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Interferonγ Activation of Raf-1 Is Jak1-dependent and p21^{ras}-independent*

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Signal transduction through the interferonγ (IFNγ) receptor involves the formation of a ligand-dependent multimolecular association of receptor chains (α and β), Janus tyrosine kinases (Jak1 and Jak2), and the transcription factor (signal transducers and activators of transcription 1α (STAT1α)) in addition to activation of mitogen-activated protein kinases (MAPK). Interactions between components of the Jak/STAT cascade and the p21^{ras}/Raf-1/MAPK cascade are unexplored. Treatment of HeLa cells with IFNγ resulted in the rapid and transient activation of Raf-1 and MAPK. Parallel activation of cells resulted in essentially no enhancement of p21^{ras} activation despite marked enhancement after treatment with epidermal growth factor. In HeLa (E1C3) and fibrosarcoma (U4A) cell lines, both of which are deficient in Jak1 kinase, Raf-1 activation by IFNγ was absent. Reconstitution of Raf-1 activity was observed only with kinase active Jak1 in both cell lines. In COS cells, transient expression of wild type or kinase-inactive Jak1 coimmunoprecipitated with Raf-1, but activation of Raf-1 activity was only observed in cells expressing kinase-active Jak1. These observations suggest that a kinase-active Jak1 is required for IFNγ activation of Raf-1 that is p21^{ras}-independent.

Interferonγ transcriptionally induces the expression of early response genes through the activation of the Janus tyrosine kinases 1 and 2 (Jak) and the latent transcription factor, signal transducers and activators of transcription 1α (STAT1α)[1] (1, 2). This cascade is initiated by the ligand-induced tyrosine phosphorylation of the IFNγ receptor, Jak1, Jak2, and STAT1α (3). Tyrosine-phosphorylated STAT1α homodimerizes, translocates to the nucleus, and binds enhancers located in the promoters of IFNγ-sensitive early response genes. In addition to the tyrosine phosphorylation of components of this pathway, data suggest that serine/threonine (Ser/Thr) phosphorylation also plays a role in transcriptional regulation of these genes (4–6). Evidence for the role of Ser/Thr kinases in interferonγ-induced responses derive from studies showing a slow (60–120 min) activation of both MAP kinase (MAPK) and protein kinase C (7), differential modulation of the expression of early response genes by Ser/Thr phosphatase inhibitors (8), and a novel Ser/Thr kinase that mediates some of the antiproliferative effects of IFNγ (9). Therefore, both tyrosine and Ser/Thr kinase activity can regulate IFNγ-induced expression of cellular genes.

IFNγ primarily activates STAT1 as the main driving force for gene expression. Although it has been suggested that MAPK activation may drive serine phosphorylation of STAT1, the relationship, if any, between activation of Jaks and MAPK is undefined. MAPK activation occurs as a result of activation of both MEK, a dual specific kinase, and Raf-1, a Ser/Thr kinase. Moreover, tyrosine phosphorylation of Raf-1 appears to modulate enzymatic activity and subcellular localization (10). Whereas activation of Raf-1 by many growth factors and cytokines is p21^{ras}-dependent, evidence exists that Raf-1 activity can also be stimulated independent of its interaction with p21^{ras} (11). To explore the potential relationship between those two signal transduction cascades, we used cell lines that lack Jak1 activity. We go on to show that Jak1 is required for Raf-1 activation and that this activity occurs independent of p21^{ras}.

EXPERIMENTAL PROCEDURES

Cells—The parental HeLa and E1C3 Jak1-deficient cell lines have been described (12), as have the parental 2fTGH human fibrosarcoma cell line and the Jak1-deficient U4A cell line (13). The Jak1-deficient cells do not support IFNα or IFNγ-induced gene expression. Cells were maintained as adherent cultures in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone). For the MAPK, Raf-1, and p21^{ras} assays, cells were placed in 1% fetal bovine serum 48 h before the experiment. For the 24–36 h immediately before cytokine treatment, the cells were cultured in serum-free media.

Electrophoretic Mobility Shift Assay—After treatment with IFNγ, cells were solubilized with cold whole cell extraction buffer (1 mM $MgCl_2$, 20 mM Hepes, pH 7.0, 10 mM KCl, 300 mM NaCl, 0.5 mM dithiothreitol, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM vanadate, and 20% glycerol). DNA-binding proteins were assayed as described previously (14). Briefly, 10 μg of protein were incubated in binding buffer with the ^{32}P-labeled oligonucleotide probe consisting of the double-stranded IFNγ activation sequence (GAS) referred to as the gamma response region (GRR) (5′-AGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAG-3′) of the promoter of the *Fcgr1* gene (15). The sample was then applied to a 6% nondenaturing polyacrylamide gel to separate free probe from probe bound to protein.

Binding of ^{125}I-rIFNγ to Cells—rIFNγ was radiolabeled to high specific activity using Bolton-Hunter reagent as described (16). Cells were incubated with the radiolabeled IFNγ for 2 h at 4 °C and assayed as described previously (16).

