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Beta Interferon and Oncostatin M Activate Raf-1 and Mitogen-Activated Protein Kinase through a JAK1-Dependent Pathway

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Activation of early response genes by interferons (IFNs) and other cytokines requires tyrosine phosphorylation of a family of transcription factors termed signal transducers and activators of transcription (Stats). The Janus family of tyrosine kinases (Jak1, Jak2, Jak3, and Tyk2) is required for cytokine-induced tyrosine phosphorylation and dimerization of the Stat proteins. In order for IFNs to stimulate maximal expression of Stat1α-regulated genes, phosphorylation of a serine residue in the carboxy terminus by mitogen-activated protein kinase (MAPK) is also required. In HeLa cells, both IFN-β and oncostatin M (OSM) stimulated MAPK and Raf-1 enzyme activity, in addition to Stat1 and Stat3 tyrosine phosphorylation. OSM stimulation of Raf-1 correlated with GTP loading of Ras, whereas IFN-β activation of Raf-1 was Ras independent. IFN-β- and OSM-induced Raf-1 activity could be coimmunoprecipitated with either Jak1 or Tyk2. Furthermore, HeLa cells lacking Jak1 displayed no activation of STAT1α, STAT3, and Raf-1 by IFN-β or OSM and also demonstrated no increase in the relative level of GTP-bound p21^{ras} in response to OSM. The requirement for Jak1 for IFN-β- and OSM-induced activation of Raf-1 was also seen in Jak1-deficient U4A fibrosarcoma cells. Interestingly, basal MAPK, but not Raf-1, activity was constitutively enhanced in Jak1-deficient HeLa cells. Transient expression of Jak1 in both Jak-deficient HeLa cells and U4A cells reconstituted the ability of IFN-β and OSM to activate Raf-1 and decreased the basal activity of MAPK, while expression of a kinase-inactive form of the protein showed no effect. Moreover, U4A cells selected for stable expression of Jak1, or COS cells transiently expressing Jak1 or Tyk2 but not Jak3, exhibited enhanced Raf-1 activity. Therefore, it appears that Jak1 is required for Raf-1 activation by both IFN-β and OSM. These results provide evidence for a link between the Jaks and the Raf/MAPK signaling pathways.

Activation of the Jak/Stat signaling pathway is required for stimulation of specific cellular genes that are responsible for the biological actions of cytokines and growth factors which utilize this signaling cascade (13). Most cytokines which activate the Jak/Stat pathway, including the interferons (IFNs), also stimulate MAPK (mitogen-activated protein kinase) (23). Maximal activation of several Stat-regulated genes requires both serine phosphorylation of these transcription factors, probably by Erk2 (p42 MAPK) and tyrosine phosphorylation (6, 27). Regulation of MAPK activity is also essential for cytokine-induced expression of immediate-early genes as well as regulation of proliferation and differentiation. Activation of MAPKs is controlled via membrane-associated signaling complexes and involves a network which includes Ras protein(s), the Raf family (Raf-1, B-Raf, A-Raf, and MEKK) of serine kinases, and MAPK kinases (MEK1 and -2) (23). The mechanisms which control Raf activation are poorly understood. Activation of Raf-1 by some mitogens is Ras dependent, while in other cases Ras activation is not required (3). Ras association with Raf is insufficient for Raf activation, suggesting that other cofactors (i.e., protein kinases) are also necessary. Recent evidence has indicated that a ceramide-activated protein kinase which is triggered by tumor necrosis factor alpha and interleukin-1 (IL-1) can enhance Raf-1 activity in vitro, and it also appears that the Src family of protein kinases stimulates Raf activity (9, 31). Transformation of cells by Src and other oncogenic tyrosine kinases also activates the Jaks and some Stat proteins (4, 32). Experiments were therefore designed to examine the potential role of the Jaks in activation of the MAPK signaling cascade.

MATERIALS AND METHODS

Cells. The parental HeLa and E1C3 Jak1-deficient cell lines (16) and the parental 2fTGH human fibrosarcoma and Jak1-deficient U4A cell lines (20) have been described elsewhere. The Jak1-deficient cells do not support IFN-α- or IFN-γ-induced gene expression. Cells were maintained as adherent cultures in Dulbecco modified Eagle medium (GIBCO/BRL) supplemented with 10% fetal bovine serum (HyClone). For the MAPK, Raf-1, and p21^{ras} assays, cells were placed in serum-free medium for the 2 or 24 h prior to cytokine treatment.

Electrophoretic mobility shift assay. After treatment with cytokine, 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, 20% glycerol). DNA binding proteins were assayed as previously described (28). Briefly, 10 μg of protein was incubated in binding buffer with the ^{32}P-labeled oligonucleotide

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probe consisting of the double-stranded IFN-γ activation sequence referred to as the gamma response region (GRR) (5′ AGCATGTTTCAAGGATTTGAGAT GTATTTCCCAGAAAAG 3′) of the promoter of the Fcγr1 gene (22). The sample was then applied to a 6% nondenaturing polyacrylamide gel in order to separate free probe from probe bound to protein.

MAPK (Erk2/p42) assay. After treatment with cytokine for various time periods, cells were lysed in lysis buffer (150 mM NaCl, 25 mM HEPES [pH 7.3], 1 mM sodium orthovanadate, 1% Triton X-100, proteinase inhibitors, 0.5 mM dithiothreitol). 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-Erk2 antibody (TR10; kindly provided by Michael Weber, University of Virginia) for 2 h at 4°C and then with protein G-Sepharose for 1 h. The immunoprecipitate was washed three times with lysis buffer, and the kinase reaction was carried out in the kinase assay buffer (20 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 20 μM ATP, 2 mM EGTA) containing [γ-^{32}P]ATP (5 μCi) and myelin basic protein (MBP; 1.5 mg/ml) at room temperature for 15 min. The proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by electrophoretic transfer to polyvinylidene difluoride membranes. The phosphorylation of MBP was quantitated by PhosphorImager (Molecular Dynamics) analysis. The membrane was subsequently probed with antiphosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc., [UBI]) followed by anti-pan-Erk monoclonal antibody (Transduction Laboratories, Lexington, Ky.). The amount of Erk2 protein on the membrane was normalized by counterimmunoblotting with an ^{125}I-labeled anti-mouse immunoglobulin G (IgG). In experiments using cells transfected with hemagglutinin (HA)-tagged p42 MAPK, a monoclonal antibody to HA, 12CA5 (Babco, Berkeley, Calif.), was used to immunoprecipitate the transfected protein, and MAPK assays were performed as described above.

