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Roles of JAKs in Activation of STATs and Stimulation of c-*fos* Gene Expression by Epidermal Growth Factor

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The tyrosine kinase JAK1 and the transcription factors STAT1 and STAT3 are phosphorylated in response to epidermal growth factor (EGF) and other growth factors. We have used EGF receptor-transfected cell lines defective in individual JAKs to assess the roles of these kinases in STAT activation and signal transduction in response to EGF. Although JAK1 is phosphorylated in response to EGF, it is not required for STAT activation or for induction of the c-*fos* gene. STAT activation in JAK2- and TYK2-defective cells is also normal, and the tyrosine phosphorylation of these two kinases does not increase upon EGF stimulation in wild-type or JAK1-negative cells. In cells transfected with a kinase-negative mutant EGF receptor, there is no STAT activation in response to EGF and c-*fos* is not induced, showing that the kinase activity of the receptor is required, directly or indirectly, for these two responses. The data do not support a role for any of the three JAK family members tested in STAT activation and are consistent with a JAK-independent pathway in which the intrinsic kinase domain of the EGF receptor is crucial. Furthermore, data from transient transfection experiments in HeLa cells, using c-*fos* promoters lacking the STAT regulatory element c-*sis*-inducible element, indicate that this element may play only a minor role in the induction of c-*fos* by EGF in these cells.

Cytokines and growth factors interact with their cognate cell surface receptors to activate one or more signaling pathways. These pathways transduce specific responses to the nucleus, culminating in the transcriptional induction of discrete sets of target genes. Cell surface receptors fall into several large families: some have intrinsic tyrosine or serine/threonine kinase activity, some are coupled to G proteins, and some couple ligand binding to tyrosine phosphorylation but lack intrinsic kinase catalytic domains (3, 33, 46, 67, 68). Multiple, overlapping intracellular signaling pathways are utilized by members of each of these families of receptors, although the biochemical details of their actions are only now beginning to be uncovered.

Growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), utilize more than one signaling pathway to direct information from the cell surface to the nucleus. Upon binding ligand, the intrinsic kinase domain of the EGF receptor (EGFR) becomes activated, leading to the phosphorylation of several tyrosine residues in its cytoplasmic tail (67, 81). These phosphotyrosines serve as docking sites for proteins such as GAP, GRB2, and Shc, which have *src* homology 2 (SH2) domains that couple the EGFR to the ras pathway (2, 8, 40). Activated ras triggers a cascade of serine/threonine phosphorylations, mediated by kinases such as the mitogen-activated protein kinases (7), leading to phosphorylation in the nucleus of specific transcription factors (22).

Recently, it has been discovered that EGF activates a second pathway (14, 21, 52) which involves rapid activation of latent cytoplasmic transcription factors called STATs (signal transducers and activators of transcription) (5). Originally identified on the basis of their function in interferon (IFN)-dependent signal transduction (6, 12, 13, 31, 59), STATs are now known to play an integral role in transducing signals initiated by a wide variety of cytokines and growth factors (57). STATs become phosphorylated on tyrosine when they interact with activated ligand-receptor-JAK complexes and then move to the nucleus where they stimulate transcription of target genes (5, 27). To date, six distinct mammalian STAT family members have been cloned (1, 13, 25, 58, 72, 80, 83). At least two of these, STAT1 and STAT3 (known also as APRF), are activated by EGF in cultured cells (14, 49, 53, 62, 82) and in the livers of mice injected with EGF (51). Immunoprecipitation experiments have suggested that STAT1 can bind directly to the EGFR (14), presumably through an SH2-phosphotyrosine interaction (14, 52, 62). Once phosphorylated, STATs dimerize and bind to a number of DNA elements in vitro (52, 71). However, the role of either STAT1 or STAT3 in EGF signal transduction has yet to be fully defined.

Cytokine receptors that lack kinase catalytic domains often bind to and activate one or more members of the JAK tyrosine kinase family (27). These receptor-associated kinases then mediate ligand-dependent STAT phosphorylation (26). JAK1, JAK2, and TYK2 are widely expressed (9, 47, 75, 76), whereas JAK3 has been found only in lymphoid and myeloid cells (30, 77). EGF stimulates phosphorylation of JAK1 on tyrosine residues (60), a function that is dependent on the intrinsic kinase activity of the EGFR (79). However, the role of JAK1 in the overall response to EGF is unclear.

