Identification of Both Positive and Negative Domains within the Epidermal Growth Factor Receptor COOH-terminal Region for Signal Transducer and Activator of Transcription (STAT) Activation*

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Ling Xia‡, Lijuan Wang‡, Alicia S. Chung‡, Stanimir S. Ivanov‡, Mike Y. Ling‡, Ana M. Dragoi‡, Adam Platé§†, Tona M. Gilmer.§, Xin-Yuan Fu§, and Y. Eugene Chin**

From the §Department of Pathology and Laboratory Medicine, Brown University School of Medicine, Providence, Rhode Island 02912, the $Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510, and the ¶Department of Cell Biology, Glaxo-Wellcome Research Institute, Research Triangle Park, North Carolina 27709

The cytoplasmic region of human epidermal growth factor receptor (EGFR) contains an intrinsic tyrosine kinase (697–955) followed by a 231-residue-long COOH-terminal tail (C-tail), which contains multiple tyrosine residues. To examine the role of the EGFR C-tail in signal transducer and activator of transcription (STAT) activation, a series of EGFR C-tail truncations were constructed. Transient transfection of 293 cells with EGFR lacking the C-tail, i.e. Y974ΔEGFR or Y992ΔEGFR, led to EGF-independent or constitutive STAT activation, whereas EGFR-dependent STAT activation was restored with truncations made COOH-terminal to the next tyrosine residue, i.e. EGFR-Y1045Δ. Transfection with the truncated form EGFR-Y954Δ resulted in the loss of STAT activation, suggesting that the sequence between Tyr974 and Tyr954 is essential for STAT activation. Phosphopeptide competition analysis revealed multiple tyrosine residues within the C-tail that can act as the docking sites for both Stat1 and Stat3. A region that negatively regulated STAT activation was also identified, extending from Tyr1114 to Glu1172, consistent with the ability of this region to recruit a suppressor of cytokine signaling factors SOCS1 and SOCS3. When cotransfected with the full-length EGFR, but not Y992ΔEGFR, SOCS1 or SOCS3 inhibited STAT activation by EGFR in 293 cells. This suggests that both SOCS1 and SOCS3 can negatively regulate EGFR activation, presumably by inducing ubiquitination-dependent EGFR degradation upon ligand binding. These findings may therefore offer clues to how the EGF receptor C-tail regulates STAT activity.

Epidermal growth factor (EGF)1 stimulates proliferation and maintains survival in a variety of normal and malignant cells. However, EGF also inhibits cell growth and induces apoptosis in some cancer cell lines with aberrant expression of EGF receptor (EGFR) (1, 2). Binding of EGF to the 170-kDa EGFR initiates a program of intrinsic tyrosine kinase activity resulting in autophosphorylation of the receptor and phosphorylation of a number of SH2 domain-containing signaling proteins, including STAT. Within the EGFR cytoplasmic domain (654–1210), the tyrosine kinase motif extends from amino acid 697 to 955 followed by a 255-amino acid distal COOH-terminal domain (C-tail). Among the C-tail tyrosine residues, Tyr992, Tyr1058, Tyr1086, Tyr1148, and Tyr1172 were demonstrated previously as autophosphorylation sites (3). However, it is generally believed that these five individual EGFR autophosphorylation sites do not stringently define all possible association motifs of SH2-containing proteins (4). Among all of the C-tail tyrosine residues, there are three YXX/L/V, four YXXP/D, and five N/D/VPX sequences, with some overlap. For many transmembrane receptors, YXXL/V and YXXP/D motifs within their cytoplasmic domains serve as the docking sites for the SH2 domain-containing proteins. ZAP-70, SHP-2, and Stat5 all have been found to recognize these sequences (5–7). The YXXQ sequence within the cytoplasmic domain of gp130 was proposed to be the Stat3 binding consensus sequence (8), whereas proteins containing phospho-tyrosine binding domains can recognize an N/D/VPXY motif (9). The features of the sequences flanking the tyrosine residues within the C-tail suggest that the EGFR may contain more than five autophosphorylation tyrosine residues that have not been recognized previously. The diversity of potential phospho-tyrosine motifs within the EGFR C-tail may be the reason why this receptor is able to recruit and activate many different signaling proteins. STAT (i.e. Stat1, 3, and 5) activation by EGF was detected in mouse liver and in some cancer cell lines such as A431 and MD-MBA-468 cells that overexpress EGFR (10–13). Although EGFR expression level was hypothesized to be critical for STAT activation, Stat1 activation by EGF was not detected in a number of cancer cell lines with abnormal EGFR expression (14–16). An EGFR-associated inhibitory factor was thus proposed to block EGF-mediated Stat1 activation (17). For those cytokine receptors lacking intrinsic kinase activity, removal or mutation of the C-tail tyrosine residues does impair the ability