Raf-1 assay. Cells were solubilized in lysis buffer (150 mM NaCl, 25 mM HEPES [pH 7.3], 1 mM sodium orthovanadate, 1% Triton X-100, protease inhibitors, 0.5 mM dithiothreitol), and the assay was carried out as described previously (8). 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) and then 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, 0.2% β-mercaptoethanol) with 1 μg of purified recombinant MEK1 added as the substrate. After MEK1 activation, 15 μCi of [γ-^{32}P]ATP and 1 μg of kinase-defective (K52R) Erk were added as the substrate for an additional 2 min. The reaction was terminated by the addition of sample buffer, the mixture was boiled for 5 min, and the proteins were separated by SDS-PAGE. The gel proteins were then transferred to a polyvinylidene difluoride membrane, on which the amount of radiolabeled K52R Erk was quantitated by a PhosphorImager. For the calculations of Raf-1 activity, the amount of Raf-1 protein on the same membrane was determined by probing the membrane with ^{125}I-labeled goat anti-mouse IgG following mouse monoclonal anti-Raf-1 blotting. 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 (CAAX), was used in a p21^{ras}-independent Raf-1 assay (18). Cells were transfected with R89LRaf-1 plasmid by using DEAE-dextran. Forty-eight hours posttransfection, lysates were prepared from either untreated cells or cells incubated for 5 min with IFN-β or oncostatin M (OSM). 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. Raf-1 assays (see Table 1) were performed on immunoprecipitates from extracts that had been incubated with the Ras monoclonal antibody LA069 (Quality Biotech, Camden, N.J.).

Ras activation assay. The activation state of p21^{ras} was determined as described elsewhere (12). HeLa cells in 100-mm-diameter dishes were labeled with 1 mCi of [^{32}P]orthophosphate per ml for 4 h at 37°C in phosphate-free Dulbecco modified Eagle medium. The cells were then treated with IFN-β, OSM, or epidermal growth factor (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 of bovine serum albumin per ml, 1 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml, 5 μg of aprotinin per ml, 1 mM vanadate, 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) 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, 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, 0.5 mM GDP). After brief centrifugation, 8 μl of supernatant was spotted onto a polyethyleneimine-cellulose thin-layer chromatography plate. After drying, the plates were washed briefly in water and air dried before development in 0.65 M KH_2PO_4 (pH 3.4) for 75 min. Radioactivity was visualized by autoradiography and quantitated by a PhosphorImager.

Expression vectors and transfections. The full-length cytomegalovirus (CMV)-driven wild-type murine Jak1 cDNA (pRK5mJak1wt) and a kinase-negative form (ATP binding site K→E; pRK5mJak1kd) were constructed as described previously (2). HA-tagged p42 MAPK driven by CMV was a generous gift from S. Gutkind (17). CMV-driven β-galactosidase was used as a control. Transfection of cDNAs into HeLa cells was performed by electroporation (10^7 cells/ml) in phosphate-buffered saline (300 V/cm, 800 μF; Cell-Porator; Bethesda Research Laboratories). To enhance expression, a plasmid (2 μg) which expresses the simian virus 40 large T antigen was included in all transfections (21). Immunoblotting for detection of expressed Jak1 showed that 9 to 14 h of incubation after electroporation gave maximal expression of Jak1 protein. Parental 2fTGH cells and the U4A variants were transfected with R89LRaf-1 plasmid by using DEAE-dextran (18). At 48 h posttransfection, cells were serum starved for 2 h prior to treatments and preparation of cell extracts. Cell extracts were incubated with monoclonal antibody 9E10, which recognizes the Myc epitope tag (GGEQKLISEEDL). Immunoprecipitates were assayed for Raf-1 kinase activity as described above. U4A cells stably expressing Jak1 and mutant Jak1 constructs were prepared as described previously (2).

RESULTS

To explore the role of the Jaks in the regulation of IFN-β and OSM activation of the MAPK signaling pathway, we used both HeLa cells and the human fibrosarcoma cell line 2fTGH because of the availability of mutant cell lines which do not express Jak1 (16, 20). IFN-β, OSM (which utilizes the gp130 protein as a component of its receptor), and EGF stimulate the Jak/Stat pathway in wild-type HeLa cells, as assayed by binding of tyrosine-phosphorylated Stats to an enhancer (GRR) present in the promoter of the IFN-induced high-affinity FcγR1 receptor gene (Fig. 1A, lanes 1 to 4). Similar results were obtained for the Jak1-deficient 2fTGH cell line U4A (data not shown) (20). Immunoprecipitation of various Stats and Jaks from both cell lines indicated that IFN-β and OSM stimulate tyrosine phosphorylation of Jak1 and Tyk2 but not Jak2 (data not shown). OSM stimulates tyrosine phosphorylation primarily of Stat3, whereas IFN-β activates Stat1 and Stat2 (1, 10, 13). EGF stimulates tyrosine phosphorylation of Jak1, Stat3, and Stat5 in wild-type HeLa cells. In the Jak1-deficient HeLa line, there was no activation of the GRR binding protein by IFN-β or OSM (lanes 5 to 7). Only EGF, whose receptor possesses intrinsic tyrosine kinase activity, activates the formation of a complex which binds to the GRR (lane 8). This result confirms reports that Jaks are not always required for activation of Stats (7, 14).

Activation of MAPK activity requires its phosphorylation on both threonine and tyrosine (23). To determine whether MAPK (p42) activation by IFN-β, OSM, and EGF was Jak1 dependent, wild-type and Jak1-deficient HeLa cells were incubated with each cytokine, cellular extracts were prepared, and MAPK (p42) was immunoprecipitated. The immunoprecipitates were resolved on SDS-polyacrylamide gels and transferred to Immobilon, and the membrane was probed with antiphosphotyrosine antibody (Fig. 1B). IFN-β, OSM, and EGF stimulated tyrosine phosphorylation of MAPK in wild-type HeLa cells (compare lane 1 with lanes 2 to 4), while in HeLa cells deficient in Jak1, no enhancement of tyrosine phosphorylation of MAPK was observed with any of the treatments (lanes 5 to 8). The basal level of tyrosine phosphorylation of p42 MAPK in Jak1-deficient HeLa cells was elevated to the same extent found in the cytokine-treated wild-type cells (compare lane 5 with lanes 2 to 4). Kinase assays with immunoprecipitated MAPK using MBP as a substrate confirmed that tyrosine phosphorylation correlated with enhanced enzymatic activity (data not shown). Increased basal activity of p42 MAPK in Jak1-negative cells was not associated with either elevated activity of Raf-1 (see below) or enhanced activity of MEK1 or MEK2 (data not shown).

