STAT Activation by Epidermal Growth Factor (EGF) and Amphiregulin

REQUIREMENT FOR THE EGF RECEPTOR KINASE BUT NOT FOR TYROSINE PHOSPHORYLATION SITES OR JAK1*

(Received for publication, November 28, 1995, and in revised form, February 8, 1996)

Michael David‡§, Lily Wong†, Richard Flavell†, Stewart A. Thompson§, Alan Wells**, Andrew C. Larner†, and Gibbes R. Johnson‡§

From the ‡Division of Cytokine Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892, †Howard Hughes Medical Institute, Yale University, New Haven, Connecticut 06510, ‡Department of Protein Chemistry and Biophysics, Berlex Biosciences, Richmond, California 94804, and **Department of Pathology, University of Alabama at Birmingham and Veterans Administration Medical Center, Birmingham, Alabama 35294

The epidermal growth factor (EGF) receptor activates several signaling cascades in response to the ligands EGF and amphiregulin (AR). One of these signaling events involves the tyrosine phosphorylation of STATs (signal transducers and activators of transcription), a process believed to require the activation of a tyrosine kinase of the JAK family. In this report we demonstrate that EGF- and AR-induced STAT activation requires the intrinsic kinase activity of the receptor but not the presence of JAK1. We show that both wild type (WT) and truncated EGF receptors lacking all autophosphorylation sites activate STAT 1, 3, and 5 in response to either EGF or AR. Furthermore, relative to cells expressing WT receptor, ligand-induced tyrosine phosphorylation of the STATs was enhanced in cells expressing only the truncated receptor. These results provide the first evidence that (i) EGF receptor-mediated STAT activation occurs in a JAK1-independent manner, (ii) the intrinsic tyrosine kinase activity of the receptor is essential for STAT activation, and (iii) tyrosine phosphorylation sites within the EGF receptor are not required for STAT activation.

The activated epidermal growth factor (EGF) receptor mediates a number of important biological responses in mammalian cells including the stimulation of cell division, migration, and differentiation (1). Six different polypeptide ligands, which derive from distinct genes, are capable of binding to the extracellular domain of the EGF receptor. These ligands include EGF, transforming growth factor-α, amphiregulin (AR), heparin binding EGF-like growth factor, epiregulin, and betacellulin (2). All of these growth factors contain a characteristic EGF-like domain, which is defined by 6 evenly spaced cysteine residues that generate 3 loops through the formation of disulfide bonds. In the case of AR it has been demonstrated that heparan sulfate proteoglycans are necessary for activation of the EGF receptor and a cellular response (3). Upon ligand binding, the intrinsic tyrosine kinase activity of the EGF receptor is augmented, resulting in autophosphorylation as well as the phosphorylation of specific intracellular substrates (4). Among the target proteins that become phosphorylated in response to EGF are mitogen-activated protein kinase, the adapter protein SHC, the GTPase-activating protein of p21ras, phospholipase C-γ, the phosphotyrosine phosphatase 1D, as well as the non-receptor tyrosine kinase jak1 (5) and members of the STAT (signal transducers and activators of transcription) family of transcription factors (6–8). Several reports have shown that the intrinsic receptor kinase activity is necessary for activation of a subset of these proteins, whereas others can be activated also via a kinase-inactive EGF receptor (9–11). Additional mutations have allowed for the identification of receptor domains that are needed for cell motility, mitogenesis, and activation of mitogen-activated protein kinase or phospholipase C-γ (12, 13). In the case of the STAT proteins it has been suggested that the phosphorylation of the receptor itself on tyrosine residues is a requirement for their activation (14). Based on mutational analysis of several cytokine receptors and the Src homology 2 (SH2) domains of the STAT proteins, it is believed that the specificity of STAT activation is conferred by an interaction between the distinct tyrosine phosphorylation sites of a certain receptor and the SH2 domains of a STAT (15, 16). In this report we show that the EGF receptor kinase activity is needed for STAT tyrosine phosphorylation, whereas the presence of jak1 is not necessary. Furthermore, we provide evidence, using a truncated receptor lacking all autophosphorylation sites, that receptor phosphorylation is not required for STAT activation by either AR or EGF.