1 The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; C-tail, COOH-terminal tail; STAT, signal transducer and activator of transcription; DBD, DNA binding domain; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; JAK, Janus kinase; SH2, Src homology 2; MAP, mitogen-activated protein; SOCS, suppressor of cytokine signaling; IFN, interferon; pY, phospho-tyrosine.
of the receptor to recruit and activate STAT, even though JAK association is unaffected (8). The involvement of the EGFR C-tail and its tyrosine residues in STAT binding and activation are controversial (18, 19). Because of the number and redundancy of tyrosine sites in EGFR, determining which sites mediate relevant biological responses is difficult.

To investigate EGFR-mediated STAT activation, EGFR with Tyr → Phe C-tail point mutations of all known autophosphorylation sites and EGFR mutants with progressively-truncated C-tails were constructed and tested for their ability to activate STAT. Moreover, tyrosine phosphopeptides representing the EGFR C-tail tyrosine residue motifs were synthesized and analyzed for interaction with STAT. Together these data revealed multiple docking sites for both Stat1 and Stat3 within the EGFR C-tail as well as a negative regulatory region for STAT activation. The observation that SOCS1 and SOCS3 were found to interact with EGFR C-tail, together with the knowledge that STAT activation is sensitive to EGFR expression levels, suggests that SOCS1 and SOCS3 inhibit STAT activation, presumably because of their induction of ubiquitination-dependent EGFR proteolytic degradation.

MATERIALS AND METHODS

Reagents, Constructs, and Cell Cultures—Recombinant human EGF was purchased from Invitrogen and IFN-γ was purchased from Genentech (San Francisco, CA). Monoclonal anti-EGFR (extracellular domain) antibody was from Sigma. Anti-Stat1 (COOH-terminal region) and anti-pY20 antibodies were from Santa Cruz Biotechnology. Anti-Stat1 NH2-terminal region antibody was from Transduction Laboratories. Both anti-phosphoStat1 (pY701) and anti-phosphoStat3 (pY705) were provided by NEB.

Wild type Stat1 cDNA was constructed into pSG5 expression vector as described previously (20). The DNA binding domain (DBD)-mutated Stat1 was created by converting Glu428Glu429 → Ala428Ala429 (pSG5-Stat1-E248E249/AA). Stat1 with a deletion mutation from Ser316 to Gly576 (Stat1-LS3) was constructed using Sall digestion. The linker domain (residues Thr462-Gly576) and the α-β motif (Lys545→Phe565) motif of the SH2 domain were deleted in this Stat1-LS3 construct. The expression construct of full-length human EGFR in pCDNA3 was supplied by G. Gill. EGFR C-tail tyrosine point mutations (Tyr → Phe) were constructed by the QuikChange PCR amplification method using appropriately designed oligonucleotide primers (Stratagene). The C-tail-truncated EGFRs were constructed using the method of Cao et al. (21). All EGFR mutant constructs were confirmed by sequencing. c-Myc-tagged SOCS factors in pCDNA3 were provided by A. Yoshimura. Both GST-SOCS and His-STAT constructs were prepared as described in our previous publication (22).