To determine whether the elevated activity of MAPK was associated with the lack of expression of Jak1 in Jak1-deficient HeLa cells or was an unrelated phenomenon secondary to mutagenesis, cells were transfected with either wild-type or kinase-inactive Jak1 and HA-tagged p42 MAPK. Transfected

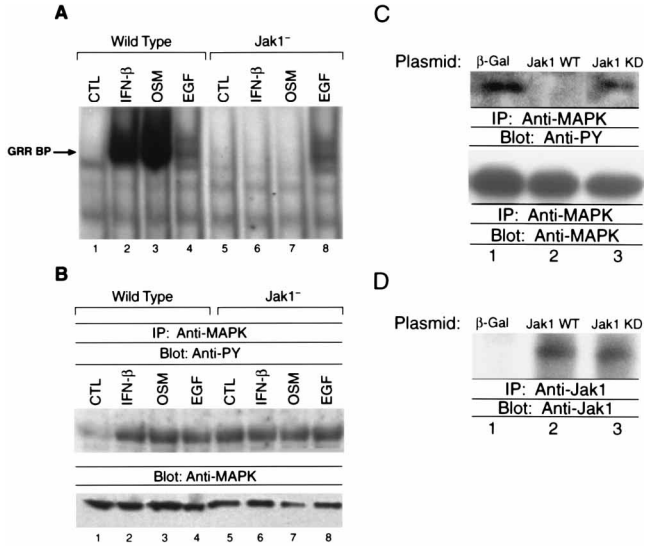

FIG. 1. IFN-β and OSM activate Stats and MAPK in wild-type but not Jak1-deficient HeLa cells. (A) Activation of Stats in HeLa cells by IFN-β, OSM, and EGF. Either wild-type (lanes 1 to 4) or Jak1-deficient (lanes 5 to 8) HeLa cells were incubated for 5 min at 37°C with IFN-β (10^3 U/ml; lanes 2 and 6), OSM (100 ng/ml; lanes 3 and 7), or EGF (50 ng/ml; lanes 4 and 8). Untreated (control [CTL]) samples are shown in lanes 1 and 5. Cellular extracts were prepared and analyzed for tyrosine phosphorylation of the Stat proteins by their ability to bind to the GRR, an enhancer present in the promoter of the high-affinity FcγR1 receptor (28). Tyrosine-phosphorylated Stats which are activated by IFN-β, OSM, and EGF bind to this enhancer with high affinity (10). The complex whose formation is stimulated in extracts from cells incubated with IFN-β, OSM, or EGF is labeled GRR BP. (B) Cytokine-stimulated tyrosine phosphorylation of MAPK in wild-type and Jak1-deficient HeLa cells. Cells were incubated for 5 min at 37°C as described above, and extracts were prepared. MAPK (42 kDa) was directly immunoprecipitated (IP) (6), and proteins were separated by SDS-PAGE (4 to 16% gels) and transferred to Immobilon (Millipore). The membranes were then incubated with a monoclonal antibody to phosphotyrosine (PY) (4G10; UBI) (top) or pan-MAPK (Transduction Laboratories) (bottom), and immunoreactive proteins were visualized by enhanced chemiluminescence. (C) Expression of wild-type but not kinase-inactive Jak1 (Jak1 KD) in Jak1-deficient HeLa cells decreases the basal tyrosine phosphorylation of MAPK. Jak-1-deficient HeLa cells were transfected with HA-MAPK and either β-galactosidase (β-Gal; lane 1), wild-type (WT) Jak1 (lane 2), or kinase-inactive Jak1 (lane 3). Twenty hours after transfection, the cells were placed in serum-free medium for 6 h, and cellular extracts were prepared. HA-tagged MAPK was immunoprecipitated with an HA monoclonal antibody, immunoprecipitates were resolved by SDS-PAGE, and the resulting blot was probed with antiphosphotyrosine antibody (top). A separate aliquot of the immunoprecipitate was also probed for the MAPK (bottom). (D) To ensure that wild-type and kinase-inactive Jak1 were expressed equally in transfected samples, the cell lysates were also immunoprecipitated with Jak1 antisera, and the resulting blots were probed for the presence of Jak1.