Some nuclear targets of growth factors, such as c-*fos*, are proto-oncogenes which are important in the mitogenic response (4). The c-*fos* promoter has multiple regulatory elements; of these, the serum response element (SRE) appears to be the major mediator of induction by serum and growth factors (10, 15, 17, 28, 65). Signals arrive at the SRE primarily

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through ras-dependent pathways (66). Serum response factor binds to the SRE constitutively (21), and the SRE-serum response factor complex in turn forms ternary complexes with other proteins, including TCF, Elk-1, or SAP-1 (22, 39), leading to activation of c-*fos* transcription following binding of growth factors to their receptors. Other regulatory elements have been identified within the c-*fos* promoter, including the binding sites for CRE and AP-1 (10, 11, 56), and a canonical STAT binding sequence, called the c-*sis*-inducible element (SIE), located ~35 nucleotides upstream of the SRE (20, 71). Stimulation with EGF results in the rapid binding of STATs to the SIE, as demonstrated by gel mobility shift (52, 62) and DNase I footprinting assays (21). The SIE contributes to the induction of c-*fos* by c-*sis* (PDGF) (50, 71), and experiments with transgenic mice have implicated the SIE in the regulation of c-*fos* expression in many tissues (50). It is known that EGF is capable of transcriptionally activating an SIE-minimal promoter construct in vitro (14). However, the role of the SIE in EGF-induced c-*fos* expression was not examined.

Here, by using EGFR-transfected cell lines lacking JAK1, JAK2, and TYK2, we show that none of these kinases are essential for EGF-dependent STAT activation. However, the intrinsic tyrosine kinase activity of the EGFR is required for the JAK-independent activation of STATs in response to EGF.

MATERIALS AND METHODS

Cell lines and EGF stimulation. 2fTGH cells and most mutant cell lines derived from them have been described elsewhere (29, 35, 42, 44, 45, 48). Mutant cell lines defective in JAK and STAT signaling components and their respective complementing proteins were as follows: U1A, TYK2; U3A, STAT1; U4A/U4C, JAK1; γ2A, JAK2. γ2A cells, which were isolated as described by Watling et al. (73), are defective in JAK2 (74). A431 and HeLa cells were obtained from the American Type Culture Collection. HER14 and K721A cells have been described previously (23, 24). All cells were cultured in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum. Cells transferred to serum-free medium for 24 h were treated with recombinant human EGF (Oncogene Science) at 200 ng/ml for 15 min (gel mobility shift experiments), 30 min (RNA analyses), or 1 h (luciferase assays).

Gel mobility shift assays, immunoprecipitations, Western blotting (immunoblotting), use of the fluorescence-activated cell sorter (FACS), and RNA analyses. Gel mobility shift experiments were performed with whole-cell extracts as described previously (52). For supershift analyses, the appropriate antiserum was added to the extracts, in the presence of 0.5 μg of poly(dI-dC) per ml, for 1 h before adding probe. Antisera to STAT1 and STAT3 were kindly provided by James E. Darnell, Jr. (Rockefeller University). High-affinity SIE (hSIE) probes were prepared according to published procedures (71).

Antisera to JAK1, JAK2, TYK2, and EGFR were purchased from Upstate Biotechnology Inc., and antiphosphotyrosine monoclonal antibodies 4G10 and PY20 were obtained from Upstate Biotechnology Inc. and Signal Transduction Laboratories, respectively. Immunoprecipitations were done as described previously (58). The precipitated proteins were resolved in an 8% polyacrylamide gel, transferred to polyvinylidene difluoride membranes, and incubated with antibodies. Bands were visualized by enhanced chemiluminescence, with Renaissance Kit reagents (DuPont).

To analyze EGFR expression, cells were incubated with an anti-EGFR monoclonal primary antibody (Oncogene Science) and then stained with a goat anti-mouse fluorescein-conjugated secondary antibody (DAKO Corp.). Analyses were performed on a Becton Dickinson FACScan instrument with the LYSYS II software package.

RNA was isolated by the Trizol method according to the manufacturer's specifications (Gibco BRL). Twenty micrograms of total RNA was separated by electrophoresis in 1.5% agarose formaldehyde gels, transferred to nylon membranes, and hybridized with c-*fos* or β-actin probes. The c-*fos* probe was a 747-bp *Acc*I restriction enzyme fragment encompassing exon 4 of the c-*fos* gene (65). The probes were labeled by random priming according to manufacturer's specifications (Amersham). Hybridizations were carried out at 42°C in 50% formamide buffer, and the membranes were washed under stringent conditions (55). RNase protection experiments were performed as described previously (36) with probes from c-*fos* (16) and glyceraldehyde-3-phosphate-dehydrogenase.