Peptide Synthesis—All of the peptides were synthesized as amides on a multiple peptide synthesizer according to the standard Fmoc (N-9-fluorenylmethoxycarbonyl) machine protocol (Abimed AMS 422, Langenfeld, Germany). Amino acid composition was verified, and molarity was calculated using a Beckman 6300 amino acid analyzer. All of the products were purified with high performance liquid chromatography to purity >80%.

Cell-free STAT Activation by EGF—Fractions of cell membrane and cytosolic proteins were prepared from A431 cells for in vitro EGFR kinase assay as described previously (17, 23). In brief, confluent A431 cells were pelleted and lysed in 20 mM Hepes buffer pH 7.9 containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each leupeptin, pepstatin A, and aprotinin. The resulting sample was saved as a cytosolic fraction. The pellet was resuspended in lysis buffer containing 150 mM NaCl and centrifuged at 10,000 × g for 30 min at 4 °C. The pellet was saved as the membrane fraction. Cell-free activation of STAT reaction (15 μl) contained 1 μl of membrane and 2 μl of cytosolic fractions. The reactions were incubated for 15 min on ice in the absence or presence of 2 μg/ml EGF. Buffer containing ATP was added, and the reactions were incubated at 30 °C for 30 min. Reactions were stopped by incubation on ice with 1 mM EDTA and brief centrifugation. Aliquots were incubated with peptides at the indicated concentrations before being subjected to electrophoretic mobility shift assay (EMSA) analysis.

EMSA, Affinity Precipitation, Western Blotting, and Luciferase Assays—For STAT-DNA binding activity analysis, M67SIE was used as a probe, and the procedure for EMSA was described in detail previously (14). For affinity precipitation and Western blotting assay, Ni2+ resin was used to conjugate His-tagged proteins (Novagen). Whole cell extracts were incubated for a period of at least 4 h with either agarose beads, conjugated antibodies, or GST-SOCS proteins or resin-conjugated His-STAT proteins. After incubation, the beads were washed extensively with lysis buffer and suspended in 2 × loading buffer. The affinity precipitates or cell extracts were electrophoresized on a SDS polyacrylamide gel (10%), transferred onto polyvinylidene difluoride membrane, and followed by immunoblotting analysis with appropriate antibodies (15). For the luciferase activity assay, 2×M67SIE-luciferase reporter was cotransfected with EGF into 293 or NIH3T3 cells using LipofectAMINE according to the manufacturer’s procedure (Invitrogen). 24 h after transfection the cells were treated with or without EGF for an additional 6 h.

RESULTS

The EGFR C-tail, Essential in Regulation of STAT Activity, Is Not Required for STAT Activation—In 293 cells, Stat1 activation by EGF was detected only when EGFR was transfected (Fig. 1, A and B), whereas MAP kinase activation by EGF was detected regardless of EGFR transfection (Fig. 1B). Although Stat3 DNA binding activity (homodimer) was barely detected...
under the conditions used, both Stat1 and Stat3 tyrosine phosphorylations were detected (Fig. 1, A and B). These results suggest that EGFR expression is crucial for STAT but not for MAP kinase ligand-induced activation.

