Epidermal Growth Factor-induced Growth Inhibition Requires Stat1 Activation

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Abstract

Epidermal growth factor (EGF) is a mitogen for most epithelial cells. Paradoxically, the growth of some cultured cell lines, containing high numbers of EGF receptors, are inhibited by EGF. Here we demonstrate that growth inhibition by EGF in several cell lines correlates with the activation of the signal transducer and activator of transcription (Stat1). In contrast, in normal fibroblasts and several cell lines that are growth stimulated by EGF, we observed no or very transient activation of Stat1. A causal association between Stat1 activation by EGF and growth inhibition was suggested by the expression of a dominant-negative Stat1 in A431 cells, resulting in the loss of Stat1 DNA binding and concomitant resistance to growth inhibition by EGF. We conclude that, in the cells examined, EGF-induced arrest of growth requires activated Stat1.

Introduction

EGF stimulates growth of many normal and malignant cell lines (1). Nevertheless, a number of tumor cell lines that contain high numbers of EGF receptors are growth inhibited by nanomolar amounts of EGF (2–7). This paradoxical phenomenon has been best characterized in A431 cells, a line derived from a vulvar carcinoma (2, 4). These cells contain a high number of EGFRs, specifically erb1 (4). EGF induces a block in the G1 phase of the cell cycle with a corresponding inhibition of CDK2 activity because of the increased production of the CDK2 inhibitor CIP1/WAF1/p21 (8, 9). The mechanisms leading to increases in p21 production in A431 cells or in other cell types have not been determined, although a number of molecules have been implicated, including p53 (10, 11), IRF1 (12), and Stat1 (9).

Exposure to EGF activates the EGF signal transduction pathway through homo- and heterodimerization of different receptors in the EGF receptor family, followed by transphosphorylation of the receptor molecules on several different tyrosine residues. Numerous proteins containing SH2 domains then bind to the tyrosine phosphates on the receptor (1, 13, 14). Once bound to the receptor, many of these proteins also become tyrosine phosphorylated, presumably by the kinase domain of the EGFR, and mediate a cascade of biochemical events. Some of these proteins that mediate EGF signaling include syp, vav, shc, grb2, PLCγ, PI3Kp85, Stat1, and Stat3 (14–20). Why growth stimulation occurs in most cases of EGF treatment and growth inhibition in other cases is not known.

To better understand EGF-mediated growth inhibition, we examined Stat protein activation in cell lines that were either growth stimulated or inhibited by EGF. Stats are latent transcription factors that upon ligand binding become tyrosine phosphorylated, form dimers, translocate to the nucleus, recognize specific Stat DNA-binding elements, and induce transcription (21, 22). Stat1 and Stat3 are tyrosine phosphorylated after treatment of A431 cells with EGF (16, 17). The phosphorylation event may be mediated by the receptor tyrosine kinase (EGFR) or by Jak1 (23, 24), both of which are activated in EGF-treated cells. We evaluated Stat phosphorylation in cell lines containing either high numbers (2 × 10⁶) of EGFR, such as the A431 cell line (4) and A431 variant cells that are not growth inhibited by EGF (8), moderate numbers (1 × 10⁵), such as the cervical carcinoma cell lines SiHa and Me180 (25), or low numbers (4 × 10⁴), as seen in primary fibroblasts (26, 27). In all cases examined, Stat1 activation by EGF correlated with growth inhibition by this ligand. When growth stimulation was observed, no Stat1 phosphorylation was detected. However, another EGF-activated pathway, the MAPK pathway, was enhanced by EGF in all of the described cell lines.