Reporter gene constructs and transfection procedures. The promoterless luciferase reporter gene plasmid, pGL2Basic (Promega), was used to construct all c-*fos* reporter plasmids. Transfections were performed by the calcium phosphate technique (32). The *Xho*I (−711)-to-*Nae*I (+45) fragment of the human c-*fos* promoter from plasmid pF4 (65) was cloned into *Xho*I-*Sma*I-restricted pBluescript II (Stratagene) to generate plasmid pBShfospro. An *Xho*I-to-*Hin*dIII fragment from pBShfospro was then cloned into pGL2Basic to generate the −711fosluc plasmid. The −360fosluc plasmid was prepared by *Pst*I digestion of the pBShfospro plasmid to liberate the c-*fos* promoter sequences from −360 to +16, which were then subcloned into *Pst*I-cut pBluescript II, to give the plasmid pBS−360fos. pBS−360fos was restricted with *Sac*I-*Hin*dIII, and the promoter fragment was cloned directionally into pGL2Basic. For the −335fosluc construct, the PCR was used to amplify the desired fragment from plasmid pBS−360fos by using the T3 primer of pBluescript II and a promoter-specific primer designed to recognize SRE sequences downstream of the SIE (5′-GAATTCTTACACAG GATGTCCATATTAGGA-3′). The expected PCR product was purified in a gel and ligated into pGL2Basic. The plasmid −222fosluc was constructed by digesting the −711fosluc plasmid with *Xho*I and *Apa*I (base −222 in the c-*fos* promoter) and treating the product with the Klenow fragment of DNA polymerase I before self-ligation. For the SIE−222fosluc plasmid, complementary oligonucleotides representing the wild-type SIE (sense, 5′-GTCGACAGTTCCCGTC AATCGTCGA-3′, and antisense, 5′-TCGACGATTGACGGGAACTGTCG AC-3′) were annealed, kinase treated, and ligated upstream of the −222 site within the −222fosluc plasmid. All reporter constructs were checked by sequence and/or restriction analysis.

A simian virus 40 promoter–β-galactosidase reporter plasmid, pCH110 (Pharmacia), was cotransfected with the test plasmids as an internal control to normalize for transfection efficiency. β-Galactosidase activity was measured in cell lysates by using the β-Galactosidase Enzyme Assay System kit (Promega).

For stable transfectants, 10 μg of the plasmid pRcCMVHER1 (69), containing the human EGFR cDNA under control of the cytomegalovirus promoter, was cotransfected with 1 μg of a puromycin resistance plasmid (pSV2Puro) by the calcium phosphate technique. Puromycin-resistant colonies were selected in 1 μg of drug per ml for 1 to 2 weeks and then pooled prior to FACS sorting for high EGFR expression.

RESULTS

STAT activation by EGF in 2fTGH and mutant cell lines. Parental 2fTGH cells and the mutant cell lines U1A, U3A, U4A, and γ2A (Materials and Methods) express EGFRs, as determined by FACS (Fig. 1A) and Western blot (34) analyses. However, stimulation of the endogenous receptors with EGF did not result in detectable STAT activation (Fig. 1B) or in an increase in c-*fos* mRNA expression (34). To obtain higher expression, a full-length human EGFR cDNA was stably transfected into 2fTGH and mutant cells. The resulting populations were enriched for cells expressing high levels of EGFR by FACS sorting (Fig. 1A). In contrast to parental cells, EGFR-transfected 2fTGH cells (2f/EGFR) exhibited strong STAT activation in response to EGF (Fig. 1B). The response of these cells was comparable to that of A431 or HeLa cells (Fig. 1B), already known to express functional EGFR (43, 78).

EGF treatment induces activation of latent cytoplasmic DNA-binding proteins that can be identified in gel shift experiments with an hSIE probe (71). Three complexes, previously designated SIF-A, SIF-B, and SIF-C (Fig. 1B), have been shown to represent STAT3 homodimer, STAT1/3 heterodimer, and STAT1 homodimer, respectively (82). The three complexes observed in EGF-treated 2f/EGFR cell extracts fit the above assignments as determined by supershifting with specific antibodies (Fig. 2). Further verification was obtained in U3A/EGFR cells, which lack STAT1 protein. As expected, SIF-A was the only complex present following EGF treatment of U3A/EGFR cells (Fig. 2).

Roles of JAK1, JAK2, and TYK2 in EGF-induced STAT activation. The tyrosine kinase JAK1 is phosphorylated in response to EGF, suggesting a possible role in activating STAT proteins (60). The tyrosine phosphorylation of JAK1 did increase in response to EGF in 2f/EGFR cells (Fig. 3A). Therefore, the response of U4A/EGFR cells, which lack JAK1 (44), was assayed. Both STAT1 and STAT3 were activated by EGF in U4A/EGFR cells at levels indistinguishable from those observed in parental 2f/EGFR cells (Fig. 2). To confirm these results, a derivative of a different JAK1-negative cell line, U4C (74), was also tested. U4C/EGFR cells also supported STAT phosphorylation and activation in response to EGF (34).

