COS cells (Law et al., 1996). Furthermore, phosphatidylinositol 3,4,5-trisphosphate and Btk are implicated in the B cell receptor-induced PLC-γ signaling (Scharenberg and Kinet, 1998). It has been shown that JAKs are phosphorylated on multiple tyrosine residues after stimulation by cytokines and in turn serve as docking sites for other signal-transducing proteins containing SH2 domains, such as CIS, JAB, and SHC (Yoshimura et al., 1995; Endo et al., 1997; Giordano et al., 1997). It is possible that JAK can also serve as a docking site for SH2-containing PLCγ2 in the NCI-H292 cells. The fact that JAK1 and JAK2 acted upstream of PLCγ2 was further demonstrated by the following findings. First, the tyrosine phosphorylations of JAK1 and JAK2 were seen after treatment with IFN-γ but not TPA. Second, the IFN-γ–induced tyrosine phosphorylations of JAK1 and JAK2 were inhibited by the JAK inhibitor AG 490 but not by the PI-PLC inhibitor U73122. Third, the tyrosine phosphorylation of PLCγ by IFN-γ was inhibited in a dose-dependent manner by the JAK inhibitor AG 490. Fourth, using immunoprecipitation with either anti-JAK1 or anti-JAK2 antibody, followed by Western blotting with the anti-PLCγ antibody, direct associations between JAK1 and PLCγ or JAK2 and PLCγ were shown to increase after IFN-γ treatment. These results showed that both the JAK1/2/PLCγ2/PKCα/c-Src/STAT1 and JAK1/2/STAT1 pathways function together in the IFN-γ–mediated induction of ICAM-1 expression in the NCI-H292 cells.

In summary, the signaling pathways involved in the IFN-γ–induced ICAM-1 expression in the NCI-H292 cells have been further explored in this study. After JAK1and JAK2 are activated by IFN-γ, they can induce the STAT1α phosphorylation either directly or indirectly through PLCγ2, PKCα, and c-Src, which is followed by the initiation of ICAM-1 expression. A schematic representation of the involvements of these two pathways in the IFN-γ–induced ICAM-1 expression in the NCI-H292 cells is shown in Fig. 10.

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