Interferon-γ-induced Epithelial ICAM-1 Expression and Monocyte Adhesion

IN Volvement of Protein Kinase C-Dependent c-Src Tyrosine Kinase Activation Pathway

Received for publication, October 13, 2001, and in revised form, December 6, 2001
Published, JBC Papers in Press, December 20, 2001, DOI 10.1074/jbc.M109924200

Yu-Jen Chang, Michael J. Holtzman†, and Ching-Chow Chen§

From the Department of Pharmacology, College of Medicine, National Taiwan University, Taipei 10018, Taiwan and the Department of Medicine and Cell Biology, Washington University, School of Medicine, St. Louis, Missouri 63110

Interferon-γ (IFN-γ) induced intercellular adhesion molecule-1 (ICAM-1) expression in human NCI-H292 epithelial cells, as shown by enzyme-linked immunosorbent assay and immunofluorescence staining. The enhanced ICAM-1 expression resulted in increased adhesion of U937 cells to NCI-H292 cells. Tyrosine kinase inhibitors (genistein or herbimycin), Src family inhibitor (PP2), or a phosphatidylinositol-phospholipase C inhibitor (U73122) attenuated the IFN-γ-induced ICAM-1 expression. Protein kinase C (PKC) inhibitors (staurosporine or Ro 31-8220) also inhibited IFN-γ-induced response. 12-O-Tetradecanoylphorbol-13-acetate (TPA), a PKC activator, stimulated ICAM-1 expression; this effect was inhibited by tyrosine kinase or Src inhibitor. ICAM-1 promoter activity was enhanced by IFN-γ and TPA in cells transfected with pIC339-Luc, containing the downstream NF-κB and γ-activated site (GAS) sites, but not in cells transfected with GAS-deletion mutant, pIC135 (JAP2). Electrophoretic gel mobility shift assay demonstrated that GAS-binding complexes in IFN-γ-stimulated cells contained STAT1α. The IFN-γ-induced ICAM-1 promoter activity was inhibited by tyrosine kinase inhibitors, a phosphatidylinositol-phospholipase C inhibitor, or PKC inhibitors, and the TPA-induced ICAM-1 promoter activity was also inhibited by tyrosine kinase inhibitors. Cotransfection with a PLC-γ2 mutant inhibited IFN-γ but not TPA-induced ICAM-1 promoter activity. However, cotransfection with dominant negative mutants of PKCs or c-Src inhibited both IFN-γ- and TPA-induced ICAM-1 promoter activity. The ICAM-1 promoter activity was stimulated by cotransfection with wild type PLC-γ2, PKCo, c-Src, JAK1, or STAT1. An immunocomplex kinase assay showed that both IFN-γ and TPA activated c-Src and Lyn activities and that these effects were inhibited by staurosporine and herbimycin. Thus, in NCI-H292 epithelial cells, IFN-γ activates PLC-γ2 via an upstream tyrosine kinase to induce activation of PKC-α and c-Src or Lyn, resulting in activation of STAT1α, and GAS in the ICAM-1 promoter, followed by initiation of ICAM-1 expression and monocyte adhesion.

Cell adhesion mediated by specific cell-surface molecules is important in establishing and maintaining inflammation, bronchial asthma, rheumatoid arthritis, atopic dermatitis, tumor metastasis, and allograft rejection (1–3). It elicits the recruitment of leukocytes from the circulation into the extravascular space, a process involving several steps (4, 5). The initial interaction between leukocytes and the endothelium appears 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), an 80–114-kDa inducible surface glycoprotein belonging to the immunoglobulin superfamily, is involved in a wide range of inflammatory and immune responses (6).

During inflammation, ICAM-1 binds to two integrins belonging to the β2 subfamily, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), both expressed by leukocytes and that promote the adhesion and transendothelial migration of leukocytes (7, 8). Similar processes govern leukocyte adhesion to lung airway epithelial cells and may contribute to the damage to these cells seen in asthma (9). ICAM-1 can be up-regulated by bacterial lipopolysaccharide, phorbol esters, platelet-derived growth factor, and inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and interferon-γ (IFN-γ) (10–13). This regulation occurs at the transcriptional level and involves the binding of specific homo- or heterodimeric complexes to target DNA sequences located along the ICAM-1 promoter (14–16). The ICAM-1 promoter has been identified and shown to contain two TATA boxes, two NF-κB sites, two AP-1 sites, two AP-2 sites, two glucocorticoid receptor element sites, and one IFN-γ-activated (GAS) site (17–19).