Two other JAK family members, TYK2 and JAK2, were also

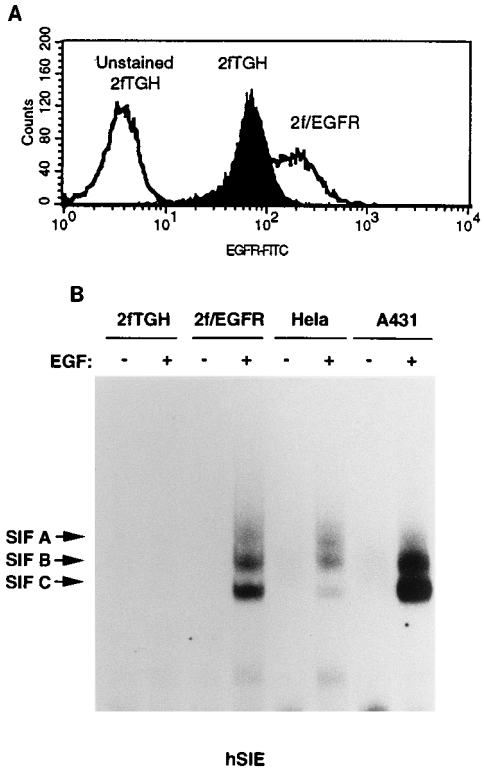

FIG. 1. Characterization of EGFR expression in 2fTGH cells. (A) FACScan analysis of cell surface EGFR expression. The level of staining of parental 2fTGH (filled profile) is compared with that of stably transfected 2f/EGFR cells that were sorted previously for higher cell surface EGFR expression (open profile). (B) Gel mobility shift analysis of EGF-induced STAT binding to a ^{32}P-labeled hSIE probe. Whole-cell extracts were prepared from 2fTGH, 2f/EGFR, HeLa, and A431 cells treated for 15 min with 200 ng of EGF per ml. Complexes SIF-A, SIF-B, and SIF-C are marked.

analyzed to determine if they might play a role in the EGF-dependent activation of STAT proteins. Although JAK2 does not become phosphorylated on tyrosine in response to EGF in A431 cells (60) and neither protein shows an increase in phosphorylation status in 2fTGH cells (34), we could not rule out the possibility that either JAK2 or TYK2 might play some role

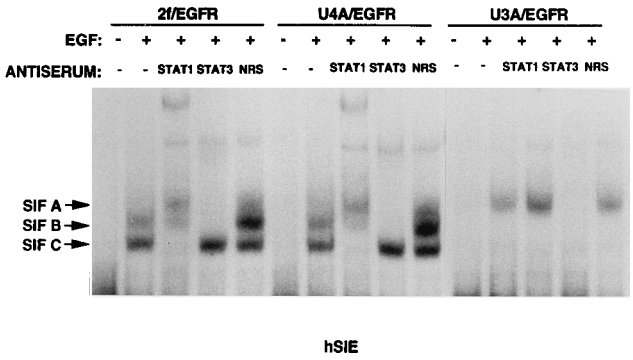

FIG. 2. STAT activation by EGF in the absence of JAK1 or STAT1. (A) Gel mobility shift analysis of STAT complexes formed in whole-cell extracts of the cell lines 2f/EGFR (lanes 1 to 5); U4A/EGFR (missing JAK1, lanes 6 to 10); and U3A/EGFR (missing STAT1, lanes 11 to 15). An hSIE probe was used. Supershift analyses were performed with antisera to human STAT1 (lanes 3, 8, and 13) and murine STAT3 (lanes 4, 9, and 14) or normal rabbit serum (NRS; lanes 5, 10, and 15).