To demonstrate a causal association between Stat1 activation and EGF induced growth inhibition, we introduced into A431 cells a Stat1 molecule lacking its terminal tyrosine 701. This molecule presumably binds to the phosphorylated EGFR through its SH2 domain but cannot be phosphorylated on residue 701 and subsequently interferes with wild-type Stat1 phosphorylation. In A431 subclones containing this construct, EGF stimulation did not lead to phosphorylation of wild-type Stat1, nor did growth inhibition occur. Thus, in A431 cells, Stat1 activation appears to be required for EGF-induced growth arrest.
Stat1 and EGF-induced Growth Arrest

Results

Effects of IFN-γ and EGF on Growth and Stat Activation in A431 Cells. A431 cells are growth inhibited by EGF and IFN-γ (4, 9), and both EGF and IFN-γ activate the phosphorylation of Stat1 protein (17–19, 28). The growth and activation of Stat1 in A431 cells in response to either IFN-γ or EGF was examined. Activated Stat1 is known to be tyrosine phosphorylated, homodimerize, binds DNA, and mediates transcriptional activation (29, 30). A431 cells were growth inhibited by both ligands, and Stat1 was activated as tested by DNA binding in response to treatment with IFN-γ and EGF (Fig. 1). There was a very weak activation by EGF of Stat3 in these cells (as seen in Figs. 1A and 4C; the slower migrating band above the Stat1 homodimer is a Stat1:3 heterodimer; data not shown). Stat3 activation by EGF was described earlier after 15 min of EGF treatment (17, 31). The activated Stat3 disappears within 60 min, the time of the experiment in Fig. 1.

Effects of IFN-γ and EGF on Growth and Stat Activation in Two Cervical Carcinoma Cell Lines: Me180 and SiHa. We next measured the growth of two cervical carcinoma cell lines in response to EGF and IFN-γ. These cell lines contain about 10-fold fewer EGFRs (1 × 10^5) than found in A431 cells (25). Me180 cells are growth inhibited by EGF, whereas SiHa cells are slightly growth stimulated by EGF (Fig. 2A). Both cell types were growth inhibited by treatment with IFN-γ (Fig. 2A). EGF stimulation of Me180 and SiHa cells resulted in the phosphorylation of the EGFR and induction of MAPK activity (Fig. 2, B and C). Thus, the EGFR can be phosphorylated in both cell types, and conservation of an EGF-activated pathway is maintained.

These cell types differ, however, in their ability to stimulate Stat1 phosphorylation in response to EGF. In the Me180 cell line, Stat1 phosphorylation (confirmed by DNA binding and Stat1 antibody interaction) was observed after treatment with EGF and was maintained for ~45 min (Fig. 2D, Lanes 4 and 6; data not shown). In contrast no Stat1 phosphorylation was observed in the SiHa cell line after treatment with EGF (Fig. 2D, Lane 5). Stat3 activation was not observed in either cell line. The Stat1 protein in the SiHa cell line could be activated because treatment of these cells with IFN-γ resulted in Stat1 phosphorylation (Fig. 2D, Lanes 11 and 13). Furthermore, the phosphorylated Stat1 protein that binds DNA was transcriptionally competent because it induced IRF1 gene transcription (Fig. 2E). Transcriptional induction of IRF1 requires Stat1 (29, 32, 33).

Effects of IFN-γ and EGF on Growth and Stat1 Activation of Primary Fibroblasts. Primary fibroblasts typically have 4 × 10^4–1 × 10^5 EGFRs and are growth stimulated by EGF (26, 27, 34, 35). Therefore, we determined the growth response of BUD8 cells (diploid, nontransformed human fibroblasts derived from skin) to EGF and IFN-γ. These cells were growth inhibited by IFN-γ and growth stimulated by EGF, as determined by cell number and tritiated thymidine uptake (Fig. 3, A and B). Nuclear extracts from EGF- and IFN-γ-treated BUD8 cells showed Stat1 DNA binding complexes after IFN-γ treatment but no Stat1 activation by EGF (Fig. 3C). Thus in this cell type, as shown in SiHa cells where EGF leads to growth stimulation, no Stat1 activation was seen. MAPK activity, however, was enhanced by EGF in these cells (data not shown).