IFN-γ, a lymphocyte effector molecule produced by T cells and natural killer cells, plays an important role in macrophage activation and is implicated in the pathogenesis of a number of inflammatory diseases of infectious or presumed autoimmune origin (20). The intracellular signaling of IFN-γ has been shown to act through the JAK/STAT pathway in several different tissues (21, 22), and the mechanism of IFN-γ-mediated gene induction has been elucidated (21–23). Following IFN-γ binding, the IFN-γ receptor oligomerizes and brings the Janus kinases (JAKs) into juxtaposition, leading to their cross-phos-
phorylation and activation. The JAKs in turn phosphorylate tyrosine residues on receptors that lack intrinsic kinase activity, thereby providing the docking site for downstream signaling proteins. The signal transducers and activators of transcription (STATs), which are recruited to the JAK-receptor complex via their Src homology 2 (SH2) domain, are phosphorylated on a conserved tyrosine residue in the C-terminal region. This phosphorylation results in STAT dimerization and forms a protein complex first identified as γ-activated factor (GAF). The GAF complex then translocates to the nucleus, where it binds to the specific promoter DNA sequence, GAS, thereby affecting the expression of multiple target genes, such as ICAM-1 (24, 25). In addition to the JAK-STAT pathway, other signaling components are involved; these include phospholipase D (PLD)-dependent arachidonic acid release to activate protein kinase C (PKC) in endothelial cells (26, 27), PC-PLC and PKC activation to induce inducible nitric-oxide synthase expression in J774 macrophages (28), or PKC activation to induce ICAM-1 in endothelial cells (29). The intracellular signaling pathways by which IFN-γ causes ICAM-1 expression are not well understood but have been suggested to be involved; these include tyrosine kinase activation (e.g. JAKs and their downstream transcriptional factors, the STATs) (25), PKC, and intracellular Ca<sup>2+</sup> concentration (29, 32). However, the relationship between these pathways is unknown. In the present study, we explored the intracellular signaling pathway involved in IFN-γ-induced ICAM-1 expression in a human alveolar epithelial cell line, NCI-H292. The results show that IFN-γ activates phosphatidylinositol-phospholipase C-γ2 (PI-PLC-γ2), resulting in the activation of PKCa, c-Src or Lyn, STAT1α, and GAS in the ICAM-1 promoter, followed by ICAM-1 expression and monocyte adhesion.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal anti-human ICAM-1 antibody (11C81) and recombinant human IFN-γ were purchased from R & D Systems (Minneapolis, MN). Rabbit polyclonal antibodies specific for p65, p91 (STAT-1α), c-Src, Lck, Lyn, or Fyn were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human IL-4 (R & D Systems, Minneapolis, MN) and dextran sulfate sodium salt (Sigma) were used as negative controls. Poly(dI-dC) (3000 U/mg) was from PerkinElmer Life Sciences (Boston, MA). Rabbit polyclonal antibodies specific for p91 (STAT1-α), c-Src, Lck, Lyn, or Fyn were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was purchased from Promega. mouse monoclonal anti-human ICAM-1 antibody, NCI-H292, was obtained from Dr. Rothman (Department of Microbiology, College of Physicians and Surgeons of Columbia University) and Dr. Levy (Department of Allergy and Immunology, San Diego, CA), respectively. The JAK1 dominant negative (PKC-γR) and c-Src (K295M),2 JAK1, JAK2, STAT1 (Y701M) or STAT3 mutant, or the empty vector. To determine the contribution of ICAM-1 to IFN-γ-induced monocytes adherence, NCI cells were treated with anti-ICAM-1 antibody at a concentration of 10 µg/ml for 30 min at 37 °C before the BACECF-labeled U937 cells were added.