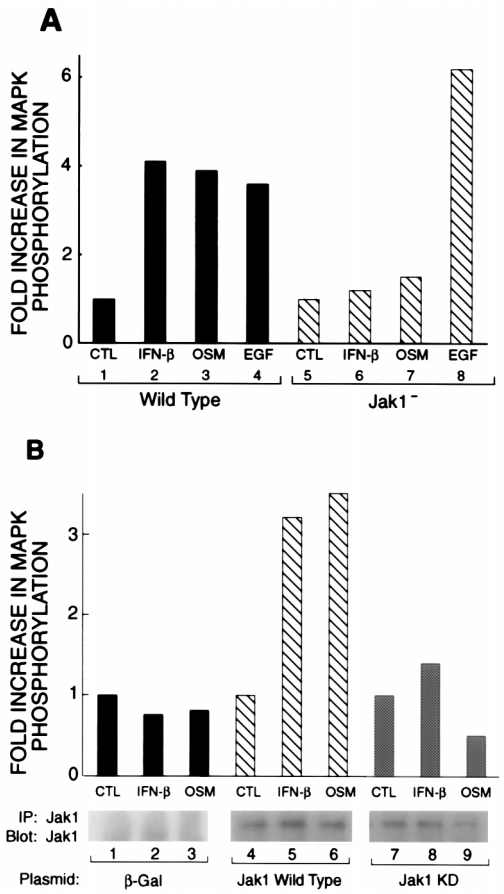

FIG. 2. Stimulation of Raf-1 kinase activity in wild-type and Jak1-deficient HeLa cells treated with IFN-β, OSM, or EGF. (A) Raf-1 is activated in wild-type but not Jak1-deficient HeLa cells. Lysates were prepared from either untreated cells (control [CTL]; lanes 1 and 5) or cells incubated for 5 min with IFN-β (lanes 2 and 6), OSM (lanes 3 and 7), or EGF (lanes 4 and 8) (all cells were serum starved for 18 h prior to treatments) and incubated with antiserum specific for Raf-1. Immunoprecipitates were assayed for Raf-1 kinase activity, using MEK1 as the Raf-1 substrate and kinase-defective MAPK as the MEK1 substrate in the presence of [γ-^{32}P]ATP (8). ^{32}P incorporation into kinase-defective MAPK was quantitated by use of a PhosphorImager (Molecular Dynamics). The fold induction of ^{32}P incorporation into MAPK was normalized to the amount of Raf-1 present in each sample assayed. The amount of Raf-1 in each sample was determined by probing the membrane with a monoclonal antibody to Raf-1 (Transduction Laboratories) followed by ^{125}I-conjugated goat anti-mouse IgG. The amount of ^{125}I was quantitated with a PhosphorImager. The increase in Raf-1 activity with IFN-β or OSM treatment of cells varied between 2- and 10-fold and was observed in at least 10 individual experiments. (B) Reconstitution of Raf-1 activation by IFN-β and OSM in Jak1-deficient cells. Jak1-deficient HeLa cells were transfected by electroporation with plasmids (5 mg) expressing β-galactosidase (β-Gal; lanes 1 to 3), wild-type murine Jak1 (lanes 4 to 6), or kinase-inactive murine Jak1 (lanes 7 to 9). Twelve hours after transfection, the cells were placed in serum-free medium for 3 h prior to incubation with IFN-β (lanes 2, 5, and 8) or OSM (lanes 3, 6, and 9) for 5 min. Raf-1 kinase activity was assayed as described in the text. The increase in Raf-1 activity with IFN-β or OSM treatment of cells varied between two- and fourfold after transfection of cells with wild type Jak1. IP, immunoprecipitation.

cells were subsequently incubated in the absence of serum for 6 h, cellular extracts were prepared, and HA-tagged MAPK was immunoprecipitated. Antiphosphotyrosine blots revealed that expression of wild-type but not kinase-inactive Jak1 decreased the tyrosine phosphorylation of HA-MAPK (Fig. 1C; compare lanes 2 and 3). Equal amounts of HA-MAPK were immunoprecipitated in all samples (Fig. 1C, lower panel). Wild-type and kinase-inactive Jak1 were expressed in equal amounts in transfected samples, as demonstrated in Fig. 1D. MBP kinase assays confirmed that the basal activity of the HA-MAPK was also decreased in cells transfected with wild-type Jak1 (data not shown).

Although the relative activity of MAPK is elevated in Jak1-deficient HeLa cells, we also wanted to determine whether Jak1 might be required for IFN-β and OSM activation of Raf kinase(s), the upstream activator of p42 MAPK (23). Raf kinase assays were performed to assess whether IFN-β and OSM stimulate Raf-1 in wild-type cells and in Jak1-deficient cells (Fig. 2A). This assay examines the ability of immunoprecipitated Raf-1 to activate a phosphorylation cascade, using recombinant MEK1 and kinase-inactive MAPK as substrates (8). Incorporation of [^{32}P]orthophosphate into kinase-inactive MAPK reflects the relative activity of the immunoprecipitated Raf-1. Incubation of wild-type cells with each of the cytokines tested resulted in a three- to fourfold increase in Raf-1 activity (lanes 1 to 4). Similar results were seen with IL-6 (data not

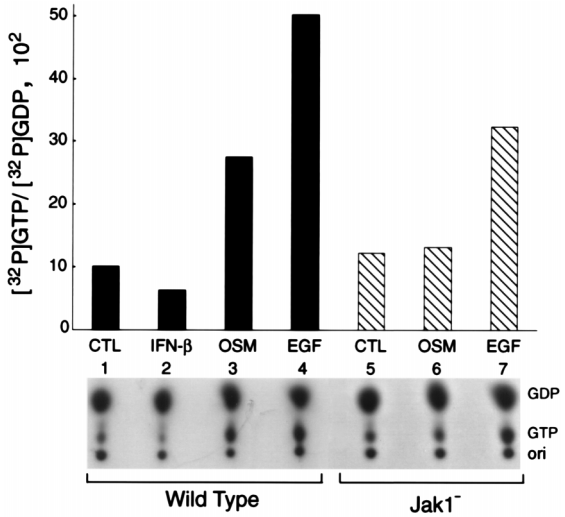

FIG. 3. Effect of IFN-β, OSM, or EGF treatment of HeLa cells on the nucleotides bound to p21*ras*. Confluent 100-mm^2 dishes of either wild-type (lanes 1 to 4) or Jak1-deficient (lanes 5 to 7) HeLa cells were cultured in serum-free medium for 48 h prior to incubation with [^{32}P]orthophosphate. Cells were incubated with cytokines for 5 min prior to preparation of cell extracts. Anti-p21*ras* monoclonal antibody Y13-259 was used for immunoprecipitations. Immunoprecipitates were spotted onto polyethyleneimine–cellulose-F thin-layer chromatography plates and developed as described previously (12). A PhosphorImager was used to quantitate the results. ori is the sample origin. CTL, control.

shown). However, only EGF stimulated Raf-1 activity in Jak1-deficient cells, suggesting that Jak1 is not required for these EGF-regulated responses (lanes 5 to 8). To reconstitute signaling in HeLa cells deficient in Jak1, transient transfections were performed with vectors which encoded either wild-type or kinase-inactive Jak1 (Fig. 2B). While expression of the wild-type protein in Jak1-negative cells permitted IFN-β and OSM to stimulate Raf-1, the kinase-inactive form of the enzyme was not capable of reconstituting a significant response.

We next examined the potential role of Ras in the activation of Raf-1 by IFN-β and OSM. EGF, which is known to activate Ras, was used as a control. Ras activation was analyzed by determining the relative amounts of GDP-bound and GTP-bound p21*ras* (Fig. 3). Both EGF and OSM caused an increase in the amount of GTP-bound p21*ras*, whereas IFN-β treatment of wild-type HeLa cells had no effect. In Jak1-deficient cells, only EGF stimulated an increase in GTP-bound p21*ras*. Interestingly, there was consistently less EGF-induced GTP loading of Ras in the Jak1-deficient cells than in wild-type cells, suggesting that EGF-induced p21*ras* activity may also be influenced by Jak1 (compare lanes 4 and 7).