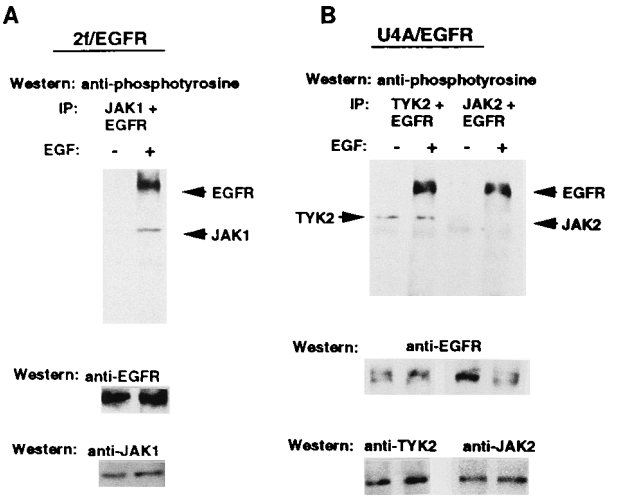

FIG. 3. Tyrosine phosphorylation of JAKs and the EGFR by EGF. (A) JAK1 and EGFR were immunoprecipitated from 2f/EGFR lysates and, after electrophoresis, transferred to a polyvinylidene difluoride membrane, which was probed with the antiphosphotyrosine antibodies 4G10 and PY20 (top panel). The blot was then stripped and reprobed with an EGFR antibody (middle panel) and then with a JAK1 antibody (bottom panel). (B) Immunoprecipitation of TYK2, JAK2, and EGFR from U4A/EGFR cell lysates. The precipitated proteins were transferred to a polyvinylidene difluoride membrane after electrophoresis and probed with antiphosphotyrosine antibodies (top panel). A low, constitutive level of TYK2 tyrosine phosphorylation has been observed previously (35). After analysis, the blots were stripped and reprobed with antisera to EGFR, TYK2, or JAK2 as in panel A.

in EGF signaling. Therefore, STAT activation was examined in U1A/EGFR and γ2A/EGFR cells, which lack TYK2 and JAK2, respectively. Activation was observed in both cell lines, suggesting that neither TYK2 nor JAK2 was essential (Fig. 4). The slightly lower level of STAT3 activation by EGF in U1/EGFR and γ2A/EGFR cells is likely to represent a lower overall level of receptor expression in these cells and is not a direct consequence of the TYK2 defect. It was important to determine whether TYK2 or JAK2 might substitute for JAK1 in its absence. To this end, their phosphorylation was examined in EGF-treated U4A/EGFR cells. As an internal control, the EGFR was also immunoprecipitated and probed with the antiphosphotyrosine antibody. The receptor was strongly phosphorylated on tyrosine in a ligand-dependent manner, whereas the phosphorylation of JAK2 and TYK2 remained unchanged in the JAK1-negative U4A/EGFR cells (Fig. 3B).

Activation of STAT proteins by EGF requires the EGFR kinase. The apparent lack of a requirement for any specific JAK kinase individually prompted us to examine the role of the intrinsic EGFR kinase. STAT activation was measured in mouse NIH 3T3 cells expressing different versions of the human EGFR (Fig. 5). These cells lack endogenous murine EGFR and therefore rely on the transfected human receptor for a response to EGF (24). In HER14 cells expressing wild-type EGFR, STAT1 and STAT3 were activated in response to EGF, as indicated by formation of SIF-A, -B, and -C (lane 2) and confirmed by antibodies. However, in K721A cells, which express a mutant EGFR lacking intrinsic protein tyrosine kinase activity, the STAT proteins were not activated in response to EGF (lane 4). As expected, these cells also failed to induce expression of c-*fos* mRNA (Fig. 5B, lane 4), which was highly inducible in HER14 cells.

Role of STATs in the EGF-stimulated expression of c-*fos*. The induction of c-*fos* mRNA by EGF was similar in 2f/EGFR, U4A/EGFR, and U3A/EGFR cells (Fig. 6). Although both the

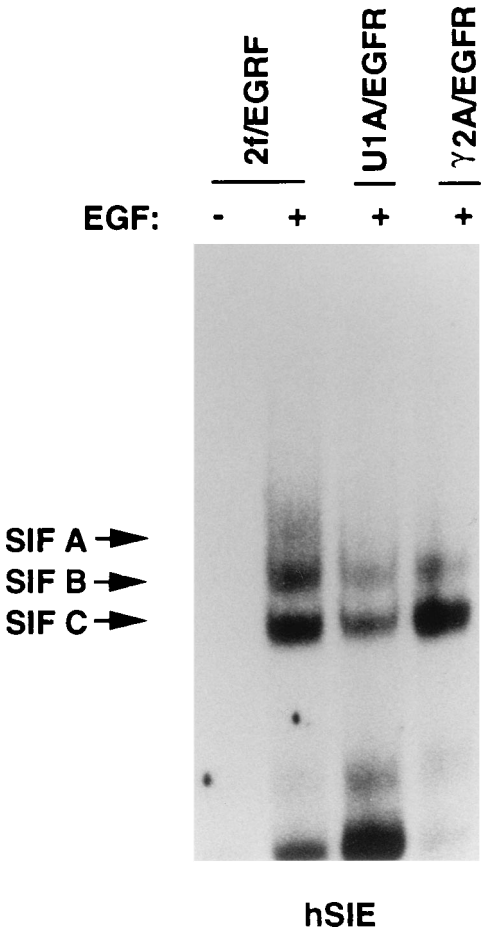

FIG. 4. STAT activation in the absence of TYK2 or JAK2. Gel mobility shift analyses of EGF-induced complexes were performed with extracts of the cell lines 2f/EGFR, U1A/EGFR (missing TYK2, lane 3), and γ2A/EGFR (missing JAK2, lane 4). An hSIE probe was used. The three SIF complexes are marked.