**Quantification of ICAM-1 Expression**—NCI-H292 cells, grown in 6-well plates, were transfected with the human ICAM-1 promoter-firefly luciferase constructs, pIC1352, pIC339, pIC135, or pIC135/ΔAP2, using Transfect™-50, as described previously (35). The following day, cells were exposed to 10 ng/ml IFN-γ or 1 µM TPA for 5 h; cell extracts were then prepared and the luciferase and β-galactosidase activities measured, and 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 and the PLC-γ2, PKCa, c-Src, JAK1, or STAT wild type plasmids, or the empty vector using SuperFect Transfection Reagent (Qiagen). Briefly, wild type 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 600 µl of serum-free RPMI 1640 medium. After 10 min of incubation at room temperature, 300 µl of serum-free RPMI 1640 medium was then applied to the cells. Eight hours later, 100 µl of FCS was added, and the cells were grown in medium containing 10% FCS. On the following day, the cell extracts were prepared. The luciferase (Promega) and β-galactosidase activities were measured, and the luciferase activity of each well was normalized to β-galactosidase activity. In PLC-γ2 (wt), PKCa (wt), c-Src(wt) and dominant negative c-Src mutant (K295M),2 STAT1(Y701M) mutant experiments, the wild types (1.5 µg) and dominant negative mutants (2.0 µg) or the empty vector were cotransfected.

**Preparation of Nuclear Extracts and the Electrophoretic Mobility Shift Assay (EMSA)**—Cells were incubated for 10 min, 1 h, or 24 h with IFN-γ and then nuclear extracts were prepared as described previously (35). Oligonucleotides corresponding to the GAS consensus sequence in the human ICAM-1 promoter (5’-CGGGTTTCCGGGAAAGCAGC-3’) were synthesized, annealed, and end-labeled with γ-[32P]ATP using T4 polynucleotide kinase, and EMSA was performed as described previously (35). The DNA-protein complexes were separated on a 4.5% polyacrylamide gel.

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In Vitro c-Src and Lyn Activity Assay—After treatment with IFN-γ or TPA for 10, 30, or 60 min, with or without pretreatment with various inhibitors for 30 min at 37 °C, the cells were rapidly washed with PBS and then 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 μM/ml of leupeptin, 20 μg/ml of aprotinin, 1 mM Na3VO4, 1% Triton X-100). A sample of total cell extract containing 50 μg of protein was incubated for 1 h at 4 °C with 0.5 μg of anti-c-Src or anti-Lyn antibody, and the antibody-bound protein was collected using protein A-Sepharose CL-4B beads (Sigma). The beads were then washed three times with lysis buffer without Triton X-100 and incubated for 30 min at 30 °C in 20 μl of kinase reaction mixture (20 mM HEPES, pH 7.4, 5 mM MgCl2, 5 mM MnCl2, 0.1 mM Na3VO4, 1 mM dithiothreitol, 5 μg of enolase, and 10 μM [γ-32P]ATP). The reaction was then stopped by addition of 20 μl of Laemmli buffer and the proteins subjected to 10% SDS-PAGE; the, phosphorylated enolase was visualized by autoradiography. Quantitative data were obtained using a densitometer with ImageQuant software.

RESULTS

IFN-γ Induces Cell Surface ICAM-1 Expression in, and U937 Adhesion to, NCI-H292 Cells—When NCI-H292 cells were treated with 100 ng/ml IL-1β, TNF-α, or IFN-γ, with 1 μg/ml lipopolysaccharide or with 1 μM TPA, only IFN-γ and TPA stimulated ICAM-1 expression, as measured by ELISA (data not shown). IFN-γ induced ICAM-1 expression in a concentration- and time-dependent manner (Fig. 1). With an exposure period of 18 h, maximal ICAM-1 expression was seen using 10 ng/ml IFN-γ (Fig. 1A), and when cells were treated with 10 ng/ml IFN-γ for various times, ICAM-1 expression was significantly increased after 5 h and was maximal at 18 h, remaining at this level for at least 40 h (Fig. 1B). Induction of ICAM-1 by IFN-γ was also demonstrated by immunofluorescence staining.