An A431-derived Cell Line Is Growth Stimulated by EGF, which Correlates with Only Transient Stat1 Activation. To further examine the mechanism of EGF-induced cell cycle arrest in cell types with identical genetic backgrounds, we used subclones of A431-P (parental) cells (8). The A431-C subclone contains approximately one-half the number of EGFRs compared with A431-P cells, as determined by 125I-labeled EGF receptor saturation binding assay and Western blot analysis (Ref. 8; Fig. 4D, bottom panel, Lane 1). Growth in response to EGF was compared with tritiated thymidine uptake analysis (Fig. 4A). Serum alone resulted in a modest increase in uptake over 72 h in both A431-P and A431-C lines; however, EGF treatment resulted in a marked decrease in uptake over time in the A431-P line and only a transient immediate decrease in uptake in the A431-C line, with a subsequent increase between 12 and 72 h.

EGF is known to induce a G1 cell cycle block in A431-P cells with an inhibition of CDK2 by the inhibitor p21/CIP1/WAF1 (8). We examined CDK2 activity in A431-P and in A431-C cells in response to EGF; the expected inhibition was observed in A431-P cells, but only transient inhibition occurred in A431-C cells that lasted less than 12 h, consistent with the thymidine uptake results (Fig. 4B and data not shown). Stat binding activity was determined in both cell lines in response to EGF at several time points (Fig. 4C). Stat1 was activated for at least 48 h in the A431-P line but only briefly in the A431-C line. Why Stat1 phosphorylation was maintained in the parental line but only transiently acti-

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4 J. Bromberg and J. E. Damell, Jr., unpublished observations.
5 Z. Fan and J. Mendelsohn, unpublished observations.
Fig. 2. Response of Me180 and SiHa cervical carcinoma cell lines to EGF and IFN-γ. In A, Me180 and SiHa cells were plated at low density in RPMI with 0.5% BCS. After 6 h, IFN-γ (γ; 15 ng/ml) or EGF (E; ▲; 20 nM) was added to the media. The average cell number from triplicate samples was determined daily (by Coulter counter). In B, whole-cell extracts were isolated from Me180 and SiHa cells treated with IFN-γ and EGF for 1 h. One hundred μg of total protein from each extract were immunoprecipitated with antisera to the EGFR, and the immunoprecipitated products were analyzed on an 8% polyacrylamide gel, transferred to nitrocellulose, and probed with an antiphosphotyrosine antibody, PY20. –, untreated; γ, IFN-γ treated; E, EGF treated. In C, whole-cell extracts were isolated from Me180 and SiHa cells treated with IFN-γ and EGF for 1 h. Two hundred μg of total protein from each extract were immunoprecipitated with antisera to ERK1 and ERK2, and the immunoprecipitated product was incubated with [γ-32P]ATP and MBP. The radiolabeled MBP was analyzed by electrophoresis. Equal amounts of ERK1 and ERK2 were immunoprecipitated and loaded (data not shown). –, untreated; γ, IFN-γ treated; E, EGF treated. In D, nuclear extracts from A431 (A), Me180 (M), and SiHa (S) cell types treated with EGF (E) or IFN-γ (γ) for 15 min were incubated with radiolabeled m67 probe and analyzed on a nondenaturing 4% polyacrylamide gel. Supershifting with Stat1 antisera (S1) but not Stat3 antisera (S3) specifies these complexes as Stat1 containing (SS; supershift). SIFC is a Stat3 homodimer, SIFB a Stat1/Stat3 heterodimer, and SICA a Stat1 homodimer. In E, total RNA from Me180 (M) and SiHa (S) cells treated with EGF (E) or IFN-γ (γ) for 4 h was resolved on a denaturing formaldehyde agarose gel, transferred to a nylon membrane, probed with radiolabeled IRF1 probe, and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. –, untreated.

vated in the A431-C line is unknown. The two cell lines have, within a factor of two, similar numbers of EGFRs, yet qualitative differences in the receptors are not known to exist.