uninduced and EGF-stimulated levels of c-*fos* mRNA were lower in U4A/EGFR cells than in 2f/EGFR or U3A/EGFR cells, the induction in response to EGF was not significantly different. Since c-*fos* expression in U4C/EGFR cells did not differ from that in 2f/EGFR cells (34), it is unlikely that the reduced level of c-*fos* mRNA observed in U4A/EGFR cells was due to a specific effect of the JAK1 defect in these cells. A surprising outcome of this experiment was that the transcriptional response of c-*fos* in U3A/EGFR cells was equivalent to that observed in the parental cells (Fig. 6, lanes 2 and 4). Only the STAT3 homodimer is induced in U3A/EGFR cells (Fig. 2), suggesting that this dimer might be sufficient for EGF-dependent c-*fos* expression. Alternatively, the STAT pathway might play only a minor role in the overall response of the c-*fos* gene to EGF.

To examine this possibility in more detail, a series of constructs in which different lengths of the human c-*fos* promoter regulate the expression of a luciferase reporter gene (Fig. 7) were made. Included were a "full-length" c-*fos* promoter (to position −711), a −360 promoter that contains all elements known to be important for regulation (including the SIE), and a −335 promoter that contains a functional SRE and all other known regulatory elements but lacks the SIE. In addition, a −222 promoter construct and an SIE-222 promoter construct were used to examine the activity of the SIE in the absence of the SRE (Fig. 7). In general, the EGF-dependent induction of the endogenous c-*fos* gene was much stronger in HeLa than in 2f/EGFR cells (12-fold versus 4-fold), as was the induction of the c-*fos* promoter-luciferase reporter constructs (sevenfold versus twofold). Therefore, the reported transfection experiments were performed in HeLa cells.

The average fold induction for each reporter construct, obtained from four independent transient-transfection experiments, each carried out in duplicate, is shown in Fig. 7. As expected from the Northern (RNA) data (Fig. 6), we did not see a significant effect of the SIE in the context of the complete regulatory region. The level of induction by EGF, which was maximal for the −360 construct (about sevenfold), was not reduced upon removal of the SIE (compare −360 with −335). When both the SIE and the SRE were eliminated, the induction in response to EGF was substantially reduced, although there was still less than twofold induction when the −222 promoter construct was used. This was enhanced by an additional twofold when a single SIE was added. The promoterless pGL2Basic plasmid displayed no basal or EGF-stimulated luciferase activity (34).

DISCUSSION

Most cytokines that signal through JAKs and STATs, including the IFNs, interact with transmembrane cell surface receptors that couple ligand binding to tyrosine phosphorylation but have no intrinsic tyrosine kinase activity themselves (3, 33). This function is provided, in most cases, by one or more JAKs, which associate with the cytoplasmic domains of the receptors and are catalytically activated following ligand binding (27). Growth factors, on the other hand, bind to and activate recep-

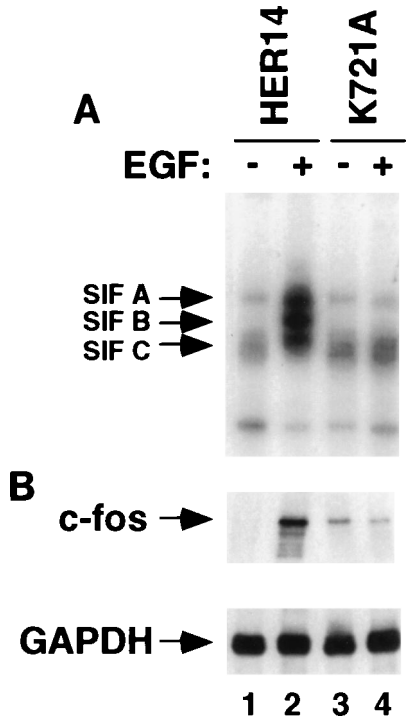

FIG. 5. STAT activation and c-*fos* induction in cells expressing wild-type or kinase-defective EGFR. (A) HER14 or K721A cells were left untreated or were treated with 100 ng of human EGF per ml for 15 min. Approximately 5 μg of nuclear extract was used in electrophoretic mobility shift assays. (B) A total of 10 μg of RNA was analyzed by RNase protection with probes for c-*fos* or glyceraldehyde-3-phosphate dehydrogenase.