As shown in Fig. 2, ICAM-1 was not seen in the basal state (Fig. 2B) but appeared on the cell surface following treatment with IFN-γ or TPA (Fig. 2, D and F). In the following ICAM-1 expression experiments, the cells were treated with 10 ng/ml IFN-γ for 18 h. Under these conditions, both the transcriptional and translational inhibitors, actinomycin and cycloheximide, inhibited the IFN-γ-induced ICAM-1 expression (data not shown).

To determine whether IFN-γ- or TPA-induced monocytes adherence to NCI-H292 cells correlated with cell-surface ICAM-1 expression, we analyzed U937 cell adhesion to NCI-H292 cells (Fig. 3). After 18 h of treatment with IFN-γ, adherence was increased by ~11-fold, and anti-ICAM-1 antibody reduced adherence to below the basal level, showing that IFN-γ-induced U937 cell adhesion to NCI-H292 cells was because of ICAM-1 expression. Similarly, 18 h of treatment with TPA resulted in a 10-fold increase in adherence, which was inhibited by 70% by anti-ICAM-1 antibody, indicating a role of ICAM-1 in TPA-induced U937 cell adhesion.

Inhibitory Effect of Tyrosine Kinase, PI-PLC, or PKC Inhibitors on IFN-γ-induced ICAM-1 Expression—To study the intracellular signaling pathway involved in IFN-γ-induced ICAM-1 expression, NCI-H292 cells were pretreated for 30 min with the tyrosine kinase inhibitors, genistein and herbimycin. Under these conditions, IFN-γ-induced ICAM-1 expression was inhibited 34, 45, or 47%, respectively, by 30 or 100 μM genistein or 1 μM herbimycin (Fig. 4A). When cells were pretreated with the PI-PLC inhibitor, U73122, at 10 or 30 μM, IFN-γ-induced ICAM-1 expression was inhibited by 31 or 50%, respectively, whereas 30 μM U73343 (an inactive analogue of U73122), 100 μM D609 (a PC-PLC inhibitor), or propranolol (a phosphatidate phosphohydrolase inhibitor) had no effect (Fig. 4B).
Because IFN-γ-induced ICAM-1 expression was inhibited by U73122, indicating involvement of the PI-PLC pathway, which increases diacylglycerol levels and then activates PKC, the PKC inhibitors, staurosporine and Ro 31-8220, were used to determine whether PKC was involved in IFN-γ-induced ICAM-1 expression. Following pretreatment of cells with 10, 30, or 100 nM staurosporine or with 0.1, 0.3, or 1 μM Ro 31-8220, IFN-γ-induced ICAM-1 expression was inhibited in a dose-dependent manner (Fig. 4C).

Because PKC had been shown to be involved, the effect of direct TPA-mediated PKC activation on ICAM-1 expression was examined. TPA (1 μM) also induced a time-dependent increase in ICAM-1 expression, which was significant at 4.5 h and maximal at 16 h and then declined after 20 h (Fig. 5A). Induction of ICAM-1 expression by TPA was also demonstrated by immunofluorescence staining (Fig. 2F) and U937 cell adhesion (Fig. 3). When cells were pretreated with 100 nM staurosporine or 10, 30, and 100 μM genistein, or 1 μM herbimycin, TPA-induced ICAM-1 expression was inhibited by 69, or 40, 62, 80, or 52%, respectively (Fig. 5B). The specific Src inhibitor, PP2 (56), inhibited IFN-γ- or TPA-induced ICAM-1 expression dose-dependently (Fig. 5C).