IFNγR α and β Chain Gene Expression—RNA was isolated by the RNAzol method as per the manufacturer's instructions. cDNAs were prepared from the RNA by reverse transcriptase, and then the amount of DNA encoding for the α and β chain of the IFNγ receptor was determined by PCR using primers specific for each gene as described previously (17). The reverse transcription and PCR were performed with the GeneAmp RNA polymerase chain reaction kit (Perkin-Elmer) according to the manufacturer's protocol. The reaction was performed

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[1] The abbreviations used are: STAT, signal transducers and activators of transcription; IFN, interferon; rIFNγ, recombinant IFNγ; IFNγR, IRNγ receptor; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK kinase; GRR, gamma response region; Erk, extracellular-regulated kinase; EGF, epidermal growth factor.

in a DNA thermal cycler (Perkin-Elmer) for 30–35 cycles: 1 min of denaturation at 95 °C and 1 min of annealing and extension at 60 °C following 2 min of an initial denaturation step at 95 °C. The primers used for the detection of IFNγRβ are as follows: IFNγRα (5′ sense, 5′-GCAGAAGGAGTCTTACATGTGTGG-3′; 3′ antisense, 5′-CTCTCT-ATTGGAGTCAGATGGCTG-3′) and IFNγRβ (5′ sense, 5′-AATGTGA-CTGTCGGGCCTCCAGAA-3′; 3′ antisense, 5′-CTCTAAGATGGGCTG-AGTTGGGTC-3′). An aliquot (10 μl) of amplified products was applied to a 2% agarose gel electrophoresis and stained by ethidium bromide. To ensure whether polymerase chain reaction bands were specific for IFNγR, we performed southern hybridization with specific cDNAs for IFNγRα and β.

MAPK (Erk2/p42) Assay—MAPK assays were performed as described using an anti-Erk2 antibody (TR10) (kindly provided by Dr. Michael Weber, University of Virginia) (6).

Raf-1 Assay—Nontransfected cells were maintained in serum-free conditions for 24–36 h before the initiation of the assay. This was required for an optimal decrease in basal Raf-1 kinase activity. For cells that underwent transfection with Jak1 constructs, the time of starvation was limited to only 2 h, since optimal Jak1 expression occurred at about 14 h after transfection. Cells were then solubilized in lysis buffer (150 mM NaCl, 25 mM Hepes, pH 7.3, 1 mM sodium orthovanadate, 1% Triton-X, 0.5% octylglucoside, 0.03% deoxycholate, 0.02% SDS, protease inhibitors, and 0.5 mM dithiothreitol), and the assay was carried out as described (18). The lysate was incubated on ice for 10 min and centrifuged at 14,000 × *g* for 10 min, and the supernatant was incubated with anti-Raf-1 antibody (polyclonal; Santa Cruz Biotechnology) followed by incubation with protein G-Sepharose at 4 °C for 1 h. The immunoprecipitates were washed twice with lysis buffer, and the kinase reaction was carried out at 30 °C for 10 min in kinase buffer (0.2 mM ATP, 30 mM $MgCl_2$, 2 mM $MnCl_2$, 40 mM sodium β-glycerophosphate, 0.2 mM sodium orthovanadate, 2 μM okadaic acid, and 0.2% β-mercaptoethanol) with 1 μg of purified recombinant MEK1 added as substrate. When the assay was carried out without the addition of MEK1, there was no measurable increase in MAPK phosphorylation. After MEK1 activation, 15 μCi of [γ-^{32}P]ATP and 1 μg of kinase-defective (K52R) Erk was added as substrate for an additional 2 min. The reaction was terminated by the addition of sample buffer and boiled for 5 min, and the proteins were separated by SDS-polyacrylamide gel electrophoresis. The gel was then transferred to a polyvinylidene difluoride membrane on which the amount of radiolabeled K52R Erk was quantitated by a PhosphorImager. The amount of Raf-1 protein on the same membrane was determined by probing the membrane with mouse monoclonal anti-Raf-1 followed by ^{125}I-labeled goat anti-mouse IgG. Data are presented as -fold increase, as explained for the MAPK assay. In 2fTGH and U4A cells, a myc epitope-tagged Raf-1 (R89LRaf-1), mutated in the p21^{ras} binding site and containing a p21^{ras} membrane localization motif (CAA*X*), was used in a p21^{ras}-independent Raf-1 assay (10). Cells were transfected with R89LRaf-1 plasmid using DEAE dextran. Forty-eight h post-transfection, lysates were prepared from either untreated cells or cells incubated for 5 min with IFNγ. Cell extracts were prepared and incubated with monoclonal antibody 9E10, which recognizes the myc epitope tag (GGEQKLISEEDL). Immunoprecipitates were assayed for Raf-1 kinase activity as described above.