To find which motif(s) within the C-tail is/are critical for STAT activation, a series of EGFR with progressive carboxyl terminus deletions were constructed. Truncations were designed so that each successive deletion resulted in the loss of one further tyrosine residue as illustrated in Fig. 2A. In Fig. 2B, EGFR-dependent Stat1 DNA binding activity was clearly detected in 293 cells expressing EGFR-Y1173Δ, a truncation missing the most COOH-terminal 14 residues of EGFR. Deletions resulting in the loss of the next two tyrosine residues, i.e. Tyr1146 (EGFR-Y1148Δ) and Tyr1148 (EGFR-Y1144Δ), resulted in a greatly reduced STAT activation response to EGF. However, STAT activation by EGF was restored when EGFR truncation was extended one tyrosine residue farther, i.e. Tyr1101 (EGFR-Y1101Δ). This result strengthened the observation made by other workers in that the EGFR C-tail may contain a negative regulatory domain (18, 24). Like Tyr1173 and Tyr1148, the tyrosine residues Tyr1086 and Tyr1068 are previously confirmed autophosphorylatable sites. Truncated EGFRs, i.e. EGFR-Y1086Δ, EGFR-Y1068Δ, and EGFR-Y1045Δ, were able to mediate EGF-induced STAT activation. Surprisingly, the C-tail truncations of the next two tyrosine residues (Tyr992, the last confirmed autophosphorylation residue, and Tyr974) led to constitutive activation of STAT in 293 cells (Fig. 2B). Thus, the maximal C-tail region that is essential for STAT activation extends from Tyr954 to Arg973, whereas residues Tyr992 to the carboxyl terminus Ala1186 appear to be inessential for STAT activation but critical for EGF-mediated regulation of STAT activation. Comparable expression levels of truncated EGFRs in 293 cells are shown in Fig. 2C.

A similar pattern of Stat1 constitutive activation by EGFR-Y992Δ and EGFR-Y974Δ was also observed in COS-7 cells (Fig. 2D, top panel). The bottom panel in Fig. 2D shows the even expression level of these EGFR mutants. In NIH3T3 cells, however, a basal level of constitutive Stat3 DNA binding activity was observed, which was enhanced by transient transfection with wild type EGFR (Fig. 2E). Both EGFR-Y992Δ and EGFR-Y974Δ were apparently more efficient than wild type EGFR in Stat3 formation and subsequent DNA binding activity as detected by EMSA (Fig. 2E). Both EGFR-Y992Δ and -Y974Δ truncations induced Stat3 tyrosine phosphorylation in NIH3T3 cells, regardless of EGF treatment (Fig. 2F). Consistently, both EGFR-Y992Δ and EGFR-Y974Δ apparently induced Stat3 tyrosine phosphorylation in NIH3T3 cells, regardless of EGF treatment (Fig. 2F). Because comparable protein levels of Stat1 and Stat3 were detected in these cell lines (data not shown), the specificity of STAT activation is unlikely to be determined by the level of EGFR or STAT in these cells but rather by another cellular activity.

Activation of Stat1 and Stat3 can have opposite implications for cell survival and growth (14–16). As expected, transient transfection with wild type EGFR or EGFR-Y992Δ in 293 cells led to apparent cell death after serum starvation for 36 h (Fig. 2G). In contrast, NIH3T3 cells transiently transfected with either wild type EGFR or EGFR-Y992Δ became resistant to induction apoptosis by serum starvation (Fig. 2G). Together these findings indicate that although the EGFR C-tail is not critical for STAT activation by EGFR it plays an important role in the regulation of STAT, including the prevention of constitutive STAT activation.

The EGFR C-tail Contains Multiple STAT Docking Sites for STAT Activity Regulation—To further identify the tyrosine module(s) corresponding to STAT docking and activation, we mutated the five previously demonstrated autophosphorylation residues (Tyr992, Tyr1068, Tyr1086, Tyr1148 and Tyr1173) either individually or in tandem. As shown in Fig. 3A, STAT activation by EGF was not impaired by any single or double mutation of these sites, in agreement with a previous observation (18). Therefore, these results indicated that either these autophosphorylatable tyrosine sites are not relevant to STAT activation as suggested by David et al. (18) or redundant tyrosine residues are involved in STAT activation as proposed by Silvennoinen et al. (19).