**Induction of ICAM-1 Promoter Activity by IFN-γ and the Inhibitory Effect of Genistein, Herbimycin, U73122, Staurosporine, Ro 31-8220, PLC-γ-2 Mutant, or Dominant Negative Mutants of PKC-α or c-Src.**

To study further the involvement of the PI-PLC-dependent PKC pathway in IFN-γ-induced ICAM-1 expression, transient transfection was performed using the human ICAM-1 promoter-luciferase constructs, pIC1352 (−1352/+1), which contains full-length human ICAM-1 promoter; pIC339 (−339/+1), which contains the downstream NF-κB and GAS sites in the ICAM-1 promoter; pIC135 (−135/+1), which contains the GAS site but not the NF-κB site; and pIC135(ΔAP2), which does not contain either the NF-κB or the GAS site but contains the proximal TATA box site. Treatment with 10 ng/ml IFN-γ or 1 μM TPA led to a 4.1- or 5.7-fold increase, respectively, using pIC1352, and a 3.7- or 5.3-fold increase, respectively, using pIC339, and a 3.5- or 3.7-fold increase, respectively, using pIC135. However, using pIC135(ΔAP2), IFN-γ, or TPA treatment only resulted in a 1.4- or 1.2-fold increase, respectively, in ICAM-1 promoter activity (Fig. 6). These results indicate that the GAS cis-acting element is responsible for mediating both IFN-γ- and TPA-induced ICAM-1 expression in NCI-H292 cells and that NF-κB may be involved in TPA- but not IFN-γ-induced ICAM-1 expression.

By using pIC339, the induction of ICAM-1 promoter activity mediated by IFN-γ was attenuated by genistein, herbimycin, U73122, staurosporine, or Ro 31-8220, but not by PDTC, whereas that induced by TPA was inhibited by genistein, herbimycin, staurosporine, or PDTC (Fig. 7A). In cotransfection experiments, the induction of ICAM-1 promoter activity by IFN-γ was inhibited by the mutant PLC-γ2 SH2(N) or by the dominant negative PKC-α/KR or c-Src(KM) mutants, whereas that induced by TPA was inhibited by the dominant negative PKC-α/KR or c-Src(KM) mutants but not by the mutant PLC-γ2 SH2(N) (Fig. 7B). By using pIC135, the IFN-γ-induced...
ICAM-1 promoter activity was also inhibited by cotransfection with the above three mutants. Furthermore, induction of ICAM-1 promoter activity by IFN-γ was inhibited by cotransfection with wild type PLCα2 (wt), PKCα (wt), c-Src (wt), JAK1 (wt) or STAT1α (Y701M) but not by STAT3 (Fig. 8A). The ICAM-1 promoter activity was enhanced by cotransfection with wild type PLC-γ2 (wt) or PKCα (wt) was blocked by cotransfection with dominant negative c-Src (KM) or STAT1 (Y701M) mutant and that by wild type c-Src (wt) was blocked by cotransfection with dominant negative c-Src (Y701M) mutant (Fig. 8C).

IFN-γ and TPA Induce STAT1α Binding to the GAS Site of the ICAM-1 Promoter—The ICAM-1 promoter contains a complex array of transactivating binding sites. To determine whether the GAS element was involved in ICAM-1 gene transcription following IFN-γ stimulation, GAF complex formation was examined by EMSA. Although no GAF-GAS DNA-protein binding was seen in nonstimulated cells, IFN-γ rapidly (10 min) stimulated GAF-GAS DNA-protein binding, with similar activation being seen after 1 and 24 h (Fig. 9A). TPA resulted in a similar pattern of GAF-GAS DNA-protein binding (data not shown). In subsequent EMSA experiments, cells were treated with IFN-γ for 1 h. To identify the specific subunits involved in the formation of the GAF-GAS complex after IFN-γ stimulation, supershift assays were performed in the presence of antibodies specific for STAT-1α (p91) or p65 (NF-κB). As shown in Fig. 9, B and C, incubation of nuclear extracts with anti-STAT-1α antibody induced attenuation of GAF-GAS DNA-protein binding (Fig. 9, B and C, lane 3), but no shift or attenuation occurred in the presence of anti-p65 antibody (Fig. 9B, lane 4), indicating that the GAF complex induced by IFN-γ contained STAT1α. Excess cold GAS probe blocked the GAF-GAS DNA-protein binding (Fig. 9C, lane 4).