MATERIALS AND METHODS

Cells—Establishment of NR6 cell lines expressing the various mutant EGF receptors has been described elsewhere (12). All NR6 cell lines were grown in α-minimum essential medium supplemented with 10% fetal bovine serum, non-essential amino acids, and l-glutamine. Characterization of the HeLa cell line lacking jak1 has been also previously published (17). These cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

Cytokines and Growth Factors—Human and murine γ-interferon (IFN-γ) were a generous gift from Genentech. EGF was purchased from Life Technologies, Inc. Recombinant human AR was expressed in Escherichia coli and purified, and homogeneity was confirmed by reverse phase high pressure liquid chromatography, Edman degradation, and electrospray mass spectrometry.2

Whole Cell Extracts—After treatment, cells were washed once with ice-cold phosphate-buffered saline and lysed on the plates with 1.5 ml of lysis buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM vanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (all from Sigma). The lysate was vortexed, incubated on ice for 10 min, and centrifuged at 18,000 × g for 10

2 S. A. Thompson, A. Harris, D. Huang, M. Ferrer, and G. R. J Johnson, manuscript in preparation.
RESULTS AND DISCUSSION

Jak1 Is Not Required for EGF Receptor-mediated Activation of STATs—It has been previously shown that EGF can stimulate tyrosine phosphorylation of STAT 1, 3, and 5 such that they can bind to the IFNγ-activatedsequence-like enhancers (6–8). EGF induces tyrosine phosphorylation of Jak1 but not of the related kinases Jak2 or Tyk2 (5). We therefore wanted to investigate whether AR, another growth factor known to signal through the EGF receptor (21), also activates STAT proteins in a manner similar to EGF. Furthermore, we sought to determine whether activation of Jak1 is a requirement for STAT activation by EGF or AR, since it has been previously reported that immunoprecipitations containing activated EGF receptor were able to cause tyrosine phosphorylation of STAT proteins in vitro (22). To address this question we utilized a HeLa cell line that has been shown to be devoid of Jak1 (17) but contains identical levels of EGF receptor compared with the wild type (WT) counterpart (data not shown). Whole cell extracts were prepared from either WT (Fig. 1A, lanes 1–4) or Jak1-deficient HeLa cells (lanes 5–8) that were either left unstimulated (lanes 1 and 5) or treated with either AR (lanes 2 and 6), EGF (lanes 3 and 7), or IFNγ (lanes 4 and 8). Cell shift assays were then performed using the SIE, a IFNγ-activated sequence-like element in the c-fos promoter. This element was identified as a STAT binding site and has been shown to be required for maximal activation of c-fos expression by EGF and platelet-derived growth factor (8, 19). The results presented in Fig. 1A demonstrate that AR and EGF can activate STAT binding to DNA to a similar extent in both cell lines, whereas IFNγ-induced formation of the DNA binding complex is absent in Jak1-deficient cells (lanes 5–8).

Intrinsic EGF Receptor Kinase Activity Is Necessary for STAT Activation—To answer the question whether the kinase activity of the EGF receptor was required for STAT activation, we employed cell lines derived from NR6 murine fibroblasts, which lack any endogenous EGF receptors. Cells expressing either the WT receptor or a mutant receptor that is kinase-inactive due to a point mutation within the ATP binding site (M721) have been generated via retrovirus-mediated transduction and were analyzed to ensure comparable receptor levels (12). Relative to the WT receptor, this point mutation does not affect the binding of either EGF or AR. As shown in Fig. 1B, the kinase-inactive EGF receptor is unable to stimulate the formation of an SIE binding complex in response to EGF or AR (lanes 6 and 7), whereas the IFNγ-activated complex was not affected (compare lanes 4 and 8). Thus, activation of STAT proteins by EGF or AR requires the kinase activity of the EGF receptor but not of Jak1.

STAT Activation by an EGF Receptor Lacking Tyrosine Phosphorylation Sites—To investigate which other regions in the EGF receptor were necessary for activation of STAT proteins, a series of deletion mutants of the EGF receptor (12) were analyzed for their ability to induce an SIE binding complex. We found that EGF receptors truncated at amino acid 1000 (Fig. 2, C1000, lanes 5–8), leaving one residual tyrosine phosphorylation site (amino acid 992), or at amino acid 973 (C973) were stimulated as described in Fig. 1A, and the lysates obtained were analyzed using EMSAs. The SIE binding complexes induced by AR/EGF or IFNγ are denoted by SIF-a or SIF-γ, respectively.