Receptor internalization and destruction is thought to be important in decreasing EGF-induced signal transduction pathways (34, 36). Therefore, we examined the fate of the EGF receptor at several time points after exposure of both cell lines to EGF (Fig. 4D). Samples of cell extracts were precipitated with anti-EGF receptor antisera, and precipitates were tested for phosphotyrosine on the EGF receptor (resolved by electrophoresis on acrylamide gels). In the A431-C line, the tyrosine phosphorylated EGFR (Fig. 4D, center panel) is maintained for 12 h but declines thereafter compared with the A431-P line, where the phosphorylated
Fig. 3. Response of primary fibroblasts to IFN-γ and EGF. In A, BUD8 cells were plated in DMEM with 10% FCS (1 × 10⁵ cells/six-well dish) and treated with 15 ng/ml of IFN-γ (γ) or 20 ng EGF. The average cell number from triplicate samples was determined after 3 and 6 days (by Coulter counter). In B, BUD8 cells were plated at 5 × 10⁵ cells/six-well dish and treated as described above. After 12 h, cells were pulsed with [³H]thymidine (1 μCi/ml; 70 Ci/mmol) for 1 h, after which time cells were washed and collected; TCA-precipitable counts were determined for each sample. □, untreated; ▣, EGF; ▤, IFN-γ. In C, nuclear extracts from untreated (-), EGF (E), or IFN-γ (γ)-treated BUD8 cells were analyzed by EMSA. Cells were treated for 30 min with their respective ligands.

receptor is maintained at high levels for 48 h. The total EGFR content of the two cell lines (determined by Western blots, Fig. 4D, bottom panel) showed a dramatically greater and more rapid loss in A431-C cells than in the parental cells. This prolonged activated EGFR may therefore be related to prolonged Stat1 activation in A431-P cells. Finally, we also examined MAPK activity in both cell types and found EGFR-induced MAPK activity in both with perhaps slight maintenance of MAPK activity in the A431-P line after 48 h (Fig. 4E), although growth inhibition was in effect at this time.

Blocking Stat1 Phosphorylation in A431 Cells Relieves EGF-Induced Growth Arrest. To attempt to determine whether activation of Stat1 was required for growth arrest of the parental A431 cells, we introduced a mutant epitope-tagged Stat1 protein (Y701F) by transfection and selected stable cell lines expressing the mutant protein. The exchange of Y701 for F produces a molecule that should still bind to the phosphorylated EGFR through its SH2 domain but cannot be phosphorylated. Such a molecule might block wild-type Stat1 from binding to the EGFR (Fig. 5A). A similar result was obtained with a Stat2 mutant, which decreased wild-type Stat2 phosphorylation at the IFN-α receptor (37), with a Y-F mutant in Stat3 in interleukin 6 signaling (38, 39) and with a Y-F mutant in Stat1 in IFNγ signaling (38). Characterization of a representative subclone, A5, that expressed the epitope-tagged mutant Y701F protein is depicted in Fig. 5. We examined EGFR expression and phosphorylation in response to EGF in these clones (Fig. 5C). Approximately equal amounts of total EGFR was found in untreated cells, but there was a significant decrease in both the total amount of receptor and phosphorylated receptor after EGF stimulation in A5 cells within 20 min of EGF treatment. The basis of rapid turnover of EGFR upon ligand addition in the cells expressing Stat1-Y-701F is unknown.

We examined Stat1 phosphorylation and binding to DNA by gel shift analysis in AP and A5 cells in response to EGF and IFNγ (Fig. 5D). Stat1 activation was not observed in A5 cells after EGF treatment but was observed after treatment with IFNγ. Thus, in some manner, Stat1Y-F can block EGFR-mediated Stat1 phosphorylation but does not significantly lower IFNγ-activation of Stat1. The EGFR in both AP and A5 cell types was capable of activating the MAPK pathway (Fig. 5E), despite the relative quick decrease of phosphorylated EGFR in the A5 cell type. Finally, we analyzed the growth response of the Stat1Y-F-containing clones to EGF and IFNγ. A5 cells are not growth inhibited by EGF, but their growth was slowed by IFNγ. In contrast, the parental A431-P cells are growth inhibited by both (Fig. 5F).