Ras Activation Assay—The activation state of p21^{ras} was performed as described elsewhere (19, 20). 100-mm dishes of HeLa cells were labeled with 1mCi/ml [^{32}P]orthophosphate for 4 h at 37 °C in phosphate-free Dulbecco's modified Eagle's medium. The cells were then treated with IFNγ or EGF for 15 min at 37 °C and lysed in 0.8 ml of lysis buffer (50 mM Hepes, 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM vanadate, and 1:10 dilution of culture supernatant of hybridoma-producing anti-Ras monoclonal antibody (Y13–259). After centrifugation at 10,000 × *g* at 4 °C for 10 min, 0.2 ml of detergent mixture (0.5% deoxycholate, 0.5% SDS, 0.5 M NaCl) and 20 μl of Protein G-Sepharose (Pharmacia Biotech Inc.) were added. The samples were incubated by rocking for 2 h at 4 °C and then washed extensively with wash buffer (50 mM Hepes, 0.5 M NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, and 0.05% SDS). The immunoprecipitates were eluted by heating at 85 °C for 3 min in the elution buffer (0.075 M KH_2PO_4, pH 3.4, 5 mM EDTA, 0.5 mM GTP, and 0.5 mM GDP). After a brief centrifugation, 8 μl of supernatant was spotted onto a polyethyleneimine-cellulose thin layer chromatography plate. After drying, the plates was washed briefly in water and air dried before developing in 0.65 M KH_2PO_4, pH 3.4, for 75 min. Radioactivity was visualized by autoradiography and quantitated by PhosphorImager.

Expression Vectors and Transfections—The full-length cytomegalovirus-driven wild type murine Jak1 cDNA (pRK5mJak1wt) and the kinase-negative form (ATP binding site K → E; pRK5mJak1kd) were constructed as described (21). Cytomegalovirus-driven pβ-galactosidase was used as a control. Transfection of cDNAs was performed by electroporation of cells (1×10^7 cells/ml) in phosphate-buffered saline (300 V/cm, 800 microfarads; Cell-Porator; Life Technologies). Immunoblotting for detection of expressed Jak1 showed that 9–14 h of incubation after electroporation gave maximal expression of Jak1 protein.

Tac Selection—Cytomegalovirus-driven interleukin 2 receptor (Tac) plasmid (provided by Dr. Bruce Howard, NIH, Bethesda, MD) was cotransfected with pRK5mJak1wt, pRK5mJak1kd, or pβ-galactosidase into the Jak1-negative mutant HeLa cells (E1C3) by electroporation. After 14 h of incubation in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, cells were incubated with magnetic beads (DynaBeads) conjugated with anti-Tac monoclonal antibody (provided by Dr. T. Waldmann, NIH, Bethesda) in medium S (phosphate-buffered saline supplemented with 4 mM EGTA, 100 μg/ml chondroitin sulfate, 10 mM Hepes, 0.5 M $MgCl_2$, 0.5 M $MgSO_4$, 8 mg/ml nonfat dry milk, and 8 mg/ml bovine serum albumin) for 15 min at 37 °C. After incubation, the mixture of cells and beads was treated with 0.25% trypsin, 0.5 mM EDTA for 2 min followed by the addition of trypsin inhibitor (Sigma). Tac-positive cells that bound to magnetic beads were separated from Tac-negative cells by magnetic field. This separation procedure was repeated three times. Selected Tac-positive cells were washed extensively by Dulbecco's modified Eagle's medium and used for further experiments.

Association of p21^{ras} with Raf-1—1D4 cells were solubilized as described above for the Raf-1 assay, and the cell lysates were immunoprecipitated with an antibody against p21^{ras} (Quality Biotech Inc., Camden, NJ) (22). The immunoprecipitates were then analyzed for Raf-1 activity and were expressed as -fold increase for each experiment, where original data represent fluorescent readings from a PhosphorImager.

Statistical Analysis—Each experiment was done three or four times independently, and data presented are the mean ± S.E. normalized to the intra-experimental control (**, $p < 0.01$; *, $p < 0.05$), except for Fig. 7 in which data of different treatment groups were pooled. Statistical significance was calculated by paired or unpaired *t* test.

RESULTS

Jak1 is required for IFNγ activation in E1C3 cells (12) (Fig. 1*A*) as measured by the inability of IFNγ to activate STAT1-dependent DNA binding to a GAS-like element referred to as the GRR, which is located in the promoter of the high affinity Fcγ receptor I gene (15) (Fig. 1*A*, *lanes 6–10*). In contrast, parental HeLa cells (1D4) respond to IFNγ by a rapid and intense activation of DNA binding activity (Fig. 1, *lanes 1–5*). Since the lack of Tyk2 was shown to affect binding of IFNα to its receptor (23), we measured the ability of radiolabeled IFNγ to bind to its receptor on the parental and E1C3 HeLa cells. The binding of ^{125}I-rIFNγ was similar in both the parental and Jak1-deficient cell lines (Fig. 1*B*). Both the maximal number of receptors (approximately 1,400/cell) and the concentration at half-saturation (2×10^{-10} M) were identical and consistent with the absence of any alteration in the affinity of IFNγ for the mutant cells. Since binding of IFNγ measures essentially only the interaction between the ligand and the α chain of the IFNγ receptor, intact binding parameters may exist, yet there may be no β chain present and thereby no signaling through the receptor (24). Moreover, the presence or absence of the β chain has not been demonstrated in the E1C3 cells. To assure that there was equal expression of both the α and β chain of the IFNγ receptor, we performed reverse transcriptase polymerase chain reaction amplification of RNA from both cell types. Both the parental and Jak1-deficient cells expressed α and β chain genes (Fig. 1*C*). Therefore, the lack of signal transduction through the IFNγ receptor in the Jak1-deficient cells was not the result of alterations in the receptor (17).