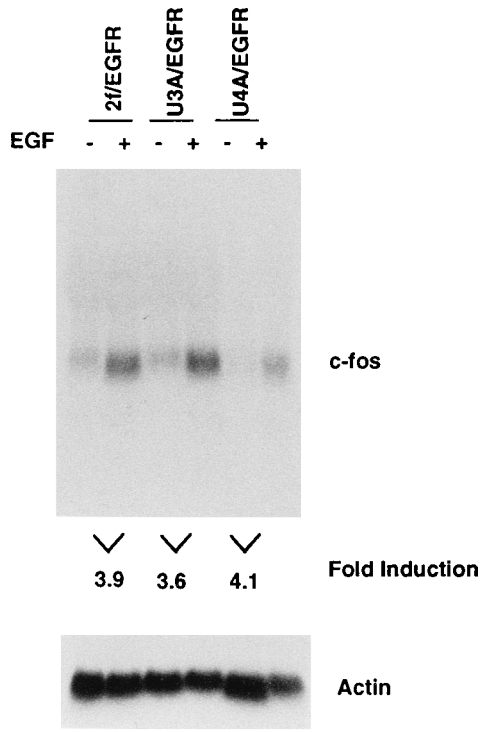

FIG. 6. Induction of c-*fos* mRNA by EGF. Total RNA (20 μg), isolated from untreated (−) or EGF-treated (+) 2f/EGFR, U3A/EGFR, and U4A/EGFR cells, was separated by electrophoresis, blotted onto a nylon membrane, and hybridized with an exon 4-specific c-*fos* probe. The blot was then stripped and reprobed with a β-actin probe to normalize for loading. Relative levels of c-*fos* induction were determined by quantitation with a phosphoimager (Becton Dickinson) and corrected for loading differences.

tors with intrinsic tyrosine kinase activities (67), bringing into question the need for additional receptor-associated kinases to catalyze STAT phosphorylation.

JAK1 is required to transduce signals initiated by several different cytokines (26, 57) and has been implicated as a mediator of EGF signal transduction, on the basis of its phosphorylation in response to EGF (60). However, our data demonstrate that the EGF-dependent activation of STATs in U4A/EGFR cells, which lack JAK1, is normal, implying either that JAK1 is not involved in EGF-stimulated STAT activation or that another JAK family member (or a related kinase) can act in a surrogate manner to phosphorylate STATs in the absence of JAK1. We think that JAK2 and TYK2 are unlikely to function in this manner, since we do not detect phosphorylation of either, even when JAK1 is missing. More importantly, we show that a kinase-defective EGFR mutant fails to activate STAT1 or STAT3 following stimulation with EGF. Together, these data indicate that no JAK is likely to be required for STAT activation in response to EGF and that the intrinsic kinase activity of the EGFR is somehow involved. Comparable results have been obtained with the PDGF receptor. Although JAK1 is activated in response to PDGF, there is no defect in SIF formation in U4A.PS1 cells (expressing PDGF receptors) (70), and kinase-defective PDGF receptors also fail to activate STATs in response to ligand (54). These data are consistent with a receptor kinase-dependent mechanism of STAT activation in which JAK kinases are either redundant or unnecessary. Nonetheless, we cannot at this time rule out the possibility that other kinases, such as src kinases, are involved in the phosphorylation and activation of STATs in response to EGF treatment.

Multiple JAKs are often phosphorylated in response to cytokines and growth factors (57), although, in many instances, the purpose of this phosphorylation remains unclear. For example, interleukin 6, CNTF, and OSM, all of which use a common gp130 receptor subunit, activate multiple JAK proteins which differ depending on the cell line examined (38, 63). Experiments with our JAK-defective mutant cells have revealed that, with interleukin 6, only the loss of JAK1 has a major effect on STAT activation, phosphorylation of gp130, or transcriptional activation of the interleukin 6-dependent IRF-1 gene (18). Growth hormone also activates both JAK1 and JAK2 in 2fTGH cells, and similarly, only JAK2 is required for STAT activation (19). PDGF induces the phosphorylation of JAK1, JAK2, and TYK2 in 2fTGH cells, but no one of these kinases is required individually for STAT activation (70). Therefore, phosphorylation of a JAK family member is insufficient evidence that it is required in a signal transduction pathway leading to activation of a particular STAT. What, if any, role JAK1 plays in the response to EGF is unclear. Our data indicate that JAK1 is unlikely to function in any aspect of the EGF response that is involved in c-*fos* expression, such as regulation of ras-dependent signaling, since we did not detect any reduction in c-*fos* gene induction by EGF in U4A/EGFR cells. We have also found that Shc phosphorylation in JAK1-negative cells is normal (34), ruling out a role for JAK1 in this early tyrosine phosphorylation event. Thus, it may be that JAK1 phosphorylation in response to EGF is spurious, although we cannot rule out the possibility that JAK1 mediates effects that do not influence c-*fos* gene expression.