As another independent assay to validate that IFN-β activation of Raf-1 does not require Ras, we examined Raf-1 activity in Ras immunoprecipitates. It is well known that Raf-1, when activated in a Ras-dependent manner, binds directly to Ras (19, 25, 26, 33). HeLa cells were untreated or treated with IFN-β, OSM, or EGF, and cellular extracts were incubated with a Ras monoclonal antibody which allows Ras–Raf-1 interactions to be detected. Immunopellets were assayed for Raf-1 activity (Table 1). It is clear that while OSM and EGF cellular extracts contained approximately four- and sixfold-elevated Raf-1 activity which precipitated with Ras, respectively, cells treated with IFN-β showed very little or no enhanced Raf-1 activity compared with untreated cells. These results are similar to those seen where GTP loading of Ras was

TABLE 1. Raf kinase activity associates with immunoprecipitated p21*ras* from OSM- and EGF-treated, but not IFN-β-treated, wild-type HeLa cells[a]

Sample	Ras-associated Raf kinase activity (cpm)	Fold induction
Control	825	1.00
IFN-β	962	1.17
OSM	3,269	3.96
EGF	5,314	6.44

[a] Cells were treated with cytokine and lysates prepared as described for Fig. 2 and incubated with 5 μg of a monoclonal antibody specific for p21*ras*. Immunoprecipitates were assayed for Raf-1 kinase activity as described for Fig. 2. The fold induction of ^{32}P incorporation into MAPK was normalized to the amount of p21*ras* in each sample assayed. The amount of p21*ras* in each sample was determined by probing the membrane with a monoclonal antibody to p21*ras* (Quality Biological, Princeton, N.J.) followed by ^{125}I-conjugated goat anti-mouse IgG. The amount of ^{125}I was quantitated with a PhosphorImager.

enhanced by both OSM and EGF but not with IFN-β treatment of HeLa cells (Fig. 3).

To confirm the requirement of Jak1 for IFN-β and OSM stimulation of Raf-1 activity, experiments were also done with the human fibrosarcoma cell line 2fTGH. This line has been used to generate a variety of mutations in the Jak/Stat pathway, including the U4A line, which does not express Jak1 (20). However, 2fTGH cells are known also to express a mutated, constitutively active Ras, which does not allow for analysis of cytokine-regulated Raf-1 activity (27). To circumvent this problem, we transfected cells with a Myc epitope-tagged Raf-1, which contains the Ras membrane localization motif (CAAX) as well as a mutation in the Ras binding domain of Raf-1 (R89LRaf-1) (18). The kinase activity of this protein is therefore independent of Ras. Transfected R89LRaf-1 was immunoprecipitated from 2fTGH and U4A cells with an antibody which recognizes the Myc epitope tag, and Raf-1 activity was assayed (Fig. 4A). While both IFN-β and OSM stimulated Raf-1 activity in wild-type cells, no activation of Raf-1 was seen in U4A cells. It is interesting that although OSM does activate GTP loading of p21*ras* in HeLa cells, this cytokine can clearly stimulate Raf-1 activity in a Ras-independent manner in 2fTGH cells.

To determine whether activation of Raf-1 by OSM or IFN-β could be detected in U4A cells expressing Jak1, we performed transient-expression assays where wild-type or kinase-inactive Jak1 and R89LRaf-1 were expressed in U4A cells (Fig. 4B). As found for HeLa cells, expression of wild-type Jak1 (lanes 2 and 3), but not the kinase-inactive form of the enzyme, permitted activation of R89LRaf-1 (lanes 5 and 6).

Permanent lines obtained from U4A cells which express either Jak1 or a kinase-inactive form of the protein were also examined for activation of R89LRaf-1. Surprisingly, U4A cells reconstituted with Jak1 demonstrated no enhanced activation of Raf-1 after incubation of cells with IFN-β or OSM (Fig. 5A), but there was a partial restoration of Raf-1 induction with these cytokines in U4A cells which express the kinase-inactive form of the protein (Fig. 5B). Previous reports have indicated that these reconstituted lines express approximately equal amounts of either wild-type or kinase-inactive protein (2). Although these results appear to contradict those seen with transient transfections of either Jak1-deficient HeLa cells or U4A cells, direct comparisons of the R89LRaf-1 activity in untreated U4A cells indicated that cells expressing wild-type Jak1 displayed approximately fivefold-greater enzyme activity than either parental 2fTGH cells or U4A cells (Fig. 5C). Cells expressing the kinase-inactive form of the protein showed a

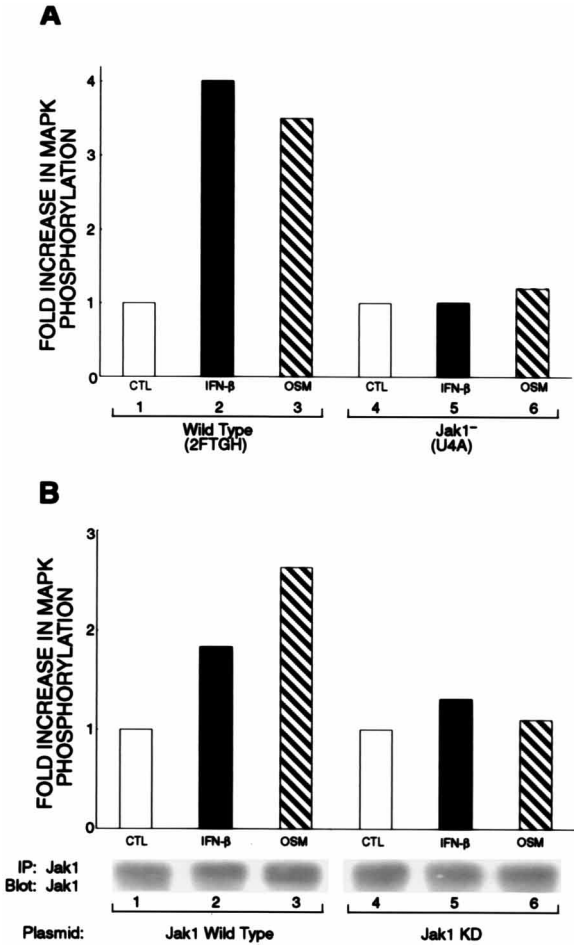

FIG. 4. Stimulation of Raf-1 kinase activity in wild-type and Jak1-deficient 2fTGH cells treated with IFN-β or OSM. (A) R89LRaf-1 is activated in 2fTGH but not Jak1-deficient U4A cells. Cells were transfected with R89LRaf-1 plasmid (18). At 48 h posttransfection, lysates were prepared from either untreated cells (lanes 1 and 4) or cells incubated for 5 min with IFN-β (lanes 2 and 5) or OSM (lanes 3 and 6). Cells were serum starved for 2 h prior to treatments. Cell extracts were prepared and incubated with monoclonal antibody 9E10, which recognizes the Myc epitope tag. Immunoprecipitates were assayed for Raf-1 kinase activity as described for Fig. 2. The increase in Raf-1 activity with IFN-β or OSM treatment of cells varied between 2- and 10-fold and was observed in at least 10 individual experiments. (B) Reconstitution of R89LRaf-1 activation by IFN-β and OSM in U4A cell lines transfected with either wild-type Jak1 (lanes 1 to 3) or kinase-inactive Jak1 (lanes 4 to 6). U4A cells were transfected as described above except that both R89LRaf-1 and wild-type or kinase-inactive Jak1 (Jak1 KD) were included in the transfections. Cells were either untreated (lanes 1 and 4) or incubated for 5 min with IFN-β (lanes 2 and 5) or OSM (lanes 3 and 6) prior to preparation of cell extracts and analysis of Raf-1 activity. The increase in Raf-1 activity with IFN-β or OSM treatment of cells varied between two- and fourfold after transfection of cells with wild-type Jak1 and was seen in two separate experiments. CTL, control; IP, immunoprecipitation.