Induction of c-Src and Lyn Activation by IFN-γ or TPA and the Inhibitory Effect of U73122, Staurosporine, or Herbimycin—Because IFN-γ or TPA-induced ICAM-1 expression was inhibited by genistein, herbimycin, and PP2 (Fig. 4A and Fig. 5, B and C), and the induced ICAM-1 promoter activity was inhibited by a dominant negative c-Src (KM) mutant (Fig. 7B), these results indicated that c-Src was involved downstream of PKC in the induction of ICAM-1 expression. Western blot analysis using antibodies against the Src family members, c-Src, Lck, Lyn, or Fyn, showed that c-Src and Lyn were expressed substantially in NCI-H292 cells (data not shown). To determine whether IFN-γ or TPA induced activation of these two tyrosine kinases, c-Src and Lyn were isolated by immunoprecipitation using anti-c-Src or anti-Lyn antibody and tested for in vitro activation by TPA.
kinase activity, using enolase as substrate. When cells were treated for 10, 30, or 60 min with 10 ng/ml IFN-γ/H9253 or 1 μM TPA, IFN-γ/H9253 induced c-Src and Lyn activation that was significant at 10 min and maximal at 60 min (Fig. 10A), although TPA also induced c-Src and Lyn activation, the kinetics were different, with activation being significant at 10 min, maximal at 30 min, and declining after 60 min (Fig. 10B). The activation of c-Src and Lyn induced by IFN-γ/H9253 was inhibited by U73122, staurosporine, and herbimycin and that induced by TPA was inhibited by staurosporine and herbimycin (Fig. 11). These inhibitors alone had no effect on basal c-Src or Lyn activity (data not shown).

**DISCUSSION**

In the present study, we have shown that IFN-γ induced ICAM-1 expression in the plasma membrane of NCI-H292 epithelial cells, and this resulted in increased adhesion of U937 cells. The transcriptional factor binding site, GAS, appears to be essential for the enhanced ICAM-1 expression seen after exposure to IFN-γ in human monocytes (36). To test whether the NF-κB or GAS site was involved in IFN-γ-induced ICAM-1 promoter activity in NCI-H292 cells, we used different deletion mutants of the ICAM-1 promoter-Luc construct, pIC1352, pIC339, pIC135, or pIC135 (ΔAP2). The results showed that the GAS site was essential for both IFN-γ- and TPA-induced ICAM-1 promoter activity and that the downstream NF-κB site...
PLC-γ2-dependent PKCα and c-Src Activation in IFN-γ-induced ICAM-1

reported to be critical for TNF-α and IL-1β to induce ICAM-1 expression (33, 34) was only involved in TPA-induced ICAM-1 promoter activity (Fig. 6). Experiments using PDTC, an NF-κB inhibitor, further supported this notion, because PDTC inhibited TPA-, but not IFN-γ-, induced ICAM-1 promoter activity. In the EMSA, IFN-γ increased GAF-GAS DNA-protein binding, indicating that the GAS site in the ICAM-1 promoter was involved in IFN-γ-mediated ICAM-1 induction. GAF, the protein complex binding to GAS sequences in IFN-γ-induced ICAM-1 expression, increased GAF-GAS DNA-protein binding, indicating the possible involvement of PLC-γ2 in IFN-γ-induced ICAM-1 expression in these cells. This was further confirmed by the result that the dominant negative PKC-α mutant, PKC-α (K/R), inhibited IFN-γ-induced ICAM-1 promoter activity (Fig. 7B), and overexpression of wild type PKC-α (wt) enhanced ICAM-1 promoter activity (Fig. 8B). PKC is activated by the physiological activator, diacylglycerol, which can be generated either directly, by the action of PLC, or indirectly, by a pathway involving the production of phosphatidic acid by PLD, followed by a dephosphorylation reaction catalyzed by phosphatidate phosphohydrolase. Normally, the PLC involved in the production of diacylglycerol is PI-PLC, but PC-PLC can also be involved (37, 38). The PI-PLC inhibitor, U73122, inhibited IFN-γ-induced ICAM-1 expression, whereas the PC-PLC inhibitor, D609, the phosphatidate phosphohydrolase inhibitor, propranolol, and the inactive U73122 analogue, U73343, did not. Tyrosine kinase inhibitors blocked IFN-γ-induced ICAM-1 expression, indicating that the PI-PLC involved might be PLC-γ, because PLC-γ contains an SH2 domain used to link phosphotyrosine-containing sequences in a receptor protein or in cytoplasmic protein tyrosine kinase to PI hydrolysis (39). In the present study, we used the PLC-γ2 N-terminal SH2 (SH2(N)) mutant (40) to further determine the role of PLC-γ and we found that it inhibited IFN-γ- but not TPA-induced ICAM-1 promoter activity, indicating the possible involvement of PLC-γ2 in IFN-γ-induced ICAM-1 expression in NCI-H292 cells. This was further confirmed by the result that cotransfection with wild type PLC-γ2 (wt) increased ICAM-1 promoter activity (Fig. 8B). Thus, IFN-γ acts through the PI-PLC-γ2 pathway, but not through the PC-PLC or PC-PLD pathway, to induce PKC activation in NCI-H292 cells. Although IFN-γ has been reported to act mainly via JAK-STAT pathway to regulate most gene expression, in some cases IFN-γ acts via PI-PLCβ to induce Ca2+ signals in neutrophils (41), and it acts via PC-PLC to induce PKC activation in macrophages (28) or via PC-PLD to