Discussion

Activation of Stat1 by IFN-α or IFN-γ leads to growth arrest in many cell types, and this appears to require wild-type Stat1. Stat1-deficient cells or cells containing a transcriptionally defective Stat1 are no longer growth inhibited by IFNs (9, 40). Prolonging the half-life of phosphorylated Stat1 also promotes growth inhibition (41). Constitutively phosphorylated Stat1, as seen in achondroplastic cells because of a constitutively activated fibroblast growth factor receptor, has also been reported to correlate with growth arrest, possibly due to the increased presence of the cell cycle inhibitor p21 (42). Thus, activation of Stat1, and especially maintenance of its activated state, can lead to growth inhibition.

EGF can induce phosphorylation of Stat1 and Stat3 (17). However, in the cell types examined in this report, we see a more pronounced and prolonged activation of Stat1 by EGF. Specifically, in response to EGF, Stat3 is phosphorylated in A431-P cells only weakly and briefly, whereas Stat1 is activated for at least 48 h. In M180 cells, only Stat1 is phosphorylated in response to EGF. Thus, in M180 and A431-P cell lines, there is a clear correlation between EGF-induced growth arrest and Stat1 activation. Furthermore, in a correspondingly matched cell line A431-C and in SiHa cells as well as BUD8 cells, where EGF does not induce growth arrest, either no or transient Stat1 activation was seen.

To demonstrate a requirement for EGF-dependent growth arrest and Stat1 activation, we introduced into A431 cells a Stat1 molecule that cannot be phosphorylated on tyrosine 701. Putative Stat docking sites on the intracellular domain of the EGFR have been determined (43). A Stat1 molecule which cannot be phosphorylated on Y-701 might dock onto
the receptor but not released as efficiently as when tyrosine phosphorylation occurred, thus decreasing the access of wild-type protein to the receptor. A similar Stat3 dominant-negative (Stat3Y705F) has been shown to block interleukin 6-mediated Stat3 activation through the gp130 receptor chain (38, 39, 44), as has a Stat2 mutation for the IFN-α receptor (37). However, activation of Stat1 by IFN-γ was not

imbued by the introduction of the Stat1 Y701F, whereas the EGF activation of the endogenous wild-type Stat was. It is therefore possible that the IFN-γ receptor-Stat1 interaction may be more transient, allowing wild-type Stat1 adequate access to the receptor. In contrast, the release of Stat1 from the EGF might require phosphorylation of the substrate. Possibly such blocked receptors are also targeted for deg-
Fig. 5. Stat1Y-F blocks endogenous Stat1 activation by EGF. A, Stat1Y-F/Flag mutant used to block Stat1 activation by EGF. In B, whole-cell extracts (100 μg of protein) from A431-P (AP) and an exemplary clone expressing the dominant-negative protein A431-S (AS) were immunoprecipitated with anti-flag antisera (M2IP) or anti-Stat1 antisera (S1IP). Immunoprecipitated proteins were resolved on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with antisera against Stat1. Proteins were visualized using ECL. In C, whole-cell extracts (100 μg of protein) from A431-P (AP) and A431-S (AS) were immunoprecipitated with anti-EGFR antisera, resolved on a 7% SDS-polyacrylamide gel, and probed with anti-phosphotyrosine antisera (PY20). The blot was stripped and reprobed with anti-EGFR antibody. Proteins were visualized using ECL. –, untreated. In D, nuclear extracts (10 μg of protein) from A431-P (AP) and A431-S (AS) cells treated with EGF (E) or IFN-γ (γ) were incubated with radiolabeled n67 probe and analyzed on a nondenaturing 4% polyacrylamide gel, as described in “Materials and Methods.” SIPC corresponds to a Stat1 homodimer as determined by supershifting (SS) this complex with antisera to Stat1 (S1). In E, extracts (200 μg of protein) from A431-P (AP) and A431-S (AS) cells treated with EGF (20 nM) for 30 min were immunoprecipitated with antisera against ERK1 and ERK2, followed by assaying MBP phosphorylation. Equal amounts of ERK1 and ERK2 were immunoprecipitated and loaded (data not shown). In F, A431-P and A431-S cells were plated at low density in DMEM with 0.5% BCS. After 6 h, the cells were adherent, and EGF (E, 20 nM) or IFN-γ (γ, 15 ng/ml) was added. The average cell number from triplicate samples was determined daily (by Coulter counter). –, untreated.

work on the requirement of Stat1 in growth restraint induced by IFNs (9, 40), strongly suggest a general role of Stat1 in balancing positive growth signals.