three- to fourfold-elevated Raf-1 activity compared to 2fTGH cells. Enhanced basal activity of Raf-1 in U4A cells which have been reconstituted with wild-type Jak1 or kinase-inactive Jak1 compared to 2fTGH cells probably accounts for the lack of or minimal activation of Raf-1 by IFN-β and OSM in the U4A lines expressing Jak1. The fact that IFN-β is not capable of inducing tyrosine phosphorylation of Stats in U4A cells reconstituted with a kinase-inactive Jak1 (2), yet kinase-inactive Jak1 is able to partially restore IFN-β activation of Raf-1, suggests that the ability of Jak1 to regulate Raf-1 activation and Stat activation may be independent. It is of note that U4A cells transfected with the wild-type kinase display constitutively tyrosine phosphorylated Jak1, even though Stat1 proteins are not tyrosine phosphorylated in untreated cells (2, 20). Since tyrosine phosphorylation of Jak1 is associated with its activation, these results suggest that Jak1 may regulate Raf-1 activity and also support the concept that regulation of Raf-1 and Stat tyrosine phosphorylation by Jak1 are independent.

Many of the components required for cytokine activation of the Jak/Stat pathway are preassociated (13). To determine whether Raf-1 was associated with the Jaks required for IFN-β and OSM signaling, immunoprecipitated Jak1 and Tyk2 were assayed for Raf-1 kinase activity (Fig. 6). Immunoprecipitates of either Jak1 or Tyk2 from HeLa cells treated with either IFN-β or OSM contained enhanced Raf-1 activity. Immunoprecipitation of Jak2, a kinase not utilized by IFN-β or OSM, did not bring down more associated Raf-1 activity (lanes 4 to 6). Although in this particular assay we were unable to detect Raf-1 protein in the Jak1 or Tyk2 immunoprecipitates, other experiments have shown Raf-1 to be associated with Jak1 (unpublished data).

The fact that U4A cells which stably expressed wild-type Jak1 showed significantly elevated Raf-1 activity (Fig. 5C) suggested that the expression of a tyrosine-phosphorylated form of Jak1 might affect Raf-1 activity in a ligand-independent manner. To examine further this possibility, we assayed Raf-1 activity in COS cells transfected with Jak1. It is known that the Jaks, when expressed in COS cells, are constitutively active in that Stat proteins become tyrosine phosphorylated in the absence of the addition of ligand (24). Jak1 or Tyk2 proteins were expressed in COS cells, endogenous Raf-1 was immunoprecipitated, and its activity was assayed (Fig. 7). The resulting blots were also probed with an antiphosphotyrosine antibody or for the presence of Jak1 or Tyk2. Expression of Jak1 or Tyk2 not only stimulated Raf-1 activity but also allowed the detection of a specific association between Raf-1 and these kinases. A modest enhancement of Raf-1 activity was also seen when kinase-inactive Jak1 was expressed in COS cells (lane 5). Similar results were observed when 293 cells were transfected with Jak1 (data not shown). Immunoprecipitations with nonspecific serum detected no tyrosine-phosphorylated Jak1 or Tyk2 in the precipitates (data not shown). Overexpression of the related kinase Jak3 did not induce Raf-1 activity, nor did Jak3 coimmunoprecipitate with Raf-1 (lanes 4 in Fig. 7A and B). However, Jak3 was active in that its expression resulted in tyrosine phosphorylation of cotransfected Stat5 (Fig. 7C, lane 2).

DISCUSSION

Most if not all cytokines and growth factors that activate the Jak/Stat signaling pathway also stimulate MAPK activity. Recent evidence has suggested that activation of MAPK by IFN-β strongly enhances the ability of Stat1 to stimulate the expression of early response genes (6, 27). The data presented here demonstrate not only that activation of MAPK by IFN-β involves a Raf-1-dependent mechanism but also that activation of Raf-1 by several cytokines requires Jak1. A model consistent with the results presented in these studies with regard to IFN-α/β activation of Raf-1 is shown in Fig. 8. Although OSM appears to activate Raf-1 by a Ras-dependent mechanism and binds to a receptor that is distinct from the IFN-α/β receptor, the basic requirement of Jak1 for activation of Raf-1 by OSM is also clear. In addition to the role that Jak1 plays in activation of Raf-1 by these cytokines, Jak1-negative HeLa cells display constitutively enhanced tyrosine phosphorylation of p42 MAPK (Fig. 1) as well as elevated activity of the enzyme.

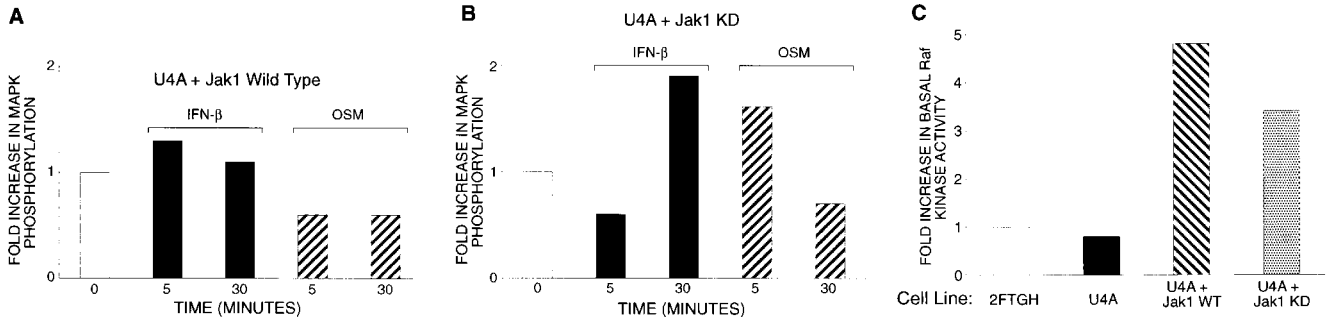

FIG. 5. IFN-β or OSM stimulation of Raf-1 kinase activity in Jak1-deficient cells which stably express either wild-type or kinase-inactive Jak1. (A and B) Reconstitution of R89LRaf-1 activation by IFN-β and OSM in U4A cell lines which express either wild-type Jak1 (A) or kinase-inactive Jak1 (Jak1 KD) (B). Cells were transfected with R89LRaf-1 as for Fig. 4 and incubated with either IFN-β or OSM for 5 or 30 min prior to preparation of cell extracts and analysis of Raf-1 activity. (C) The activity of R89LRaf-1 is elevated in U4A cells reconstituted with wild-type (WT) Jak1 compared with kinase-inactive Jak1. Cell lines were transfected with R89LRaf-1 as described above, and Raf-1 kinase was assayed 48 h posttransfection. The activity of the transfected R89LRaf-1 in 2fTGH cells was arbitrarily given a value of 1.