Increased enzymatic activity of Raf-1 is the primary stimulus for MEK activation, which is responsible for the phosphorylation of MAPK. Although HeLa cells demonstrated MAPK activation in response to IFNγ, no MAPK activation was measurable in the E1C3 cells lacking Jak1 (data not shown). We

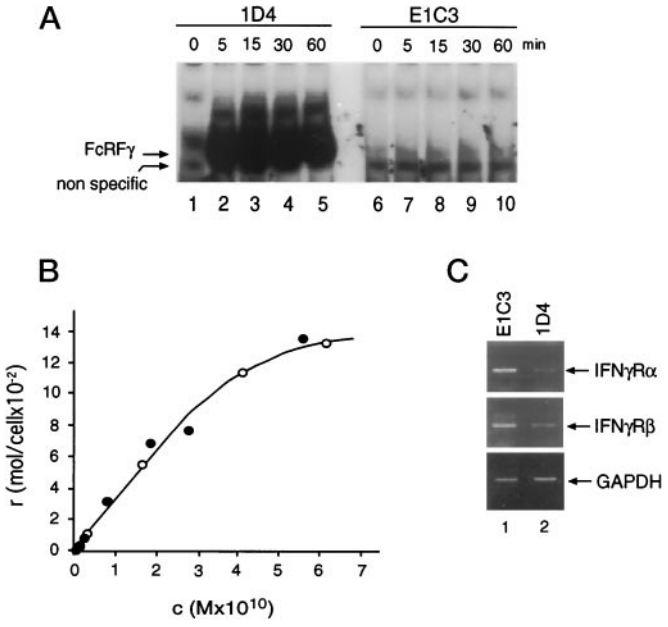

FIG. 1. **Interferonγ signal transduction in HeLa and ID4 cells.** *A*, incubation of Jak1-deficient HeLa cells with IFNγ fails to stimulate GRR binding activity. Parental (ID4) (*lanes 1–5*) and Jak1-deficient HeLa cells (E1C3) (*lanes 6–10*) were treated with IFNγ (10 ng/ml) for the indicated times, and GRR binding activity was assessed by electrophoretic mobility shift assay. *FcRF*γ, IFNγ-induced GRR binding activity. *B*, saturation binding of ^{125}I-IFNγ to parental HeLa (1D4) and Jak1-negative cells (E1C3). Cells were incubated with increasing amounts of ^{125}I-rIFNγ, and the amount of bound and free IFNγ was determined. *r*, IFNγ specifically bound in molecules/cell; *c*, concentration of unbound IFNγ. *Open circles*, 1D4; *closed circles*, E1C3. *C*, messenger RNA expression of α (IFNγRα) and β (IFNγRβ) chains of the IFNγ receptor. Reverse transcriptase-polymerase chain reaction analysis was performed using specific primers for α and β chains of IFNγ receptor. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control.

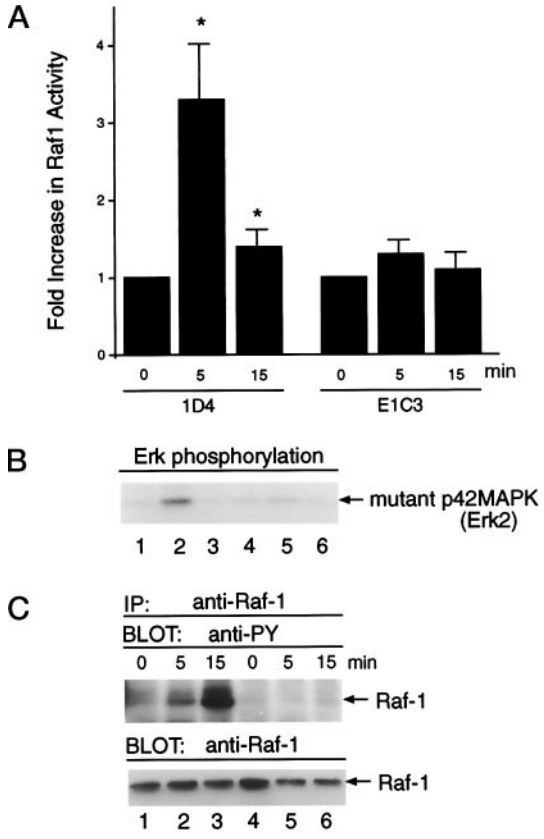

FIG. 2. **Activation of Raf-1 kinase by IFNγ.** Parental cells (1D4) (*lanes 1–3*) or Jak1-deficient cells (*lanes 4–6*) were treated with IFNγ, and the cell lysates were assayed for Raf-1 kinase activity. The immunoprecipitates were also analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. *A*, the *bar graph* presents the data as -fold increase of normalized Raf-1 activity over the value of untreated control (zero time point). *B*, the *panel* represents the phosphorylation of mutated Erk2 as a substrate of MEK1 in the kinase reaction. *Lanes 1* and *4*, *2* and *5*, and *3* and *6* represent 0, 5, and 15 min, respectively. *C*, the *upper panel* represents the membrane probed with anti-phosphotyrosine (*anti-PY*). The *lower panel* represents the membrane reprobed with anti-Raf-1. The *asterisks* represent differences at a $p < 0.05$ compared with control. *IP*, immunoprecipitated.