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blasting analysis using anti-phosphotyrosine antibodies confirmed the absence of phosphorytrosine in AR- or EGF-stimulated C991 and C973 receptors (data not shown). These results clearly demonstrate that autophosphorylation sites in the EGFR receptor are not required for STAT activation by AR or EGF and that the region between amino acids 991 and 973 may contain a negative regulatory domain.

Enhanced Ligand-induced Tyrosine Phosphorylation of STAT 1, 3, and 5 by an EGF Receptor Lacking Autophosphorylation Sites—to better understand how EGF and AR are able to induce the formation of an SH2 binding complex in NIH 3T3 cells that express either the WT or the truncated C973 receptor, we analyzed which STAT proteins become tyrosine-phosphorylated in response to AR or EGF. Since it has been shown that STAT 1, 3, and 5 are activated in response to AR or EGF (6–8), we decided to study the tyrosine phosphorylation of these STAT proteins in cells treated with either ligand. Dose-response analyses were performed using 0.1, 0.5, 5, and 20 nM AR or EGF (Fig. 3). Both AR and EGF stimulated the tyrosine phosphorylation of STAT 1, 3, and 5 in cells expressing the WT receptor (panels A, C, and E) as well as in cells expressing the C991 truncated receptor (panels B, D, and F). In both cell lines EGFR was found to be a more effective activator of all three STAT proteins relative to AR. Receptor binding studies have demonstrated that EGFR has an approximate 5.5- and 6.8-fold greater activity for the WT and C973 receptor, respectively, relative to AR. Therefore, in general, the reduced ability of AR to activate the STATs appears to correlate well with its weaker affinity for the receptors. Interestingly, ligand-induced tyrosine phosphorylation of all three STAT proteins was significantly increased in cells expressing the C973 receptor when compared with WT cells. WT and C973 cells express virtually identical numbers of cell surface receptors with very similar affinities for EGF (12). Further, Western blotting analyses of whole cell lysates for STAT 1, 3, and 5 indicate that the two cell lines express comparable levels of these STAT proteins. Thus, the enhanced tyrosine phosphorylation observed in C973 cells can be attributed solely to the loss of the COOH-terminal region of the receptor. It is important to note that relative to the WT receptor, C973 does not internalize in a ligand-dependent manner (23). This characteristic of the C973 receptor may partially explain the higher level of STAT tyrosine phosphorylation that we have observed.

In summary, our results demonstrate that the EGF receptor tyrosine kinase is necessary and sufficient to activate several members of the STAT family of transcription factors and that this activation can occur even in the absence of Jak1. As it has been previously shown that the EGF receptor tyrosine kinase can phosphorylate STAT proteins on the correct site in vitro (22), these findings suggest that STAT activation does not require a member of the Jak family of tyrosine kinases. However, we cannot rule out the possibility that an as yet undiscovered JAK-like kinase is involved in EGF receptor-mediated activation of the STATs. We also provide evidence that autophosphorylation sites in the EGFR receptor are not required for STAT activation, which is in contrast with the interpretation of results obtained by Silvennoinen et al. (14). Although we have been able to reproduce the impaired STAT activation observed with EGF receptors truncated at amino acid 991, which lacks all known autophosphorylation sites, we found that this hindrance is based on the presence of amino acids 973–991 rather than on a loss of receptor phosphorylation sites.

Our results demonstrate that the STATs do not need SH2 domain docking sites within the EGF receptor to become tyrosine-phosphorylated in response to ligand. We have previously shown that both AR and EGF induce in vivo tyrosine phosphorylation of the EGF receptor-related tyrosine kinase erbB2 (21), and similar results have been obtained in NR6 WT, C973, and M721 cells (data not shown). Therefore, it is theoretically possible that other EGF receptor-associated proteins such as erbB2 may provide the docking sites for the SH2 domain-containing STATs. However, the inability of the kinase-inactive M721 receptor to generate an SH2 binding complex indicates that erbB2 cannot substitute for the requisite EGF receptor kinase activity. The fact that truncation of the EGF receptor at residue 973 results in increased ligand-dependent phosphorylation of the STATs is consistent with the previously proposed concept that the COOH-terminal region of the EGF receptor performs an inhibitory function with regard to tyrosine phosphorylation of cellular substrates (24).
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J. Biol. Chem. 1996, 271:9185-9188.
doi: 10.1074/jbc.271.16.9185

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