Jak1-deficient HeLa cells transfected with wild-type but not kinase-inactive Jak1 demonstrate that the basal activity of MAPK is inhibited when the enzyme is expressed. These results suggest that expression of Jak1 can also function as a negative regulator of MAPK activity in a Raf-1-independent manner. Initial results indicate that the activities of MEK1 and MEK2 are not altered in HeLa cells deficient in Jak1 expression (data not shown), implying that the activity of either the dual-specificity phosphatases which inactivate MAPK or another MEK-independent pathway which activates MAPK may be regulated by Jak1.

Although we have not directly demonstrated that IFN-β activation of Raf-1 is p21^{ras} independent, the ability of Jak1 to permit activation of Raf-1 does not appear to be influenced by Ras, since OSM, which stimulates GTP loading of p21^{ras}, and IFN-β, which does not, both require Jak1. Recent reports have indicated that Jak2 may also be required for Raf-1 activation by growth hormone and IFN-γ, but these results suggest that this may be a p21^{ras}-dependent process (29, 30). There are several possible explanations for this apparent discrepancy, including the different cell lines used for the analysis, the different cytokines used to stimulate Raf-1, and the different Jaks being examined.

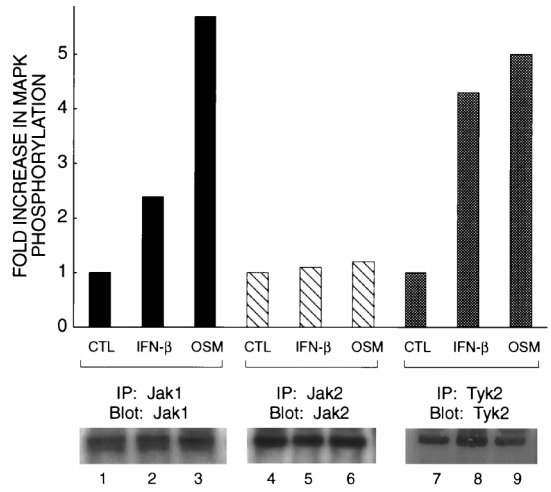

FIG. 6. Association of Raf-1 activity with Jak1 or Tyk2. Shown is coimmunoprecipitation of enhanced Raf-1 kinase activity from wild-type HeLa cells incubated with IFN-β or OSM. Lysates from either untreated (control [CTL]) cells (lanes 1, 4, and 7) or cells treated 5 min with IFN-β (lanes 2, 5, and 8) or OSM (lanes 3, 6, and 9) were prepared (6) and incubated with antiserum specific for Jak1 (5), Jak2 (UBI), or Tyk2 (5). Collected immunoprecipitates were assayed for Raf activity as described for Fig. 2. To ensure that equal amounts of Jak1, Tyk2, and Jak2 were present in each immunoprecipitate, the membranes were probed for the presence of each kinase (lower panels). IP, immunoprecipitation.

Only a kinase-active form of Jak1 can reconstitute IFN-β and OSM activation of Raf when transiently expressed in Jak1-deficient HeLa or U4A cells. However, stable lines of U4A cells which express the kinase-inactive Jak1 but not wild-type Jak1 permitted Raf-1 to be activated by both IFN-β and OSM, albeit at a much reduced level. Stable expression of either the wild-type or the kinase-inactive form of the enzyme in U4A cells caused a significant increase in the basal activity of Raf-1 compared to 2fTGH cells. There are several possible explanations for these apparent discrepancies. Jak1 may play a structural role in the IFN-β- and/or OSM-stimulated responses either in terms of functioning as a docking protein for kinase(s) involved in direct activation of Raf-1 and/or to permit receptor activation coupled to downstream signaling events. The latter possibility appears to be less likely with regard to OSM activation of downstream signaling events, since these are at least partially intact for IL-6 activation of U4A cells when they express a kinase-inactive form of the enzyme (11). IL-6 also uses gp130 as a component of its receptor. In addition, results from this lab indicate that OSM treatment of both parental and Jak1-deficient HeLa cells stimulates the synthesis of several polypeptides which have common mobilities by two-dimensional gel electrophoresis (data not shown). The role that Jak1 may play in the structural integrity of the IFN-α/β receptor independent of its activity as a protein kinase is less clear, since tyrosine phosphorylation of either chain of the receptor or of Jak1 or Tyk2 has not been well examined when U4A cells are reconstituted with kinase-inactive Jak1. It is known that IFN-α-stimulated tyrosine phosphorylation of Stat proteins is not seen in U4A cells which express kinase-inactive Jak1 (2). Clearly IFN-γ-stimulated tyrosine phosphorylation of the α chain of its receptor, Jak2, and Stat1 does occur in such cells (2). It is therefore possible that in U4A cells which express kinase-inactive Jak1, IFN-β may be able to activate weakly Tyk2, which could then activate Raf-1. Tyk2 which is constitutively activated when expressed in COS cells does increase Raf-1 activity (Fig. 7).