FIG. 9. Kinetics of IFN-γ-induced GAF-GAS DNA-protein binding in NCI-H292 epithelial cells. A, cells were treated for 10 min, 1 h, or 24 h with 10 ng/ml IFN-γ, and then nuclear extracts were prepared and tested using the GAS oligonucleotide probe to measure the DNA-protein binding activity by EMSA as under “Experimental Procedures.” B, supershift assays were performed using 2 μg of the indicated antibodies as described under “Experimental Procedures.” C, excess cold GAS probe was used as competitor as described under “Experimental Procedures.” NS, nonspecific binding.

FIG. 10. Time-dependent activation of c-Src or Lyn tyrosine kinase activity by IFN-γ or TPA in NCI-H292 cells. Cells were treated for 10, 30, or 60 min with 10 ng/ml IFN-γ (A) or with 1 μM TPA (B), and then whole cell lysates were immunoprecipitated (IP) with anti-c-Src or anti-Lyn antibody, followed by autoradiography of phosphorylated enolase as described under “Experimental Procedures.” The amount of c-Src or Lyn in immunoprecipitates was determined by Western blot (WB) using anti-c-Src or anti-Lyn antibody.
determined by Western blot (Fig. 8). The induction of ICAM-1 promoter activity by IFN-γ and TPA is seen, and this effect is inhibited by inhibitors of PKC and c-Src, indicating that the phosphorylation of STAT1 at Tyr-701 by IFN-γ and TPA is seen, and this effect is inhibited by inhibitors of PKC and c-Src, indicating that the phosphorylation of STAT1 at Tyr-701 by IFN-γ and TPA is seen, and this effect is inhibited by inhibitors of PKC and c-Src, indicating that the phosphorylation of STAT1 at Tyr-701 by IFN-γ and TPA is seen, and this effect is inhibited by inhibitors of PKC and c-Src, indicating that the phosphorylation of STAT1 at Tyr-701 by IFN-γ and TPA is seen, and this effect is inhibited by inhibitors of PKC and c-Src. Thus, c-Src can directly phosphorylate STAT1 in NCI-H292 cells. JAK1 is also involved in IFN-γ-induced ICAM-1 expression (Fig. 8, A and B). However, its role in c-Src-induced STAT1 phosphorylation is unknown and is currently under investigation. The IFN-γ-induced ICAM-1 expression in NCI-H292 cells was not affected by the mitogen-activated protein kinase inhibitor, PD98059, or the p38 inhibitor, SB203580 (data not shown), excluding the involvement of p44/42 mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor, PD98059, or the p38 inhibitor, SB203580 (data not shown), excluding the involvement of p44/42 mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor, PD98059, or the p38 inhibitor, SB203580 (data not shown), excluding the involvement of p44/42 mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor, PD98059, or the p38 inhibitor, SB203580 (data not shown), excluding the involvement of p44/42 mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor, PD98059, or the p38 inhibitor, SB203580 (data not shown), excluding the involvement of p44/42 mitogen-activated protein kinase/extracellular signal-regulated kinase 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