therefore evaluated whether IFNγ could enhance Raf-1 activity and whether this was Jak1-dependent. Cells were incubated with IFNγ and solubilized, and the extracts were incubated with anti-Raf-1 antibodies. The amount of Raf-1 in both the 1D4 and E1C3 cells were comparable, as determined by immunoblotting of cell extracts (data not shown). The Raf-1 immunoprecipitates were analyzed for enzymatic activity by incubating in the presence of ATP and purified recombinant MEK1, yielding an activated MEK1. [^{32}P-γ]ATP was then added in the presence of an enzymatically inactive form of Erk2 (p42^{MAPK}). The assay measures the ability of Erk2 to be phosphorylated by MEK1. In parental cells, there was a marked increase in the phosphorylation of Erk by Raf-1 in response to IFNγ (Fig. 2, *A*, 1D4, and *B*, *lanes 1–3*). Raf-1 kinase activity was maximal (greater than 3-fold enhancement) after 5 min and then decreased to base-line levels after 15 min (Fig. 2*A*, 1D4). Baseline Raf-1 activity was similar in both cells lines, as determined by the extent of basal phosphorylation of Erk (Fig. 2*B*, *lanes 1* and *4*). In the Jak1-deficient cells (E1C3), there was no induction of Raf-1 activity (Fig. 2*A*). An increase in tyrosine phosphorylation of Raf-1 occurred in response to IFNγ (Fig. 2*C*, *lanes 1–3*) only in the parental cells. Maximal tyrosine phosphorylation of Raf-1 occurred after 15 min, although detectable increases in phosphorylation were evident after 5 min. In contrast, there was no increase in Raf-1 phosphorylation in response to IFNγ in the Jak1-deficient cell line (Fig. 2*C*, *lanes 4–6*). There was equal loading of Raf-1 protein in all *lanes* (Fig. 2*C*, anti-Raf-1 immunoblot, *lanes 1–6*) for both cells, 1D4 and E1C3. When E1C3 cells were treated with EGF, there was a 6.0-fold enhancement of Raf-1 activity (Table I). We also examined Raf-1 activation in the 2fTGH cell line, which is the parental cell line for the U4A cells that lack Jak1. Since the 2fTGH cells express a constitutively activated p21^{ras}, they were transfected with a myc epitope-tagged Raf-1, which contains a mutation in the p21^{ras} binding domain (R89LRaf-1) (10). IFNγ enhancement in kinase activity of this Raf-1 should be independent of endogenous p21^{ras}. In the parental cells (2fTGH) IFNγ treatment resulted in a 2.1 ± 0.42 (mean ± S.D., n = 2)-fold increase in Raf-1 activity, as determined by measuring activation of R89L-Raf-1 (see "Experimental Procedures"), whereas there was no increased activity in the U4A cells (0.45 ± 0.7, mean ± S.D., n = 2).

Reconstitution of IFNγ-induced Raf-1 activity was carried out in both the E1C3 cells that were transfected with Jak1 along with Tac and T Ag as a cell selection system and the U4A cell lines, which were transfected with both Jak1 and myc epitope-tagged Raf-1. There was a 1.5-fold increase in Raf-1 activity in E1C3 extracts that were prepared from IFNγ-treated cells transfected with kinase-active Jak1 that was statistically significant ($p < 0.05$, paired t test) when compared with control cells transfected with Tac and T Ag alone (Table I). EGF treatment of cells, which was used as a control for the assay, revealed a 6.0 ± 1.7-fold increase in Raf-1 activation. The amount of transfected Jak1 in the E1C3 cells compared with endogenous Jak1 in the parental cells is shown in Fig. 3.

TABLE I
Reconstitution of Raf-1 activation in Jak1-deficient cells with wild type Jak1

°$p < 0.05$, compared to no treatment, paired t test.

Cells (treatment)	No. of experiments	-fold increase over unstimulated cells
E1C3 (no treatment)	4	1.0
E1C3 (IFNγ)	4	1.5 ± 0.2° (mean ± S.E.)
E1C3 (EGF)	4	6.0 ± 0.2° (mean ± S.E.)
U4A (no treatment)	2	1.0
U4A (IFNγ)	2	2.1 ± 0.4 (mean ± S.D.)

There is less Jak1 available in the E1C3 cells compared with the 1D4 wild type cells (Fig. 3, *upper panel, lane 1 versus lane 3*). This may account for the relatively modest IFNγ activation of Raf-1 in these transfected cells. To reconfirm the ability of Jak1 to facilitate the activation of Raf-1 in E1C3 cells, we examined the ability of U4A cells (see above) to respond to stimulation with IFNγ upon reconstitution. Reconstitution of R89L Raf-1 activity in response to IFNγ occurred following transfection with the wild type Jak1 to levels observed in the parental cell line (Table I). Both the wild type 2fTGH and the transfected U4A cells responded to IFNγ with an approximately 2-fold increase in Raf-1 activity. Therefore, two separate cell lines deficient in Jak1 responded to reconstitution with Jak1 for IFNγ-induced activation of Raf-1.