The mechanism by which stable expression of either wild-type or, to a more modest extent, kinase-inactive Jak1 activates Raf-1 in U4A cells in the absence of ligand also requires

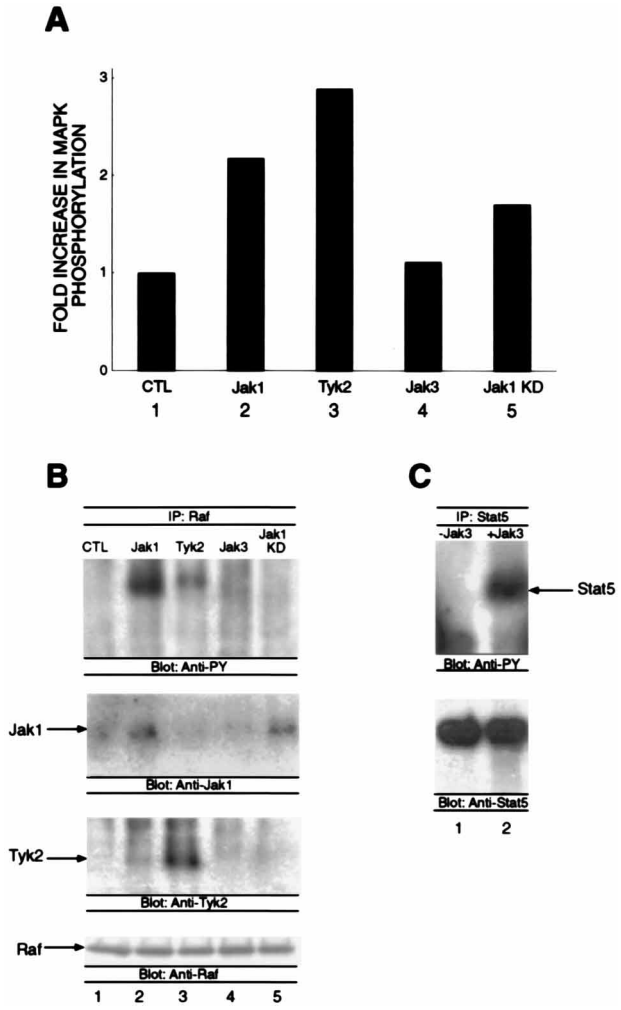

FIG. 7. Expression of Jak1 and Tyk2 in COS cells activates Raf-1. (A) Enhanced activity of Raf-1 in cells transfected with Jak1 or Tyk2. COS-7 cells were transfected by the DEAE-dextran technique with either β-galactosidase (lane 1), Jak1 (lane 2), Tyk2 (lane 3), Jak3 (lane 4), or kinase-inactive Jak1 (Jak1 KD; lane 5). Two days after transfection, the cells were cultured in serum-free medium for 3 h prior to preparation of cell lysates. Raf-1 was immunoprecipitated from the lysates, and activity was assayed as described for Fig. 1. The increase in Raf-1 activity after transfection of cells with wild-type Jak1 or Tyk2 varied between two- and threefold compared with cells transfected with CMV β-galactosidase. CTL, control. (B) Jak1 and Tyk2 immunoprecipitate with Raf-1. The membranes from Fig. 6 were probed with antiphosphotyrosine (Anti-PY), anti-Jak1, anti-Tyk2, or anti-Raf-1 antibody as indicated and developed with enhanced chemiluminescence. It should be noted that the weak signal seen in cells transfected with Jak3 and probed with antiphosphotyrosine (upper panel, lane 4) was probably due to the presence of Jak1 which may have been very weakly activated as a consequence of the expression of Jak3. This is consistent with the presence of a weak signal corresponding to Jak1 seen in lane 4 of the middle panel. Probing of the Raf-1 immunoprecipitate (IP) with Jak3 antiserum revealed no Jak3 (data not shown). (C) Expression of Jak3 stimulates tyrosine phosphorylation of Stat5. COS-7 cells were transfected with Stat5a (15) and β-galactosidase (lane 1) or Jak3 and Stat5a (lane 2) as described for Fig. 5A. Stat5 was immunoprecipitated, and the resulting blots were probed with either antiphosphotyrosine (top) or anti-Stat5 (bottom).

clarification (Fig. 5). Although in U4A cells the amount of Jak1 kinase protein that is expressed is not significantly different from the amount of endogenous Jak1 in 2fTGH cells, it is reported to be tyrosine phosphorylated in cells not exposed to cytokines (2). Thus, it is conceivable that although the kinase responds appropriately to cytokines with respect to activation of the Jak/Stat pathway, it may be constitutively activated with

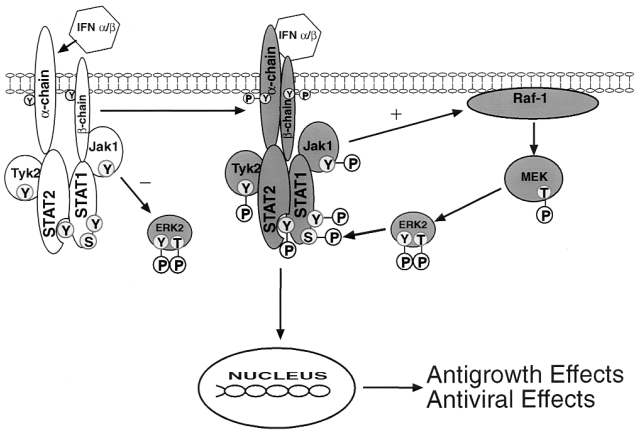

FIG. 8. Representation of the role of Jak1 in the regulation of activation of Raf-1/MEK/MAPK signaling cascade by IFN-α/β. Binding of IFN-α/β to its receptor induces dimerization of the two subunits and activation of Jak1 and Tyk2. Activation of Jak1 permits Raf-1 activation either directly or indirectly as well as tyrosine phosphorylation of Stat1 and Stat2. Activated Raf-1 phosphorylates MEK, which phosphorylates and activates Erk2. Serine phosphorylation of Stat1, possibly by Erk2, is required for this protein to maximally activate IFN-stimulated genes. In addition, the expression of Jak1 appears to inhibit basal Erk2 activity without affecting the activities of Raf-1 and MEK.

respect to regulation of Raf-1. The mechanism by which kinase-inactive Jak1 expressed in U4A cells results in modestly elevated Raf-1 kinase activity in the absence of cytokine stimulation is consistent with the possible structural role that this protein may play in either allowing the formation of functional receptors or serving as a docking molecule for other proteins which are directly responsible for activation of Raf-1.

The facts that (i) the kinase-inactive form of Jak1 expressed in U4A cells permitted IFN-β to activate Raf-1 but does not permit this cytokine to induce tyrosine phosphorylation of the Stat proteins (2) and (ii) wild-type Jak1 expressed in U4A cells (which is constitutively tyrosine phosphorylated) enhances Raf-1 activity in the absence of IFN-β but shows no Stat tyrosine phosphorylation argue that the abilities of this kinase to regulate Raf-1 and Stat activation may be independent. It needs to be clarified whether the domains in Jak1 which regulate Raf-1 activity and Stat tyrosine phosphorylation are distinct or overlapping. However, the ability of Jak1 and other members of this family of kinases to mediate both Jak/Stat and Raf-1/MAPK activation allows for optimization of two cytokine-stimulated signaling cascades, such that rapid and maximal induction of early response genes occurs.

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