To distinguish these two possibilities peptides of 13 amino acids, each containing one of the tyrosine residues of the EGFR C-tail in non-phosphorylated and phosphorylated forms, were synthesized (Fig. 3B). The approach of using synthesized peptides in a competition assay has been successfully used to identify sequences of different cytokine receptors important in STAT activation (25, 26). In cell-free assays, fractions of A431 cell membrane and cytosome were incubated with EGF. Different concentrations of synthesized peptides were subsequently added into the reactions before incubating with γ32P-labeled M7SIE probe. The EMSA results shown in Fig. 3C indicate that among all of the phosphopeptides tested, phosphopeptide pY1148, containing a NDPYQQD motif, abolished Stat1 binding to DNA without affecting Stat3-DNA complex formation. This suggests that Stat1 may preferentially interact with Tyr1148 within the EGFR C-tail. Phospho-Tyr1045 (LQRYSSD) and pY992 (ADEYLP) peptides inhibited both Stat1 and Stat3 DNA binding markedly, indicating that residues Tyr1045 and Tyr992 might be involved in the recruitment of both Stat1 and Stat3 to EGFR. To a much lesser extent, pY1173 peptide inhibited Stat1 DNA binding activity.

Phospho-Tyr1086 (pY1086) and pY1068 peptides blocked Stat3-DNA complex formation without any effect on Stat1-DNA interaction (Fig. 3C). Thus, Stat3 but not Stat1 preferentially interacts with both Tyr1086 and Tyr1068 residues, which are both tyrosine residues found within a XXXQ motif. The experimental concentration of peptides (300 μM) required for competition was relatively high. Decreasing concentrations of these peptides (150 and 75 μM) were tested to determine the relative affinity of each peptide. Tyr1148, Tyr1086 and Tyr1068 showed much weaker effects at a concentration of 150 μM. At high concentrations (300 μM), unphosphorylated peptides showed no competition (data not shown). These data further support the notion that both Stat1 and Stat3 interact with the EGFR C-tail at multiple tyrosine residues and that C-tail docking is important for regulation of EGF-induced STAT activation. The striking effect of pY1045 peptide on STAT-DNA complex formation suggests that Tyr1045 is a potentially phosphorylatable tyrosine residue.

The relatively high dose of phosphopeptides used in the above competition assay suggests that EGFR may interact with STAT not only within its SH2 domain but also within other domains. To delineate the regions in Stat1 that interact with EGFR, His6 fusion proteins of different regions of Stat1 were constructed (Fig. 4A). Bead-conjugated His6 fusion proteins were incubated with whole extracts of EGF-treated A431 cells. As shown in Fig. 4B, in addition to the full-length Stat1 both the His6-SH2 domain and the His6-DBD pulled down EGFR. His6 fusion proteins were detected in a Western blot with anti-His antibody (Fig. 4C). These results indicate that EGFR interacts with STAT via both the SH2 and the DNA binding domains. In 293 cells, EGFR was co-transfected along with wild type Stat1, a Stat1 DBD mutant (Glu428-Glu429→Ala-Ala (Stat1-LS, Stat1-RQ), and the linker-SH2 domain Stat1 truncation (Stat1-LSΔ), Stat1-RQ but not Stat1-LSΔ was recovered by anti-
FIG. 2. C-tail-truncated EGFR constitutively activates STAT. A, schematic structures of the human EGFR truncation mutants used in this study. The five previously demonstrated autophosphorylation residues are indicated as *Y. B, 293 cells were transiently transfected with full-length and C-tail-truncated EGFR followed by EGF treatment for 30'. Whole cell extracts were incubated with labeled M67SIE probe to reveal DNA binding complexes. C, the whole cell lysates of above EGFR transfectants were blotted with anti-EGFR (extracellular domain) antibody. D, COS-7 cells were transfected with truncated EGFR as indicated, and STAT activation was examined by EMSA (top panel). EGFR protein levels in the COS-7 cell lysates were examined with anti-EGFR antibody (bottom panel). E, whole lysates of NIH3T3 cells transiently transfected with EGFR of wild type, EGFR-992Δ, and EGFR-974Δ were subjected to EMSA using 32P-M67SIE as the probe. Stat1 activation by EGF in 293 cells transfected with wild type EGFR (WT) was compared as control. F, whole extracts prepared from above NIH3T3 transfectants were analyzed in Western blot with anti-phospho-Stat3 (pStat3(Y705)) and anti-Stat3 (Stat3) antibodies. G, both 293 and NIH3T3 cells were transfected with empty vector, wild type EGFR, and EGFR-Y992Δ followed by serum starvation for a period of 24 h in 293 cells and 36 h in NIH3T3 cells, respectively. Dead cells were counted with trypan blue exclusive assay. Three independent experiments were performed with means ± S.D.
EGFR immunoprecipitation, indicating a tighter binding between the DBD and EGFR than that between the SH2 domain and EGFR (Fig. 4D). Consistently, COOH-terminal linker/SH2 domain-truncated Stat1 was also recovered from EGFR immunoprecipitates. As expected, endogenous Stat1 was also pulled down by anti-Stat1 in all samples. These data further support the hypothesis that both the SH2 and DNA binding domains of STAT1 are involved in the interaction with EGFR.