Since one of the major mechanisms for the activation of Raf-1 is through $p21^{ras}$, we measured the ability of IFNγ to activate $p21^{ras}$ as measured by the increase in $p21^{ras}$-bound GTP. Cells were labeled with $^{32}P_i$ and then treated for 15 min with IFNγ. Cell extracts were made, $p21^{ras}$ was immunoprecipitated, and the GTP loading of $p21^{ras}$ was measured by thin layer chromatography. Whereas there was no evidence for increased GTP-bound $p21^{ras}$ after treatment of cells with IFNγ, a marked increase occurred following treatment of cells with EGF (Fig. 4). There was no measurable increase in $p21^{ras}$ activation in cells incubated with IFNγ at other time points (2.5, 5, and 10 min, data not shown). Therefore, the activation of Raf-1 by IFNγ appears to occur at a time when there is no measurable activation of $p21^{ras}$. Since it is known that GTP-bound $p21^{ras}$ binds Raf-1 (25), we next examined Raf-1 activity bound to $p21^{ras}$ in HeLa cells treated with IFNγ or EGF. Treatment of HeLa cells with EGF resulted in a 6-fold increase in Raf-1 activity bound to immunoprecipitated $p21^{ras}$ (Table II). This enhancement was nearly identical to the 6.5-fold increase in EGF-stimulated GTP binding of $p21^{ras}$ (Fig. 4). Under the same conditions, the $p21^{ras}$ activity associated with $p21^{ras}$ in IFNγ-treated cells showed no or very little enhancement (Table II).

To obtain more information regarding the ability of the Jak kinases to associate with and activate Raf-1, we performed coimmunoprecipitation experiments in HeLa cells and in COS cells transfected with either wild type or kinase-inactive Jak1. There was constitutive association of Raf-1 with Jak1 in HeLa cells (Fig. 5*A*, *lanes 4–6*). Previous studies have indicated that Jaks expressed in COS cells are constitutively activated and will tyrosine-phosphorylate STATs in the absence of IFNs. To determine the role of Jak1 stimulation on Raf-1 activity without ligand receptor interaction, COS cells were transfected with wild type or kinase-inactive Jak1. In COS cells transfected with wild type (*wt*) Jak1, activated Raf-1 enzyme activity measured approximately 2-fold (Fig. 5*B*) over a β-galactosidase control transfection. In contrast, transfection with a kinase-inactive (*kd*) construct resulted in no enhancement of Raf-1 activity (Fig. 5*B*). When Raf-1 was immunoprecipitated and the immunoblot membrane was probed for Jak1, both wild type and kinase-inactive Jak1 were observed to be associated with Raf-1 (Fig. 4*C*, *lanes 5* and *6*). There was no association of Jak1

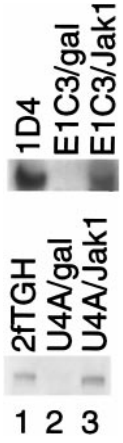

FIG. 3. **Jak1 expression in wild type and Jak1-deficient cells.** *Upper panel*, 1D4 and E1C3 cells. Equal aliquots of cells from either 1D4 (wild type) or E1C3 transfected with a β-galactose (*gal*) control or Jak1 plasmid were processed for immunoblot analysis and probed with an anti-Jak1 antibody. The membrane shown in the *upper panel* was processed by enhanced chemiluminescence. *Lane 1* represents wild type cells. *Lane 2* represents control cells transfected with the galactose plasmid. *Lane 3* represents cells transfected with Jak1. *Lower panel*, 2fTGH and U4A cells. These cells were processed as described above. The immunoblot was processed with nitro blue tetrazolium chemistry. The *lanes* are as described above.

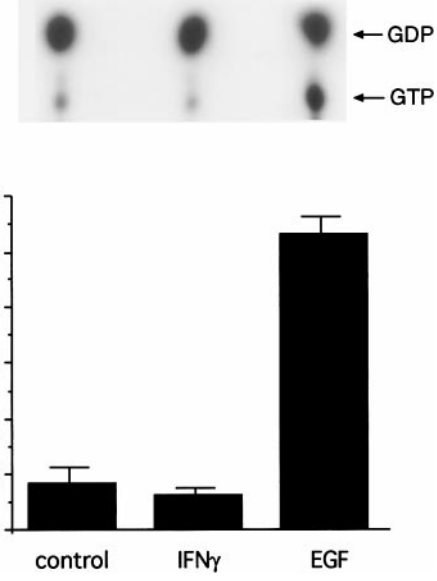

FIG. 4. **$p21^{ras}$ is not activated by IFNγ.** Cells were incubated with $^{32}P_i$ and then treated with either IFNγ or EGF for 15 min. $p21^{ras}$ activation state was performed as described. Radioactivity of GTP and GDP was quantitated and shown as GTP/GDP × 100.