Materials and Methods
Cell Lines, Culture Conditions, and Ligands. A431, Me180, SiHa, and BUD8 cell lines were obtained from the American Type Culture Collection. The A431-C cell line was isolated as described (8). A431-P and A431-C
cells were grown in DMEM with 0.5% or 10% Cosmic calf serum (Hy-Clone). Mel180 and S1Hs cervical carcinoma cell lines were grown in RPMI with 0.5% or 10% Cosmic calf serum. BUD8 cells were grown in DMEM with 10% FCS (HyClone). A431-P cells were transfected with Stat1Y701-F or a control vector, RsCMV (Invitrogen), using standard procedures with calcium phosphate (45). Stable clones were selected for in DMEM with 10% Cosmic calf serum containing 200 μg/mL of G418 sulfate (Geneticin; Life Technologies, Inc.). Growth rate analysis and thymidine uptake studies were performed essentially as described previously (40). Cells were plated in six-well dishes at 20–30% confluence (typically 5 × 10⁴–5 × 10⁵ cells/well, depending on the cell type), permitted to adhere, and then treated with either no additional ligand or EGF (R&D Systems) at 20 ng or IFNα (a generous gift from Amgen) at 15 ng/mL. At various times, cells were trypsinized and counted using a Coulter counter. Alternatively, cells were pulsed with [3H]thymidine (1 μCi/mL; 70 Ci/mmol) for 30 min to 1 h. The cells were washed and collected, and TCA-precipitable counts were determined for each sample.

Northern, Western, Immunoprecipitations, and EMSAs. Total RNA from untreated or EGF- or IFNα-treated cells was isolated using TRIzol reagent (Life Technologies, Inc.). RNAs were resolved on a denaturing formaldehyde agarose gel (45), transferred to a nylon membrane (Zetabase; Bio-Rad), and probed with radiolabeled (Random Primer; Stratagene) IRF1 and glyceraldehyde-3-phosphate dehydrogenase. Whole-cell extracts were isolated as described previously (46). Nuclear extracts were isolated as described previously (47, 48). EMSAs were performed essentially as described previously (49, 50) using a radiolabeled, 67-mer double-stranded probe (5′GATTCGGGTTAAACCCTTTATCAT; the consensus Stat binding site is underlined) and nuclear extracts. Immunoprecipitations were carried out by incubating, at 4°C, 100–200 μg of total protein from whole-cell extracts with appropriate antisera: EGFR-528, CDK2(M2), ERK1, ERK2 (Santa Cruz Biotechnology), and agarose A beads (Oncoogene Science) for several hours, followed by several washes in whole-cell extract buffer. Phosphotyrosine blots were carried out using PY20 (Transduction Laboratories). Immunoblots were visualized using ECL (DuPont NEN, Life Sciences). CDK2 assays were performed essentially as described (8, 40). MAPK assays were performed as for the CDK2 assay substituting MBP (Sigma) for histone H1. Radiolabeled MBP was resolved on a 12% polyacrylamide gel, transferred to nitrocellulose, and visualized by autoradiography. For both CDK2 and MAPK assays, one-half of the immunoprecipitated products were resolved on 8% gels transferred to nitrocellulose and probed with either CDK2 or ERK1 and ERK2 to assess variability in immunoprecipitation and loading. Stat1 antisera (51) to the COOH terminus of the protein was used for supershifting Stat1-containing DNA complexes. Stat3 antisera (17) was used as control serum. The Flag-tagged Stat1-Y701-F (46) was produced by PCR and is subcloned into the Apa/NcoI sites of RsCMV (Invitrogen).

Acknowledgments
We thank Lois Cousseau for preparing the manuscript.

References