c-*fos* is one of only a few EGF-induced genes known to have a STAT binding element (the SIE) in its promoter region. In the case of the IFNs, ISRE or GAS elements are both necessary and sufficient to confer responsiveness (5). In contrast, the c-*fos* promoter has many regulatory elements, some of which have not yet been characterized fully in terms of their contributions to expression. We found that the SIE plays only a minor role in the EGF-dependent inducibility of a c-*fos* promoter-luciferase reporter gene construct in HeLa cells (Fig. 7). These data are in agreement with earlier work that had implicated the SRE as the primary mediator of c-*fos* responsiveness to EGF in HeLa cells (10) or to PDGF (c-*sis*) in murine 3T3 cells (37, 61). Others found the SIE to contribute significantly to the PDGF response in 3T3 cells, although those studies concluded that the SRE was the stronger of the two elements

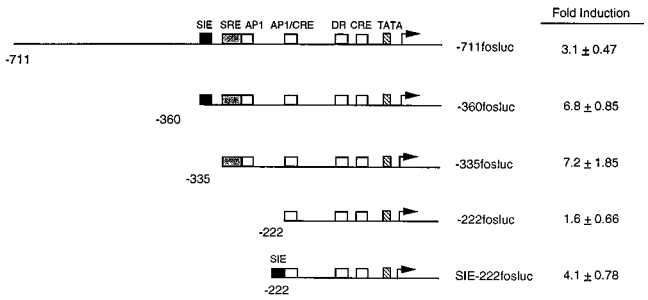

FIG. 7. Responses of c-*fos* promoter constructs to EGF in HeLa cells. In each experiment, the cells were transiently transfected, in duplicate, with equimolar amounts of c-*fos* gene promoter-luciferase reporter gene constructs. A simian virus 40–β-galactosidase expression plasmid was cotransfected as an internal control. After transfection and recovery (12 h), the cells were placed into serum-free medium for 24 h before treatment with EGF (200 ng/ml, 45 min). Luciferase and β-galactosidase activities were measured in cell lysates, and the fold induction in response to EGF (normalized for transfection efficiency) was determined. The numbers reported are the combined results of three to five independent experiments for each construct (± standard error of the mean).

(71). Transgenic mouse studies have demonstrated that the SIE is an important regulator of c-*fos* expression in vivo, as determined by examining the regulation of a β-galactosidase reporter gene under the control of a c-*fos* promoter containing either wild-type or mutant SIE (50). These same studies also indicated that the SIE was important for PDGF-mediated c-*fos* induction in cultured primary fibroblasts, but the role of the SIE in the EGF response was not examined (50). Although they proposed that the SIE plays an important role in EGF-dependent transcriptional activation of c-*fos*, the SIE was not examined in the context of the c-*fos* promoter by Fu and Zhang (14). In our hands, the SIE did function as an EGF-dependent enhancer element but only when the SRE was absent (Fig. 7), suggesting that the SRE is dominant to the SIE in the cells we studied. Nonetheless, we cannot rule out the possibility that the SIE plays an important role in c-*fos* induction in other cell types or in a different physiological setting. Different cell lines have been shown to exhibit varying degrees of dependence on the SRE for the serum inducibility of c-*fos* gene expression (61). Differences in relative levels of STAT activation, e.g., the ratio between activated STAT1 and STAT3, may also contribute to the specificity of the responses to EGF or other factors. Furthermore, specific STAT family members may be expressed in a cell-specific manner. Indeed, a combination of these variables is likely to be important in determining the overall significance of the STAT pathway in response to a given ligand such as EGF.

Because of the minimal role played by STATs in the EGF-dependent induction of c-*fos* in 2fTGH cells (Fig. 7 and data not shown), we have been unable to determine the relative importance of STAT1 and STAT3 in the pathway. However, our experiments have revealed that STAT3 activation occurs independently of STAT1 (Fig. 2). Previous cell-free experiments have shown that STAT1 interacts directly with the EGFR, suggesting that STAT1 binding and activation are independent of STAT3 (14). Together, the results suggest that STAT1 and STAT3 may each interact directly with the EGFR. In contrast, STAT1 phosphorylation in response to alpha IFN (IFN-α) depends on STAT2, suggesting an ordered binding of these two proteins to the IFN-α receptor (35).

The role of STATs in EGF signaling remains unclear. c-*fos* is not the only gene induced by EGF (41, 64), and it is possible that other targets may utilize STATs alone for activation. Some tissue-specific genes may also be primarily STAT responsive, much as c-*fos* appears to be primarily responsive to the ras-dependent pathways in HeLa cells. More work will be required to identify additional EGF-responsive genes and to determine how they are activated, emphasizing the relative contributions of these two distinct signaling pathways.

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