TABLE II
Raf1 activation when coimmunoprecipitated with $p21^{ras}$

Treatment (No. of experiments)	-fold increase over control (mean ± S.E.)
Control ($n = 4$)	1.00
IFNγ ($n = 2$)	1.24 ± 0.04
EGF ($n = 3$)	5.85 ± 1.39

with Raf-1 when either the β-galactosidase cDNA was transfected (Fig. 5*C*, *lane 4*) or when control normal rabbit IgG was used for the immunoprecipitations (Fig. 4*C*, *lanes 1–3*). Only in cells transfected with kinase-active Jak1 was tyrosine-phosphorylated Jak1 associated with Raf-1 (Fig. 4*C, lower panel, lane 5 versus lane 6*). This supports the contention that kinase-

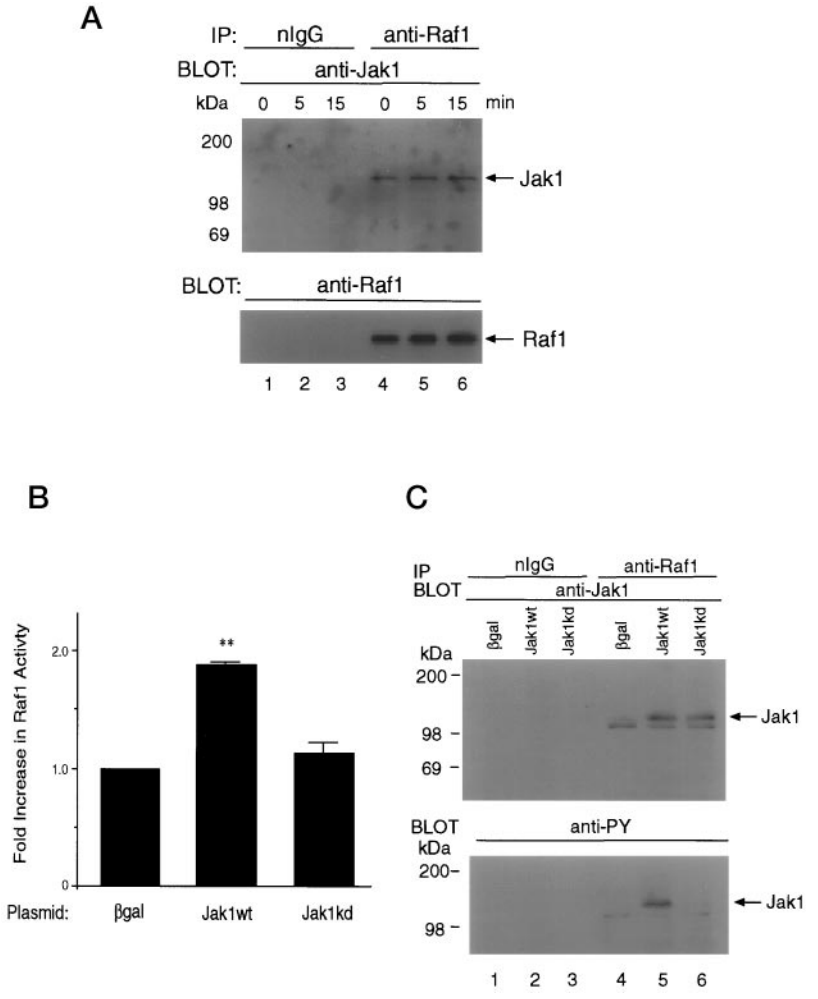

FIG. 5. **Jak1 associates with endogenous Raf-1 in HeLa and activates Raf-1 in COS cells.** *A*, HeLa cells were solubilized, Raf-1 was immunoprecipitated (*IP*), and the immunoprecipitates were analyzed by immunoblotting with anti-Jak1 or anti-Raf-1. *Lanes 1–2, nIgG*, normal rabbit IgG; *lanes 4–6*, anti-Raf-1. *B*, constructs of kinase-active Jak1 (*wt*), kinase-inactive/dead (*kd*) Jak1 and β-galactosidase genes were transiently transfected in COS cells by electroporation. After 48 h, cells were lysed and subjected to Raf-1 kinase assay after immunoprecipitation with anti-Raf-1 antibody. Data are shown as -fold increase of Raf-1 activity over that of the β-galactosidase transfectant (negative control). *C*, lysates of cells transfected with cDNAs for each of the Jak1 kinases (wt and kd) and β-galactosidase (control) were subjected to immunoprecipitation with normal rabbit serum (*nIgG*) (*lanes 1–3*) or anti-Raf-1 (*lanes 4–6*). The immunoblots were then probed with anti-Jak1 (*upper panel*) or anti-phosphotyrosine (*lower panel*).

active Jak1 must be present for Raf-1 to be activated as observed in Fig. 4*B*.

DISCUSSION

Activation of the Jak/STAT pathway by cytokines such as IFNγ allows not only for the induction of early response genes such as *IRF1* and the high affinity *Fcgr1*, but also for genes that are delayed in their expression, such as human major histocompatibility class II antigens. A deficiency in any individual component of the pathway leads to disruption of the entire signaling cascade. On the other hand, under certain circumstances cytokine-induced proliferation and/or differentiation may require more than just activation of STAT proteins (26, 27). These findings suggest that other signaling pathways may operate in parallel or integral to the Jak/STAT pathway to enable full phenotypic responses of cells exposed to extracellular ligands. In this report, we explored the relationship between two pathways utilized by cytokines to activate cells: the Jak/STAT and the $p21^{ras}$/Raf-1/MEK/MAPK pathway. We show that treatment of cells with IFNγ leads to rapid activation of Raf-1 in a $p21^{ras}$-independent manner. In cells lacking Jak1, no activation of Raf-1 occurs. Therefore, Jak1 appears to interact with this cascade at least at the level of Raf-1.