The EGFR C-tail Recruits SOCS1 and SOCS3 to Degrade EGFR and Block STAT Activation

We next wanted to answer the question of how the EGFR C-tail terminates or inhibits STAT activation. SOCS is a family of small SH2-containing signaling factors turned on by STAT and engaged in the negative feedback control of JAK (28–30). In A431 cells, both Stat1 and Stat3 can be immediately activated by EGF stimulation (10, 15). Both SOCS1 and SOCS3 proteins were detected prior to treatment, but their levels were greatly elevated 2 h after the addition of EGF (Fig. 5A). In contrast, STAT activation by EGF in HeLa cells has been barely detectable (16), and SOCS1 and SOCS3 protein levels were not markedly affected by EGF treatment (Fig. 5A). When cotransfected with EGFR, both SOCS1 and SOCS3 were recovered from the immune complex precipitated by anti-EGFR in 293 cells (Fig. 5B), suggesting that EGFR can recruit these SOCS factors. This potential interaction between EGFR C-tail and SOCS1 or SOCS3 was confirmed by the ability of GST-SOCS1 and GST-SOCS3 to pull down full-length EGFR but not EGFR-Y992Δ (Fig. 5C, top panel). Both GST-SOCS1 and GST-SOCS3 were able to pull down EGFR-Y1114Δ and EGFR-Y1148Δ but not EGFR-1101Δ, suggesting that SOCS proteins may bind within the 13 residues found between Tyr1101 and Tyr1114 of EGFR. This finding is in agreement with the observation of an inhibitory domain of STAT activation within this same region of the C-tail (Fig. 2B).

Bacterial-prepared GST fusion proteins used in these pull-down experiments are shown in Fig. 5D.

Overexpression of SOCS proteins has been reported to cause tyrosine kinase JAK proteolytic degradation (31, 32). We therefore examined the possible effect of SOCS1 and SOCS3 on EGFR degradation. 293 cells were cotransfected with EGFR, ubiquitin, and SOCS1 or SOCS3 and were treated with EGF for 6 h. The level of EGFR was greatly reduced in the cells cotransfected with either SOCS1 or SOCS3 (Fig. 6A). In the presence of lactacystin, the proteosome-specific inhibitor, EGFR degradation was reduced, indicating that SOCS-induced EGFR degradation is a proteosome-mediated process (Fig. 6A). EGFMediated Stat1 tyrosine phosphorylation was markedly reduced in 293 cells when wild type EGFR was cotransfected with SOCS1 or SOCS3 (Fig. 6B). Stat3 phosphorylation status was affected to a lesser extent by these SOCS factors (Fig. 6B). However, Stat1 constitutive activation induced by C-tail-truncated EGFR (EGFR-Y992Δ) was not affected by the cotransfection with either SOCS1 or SOCS3 (Fig. 6C). These data suggest
that the interaction between these SOCS proteins and the EGFR C-tail is crucial for the SOCS factors to inhibit STAT activation by EGFR. To test the effect of SOCS1 and SOCS3 on STAT-mediated gene activation, we performed a luciferase reporter assay. Cotransfection of EGFR with either SOCS1 or SOCS3 resulted in a 4–5-fold inhibition of STAT-mediated gene activation, indicating the importance of the interaction between SOCS and EGFR.