Although previous reports have determined that IFNγ activates MAPK activity and in some systems protein kinase C activity (7), our data confirm and extend these observations by showing that IFNγ can directly activate Raf-1 that is dependent upon the presence of intact Jak1 activity but independent of $p21^{ras}$ activation. The lower activation of Raf-1 in the transfection experiments with the E1C3 cells may indeed be a function of the reduced ability to express Jak1 in these cells along with its increased lability. The need for Jak1 suggests that an association between Jak1 and Raf-1 may be necessary for activation of Raf-1. Since Jak2 is present in these cells and in U4A cells, Jak2 apparently cannot substitute for this function. This is in contrast to growth hormone signaling in embryonic kidney cells in which $p21^{ras}$, Raf, and Jak2 (but not Jak1) are required for MAPK activation (28). The role, if any, tyrosine phosphorylation plays in the regulation of Raf-1 activity is incompletely understood. We have shown that IFNγ-induced tyrosine phosphorylation of Raf-1 is maximal 15 min after treatment, whereas Raf-1 enzyme activity becomes maximal after 5 min and is substantially reduced by 15 min. Increased Raf-1 activity has been associated with or without tyrosine phosphorylation, depending upon the activating stimulus (29). Tyrosine phosphorylation can be observed and is thought to be involved in either membrane targeting or release from receptors (30–33). Furthermore, serine/threonine phosphorylation of Raf-1 plays both an activating role, as observed for ceramide-induced activation and phosphorylation on Thr-269 (34), and an inhibitory role, as observed for protein kinase A-induced inhibition and phosphorylation on Ser-612 (35).

Since we observe that both Jak1 and Raf-1 can be coimmunoprecipitated when Jak1 is overexpressed, this suggests a direct interaction between Jak1 and Raf-1, possibly at the level of the IFNγ receptor. Dimerization of Raf-1 through a receptor-independent or -dependent mechanism is sufficient for Raf activation (36, 37). It is conceivable that a pool of Raf-1 associates with Jak1 (and thereby the α chain of the IFNγ receptor),

dimerizes upon binding of IFNγ (a bivalent homodimer), and becomes activated. However, this does not exclude the possibility that Jak1 may function through an intermediary kinase that may be responsible for the activation of Raf-1. Although it has been recently suggested that Jak2 can be coimmunoprecipitated with Raf-1 and $p21^{ras}$, this was observed by overexpression in nonmammalian insect cells (38). However, the authors also comment on their inability to show co- immunoprecipitation of Jak2 and Raf-1 in mammalian cells treated with IFNγ and fail to address the issue of $p21^{ras}$-dependence of Raf-1 activation. The absence of $p21^{ras}$ activity for cytokine stimulation of Raf-1 has been well documented to occur for other cytokines (11). Although the use of dominant negative $p21^{ras}$ mutants can in some instances confirm this notion, transfections with these constructs have not yielded suitable results for a measurable dominant negative effect (data not shown).

IFNγ-induced anti-viral activity requires the expression of Jak1 with intact kinase activity. No anti-viral activity is seen in the Jak1 kinase-inactive transfectants (39). One interpretation of these findings is that Jak1 activity is required for the activation of other systems in addition to just STAT activation (which occurs in a delayed fashion in the kinase-inactive transfectants). Our data reveal that kinase-active Jak1 may be required to obtain full Raf-1 activation and that possibly Raf-1 activity may be required for optimal anti-viral effects of IFNγ. It is of interest that treatment with IFNγ does not result in the activation of $p21^{ras}$, since activated $p21^{ras}$ is known to inhibit phosphokinase R activity, an enzyme critical for anti-viral activity (40).

There have been several reports on the activation of protein kinase C, calcium fluxes, and Ser/Thr phosphorylation in mediating the effects of IFNγ on a variety of cell types (41). These pathways appear to control activation of genes such as the major histocompatability complex class II antigens, enhancement of antigen presenting activity, and *fas* gene activation, which potentially play a role in the ability of IFNγ to induce an anti-proliferative, differentiative, or apoptotic state. Cell specificity can also play a role in modulating IFNγ-induced gene expression. This was recently demonstrated by studying the effects of IFNγ on myocytes and microvascular endothelial cells (42). Although IFNγ can activate STAT1 in both cells, only in the myocytes is MAPK activity and inducible nitric oxide synthetase gene expression observed in response to IFNγ. The authors suggest that MAPK activation along with STAT1 activation was necessary for inducible nitric oxide synthetase gene expression in myocytes. Therefore, selective expression of IFNγ-induced genes depends upon the activation of specific signaling cascades whose modulation allows for multiple layers of control in mediating IFNγ-regulated responses.

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