**Fig. 4.** Both SH2 domain and DBD of STAT interact with EGFR. A, His6-Stat1 proteins, in full-length (1–750) Linker (489–576), DBD (316–488), N-region (1–315), and SH2/TA (577–750) domains are illustrated. B, whole extracts prepared from EGF-treated A431 cells were incubated with bead-conjugated His6-Stat1 fragments. His6 fusion protein precipitates were subjected to 10% SDS-PAGE and blotted with anti-EGFR. C, N-region (1–315), and SH2/TA (577–750) domains are illustrated. D, in 293 cells EGFR was transfected along with Stat1 wild type (Stat1WT), Stat1-EA (Glu 428Glu429: AlaAla), Stat1-RQ (Arg 602: Gln), empty vector (EV), and Stat1-CA, respectively. Anti-EGFR immunoprecipitates were separated in 10% SDS-PAGE and probed with anti-Stat1 antibody (top panel). The bottom panel shows expression levels of Stat1 in the above transfectants.

**Fig. 5.** EGFR recruits SOCS1 and SOCS3 proteins. A, A431 and HeLa cells were treated with or without EGF for 2 h. Whole cell lysates prepared from these cells were then submitted to Western blotting with anti-SOCS1 (top panel) or SOCS3 antibody. B, in 293 cells, EGFR was transiently cotransfected with empty vector, c-Myc-tagged SOCS1, or c-Myc-tagged SOCS3 as indicated. EGFR were precipitated with anti-EGFR-agarose beads from the whole cell extracts of these transfectants treated with or without EGF. Anti-EGFR precipitates were then subjected to 10% SDS-PAGE followed by Western blotting with anti-c-Myc antibody (top panel). A Western blot with anti-c-Myc antibody demonstrated the expression levels in the transfected cells (bottom panel). C, in the top panel, GST control, GST-SOCS1, and GST-SOCS3 were incubated with cell lysates prepared from 293 cells transfected with empty vector (mock), wild type EGFR, or EGFR-Y992Δ as indicated. GST fusion protein precipitates were separated in SDS-PAGE and probed with anti-EGFR antibody in Western blot. In the bottom panel, 293 cells were transfected with C-tail-truncated EGFR (Y1101Δ, Y1114Δ, and Y1148Δ). GST-SOCS1 (S1) or GST-SOCS3 (S3) were then used to pull down EGFR from the whole cell lysates prepared from these 293 transfectants. D, purified GST recombinant proteins used in C were blotted with anti-GST antibody.
induction of luciferase expression in response to EGF stimulation (Fig. 6D). Together these results indicate that SOCS1 and SOCS3 may attenuate STAT-mediated gene regulation presumably via a reduction in the cellular level of EGFR, which in turn reduces or shuts down STAT activation.

**DISCUSSION**

For non-receptor tyrosine kinases such as Src and JAK, the amino-terminal SH2 domains are responsible for interacting with pTyr substrates and regulating the activity of the COOH-terminal kinase domain. Similarly, transmembrane receptor kinases such as EGFR may use the pTyr-rich C-tail to recruit SH2-containing substrates and regulate the intrinsic tyrosine kinase. In this work, through progressive truncation analysis we have demonstrated that a 20-amino acid sequence from Tyr954 to Arg973 of the EGFR C-tail is critical for the activity of the intrinsic tyrosine kinase of EGFR in STAT activation. The next immediate 17 amino acids from Tyr974 to Glu991 seem neither important for STAT activation nor related to STAT regulation because both EGFR-Y954A and EGFR-Y992A induced constitutive STAT activation. Although multiple tyrosine residues from Tyr992 to Ala1186 of the EGFR C-tail may serve as the binding sites for STAT proteins, the entire length of the C-tail is not necessarily involved in STAT activation. Instead, the C-tail appears to play an important regulatory role in STAT ligand-dependent activation.

Five tyrosine residues within EGFR C-tail (Tyr992, Tyr1068, Tyr1086, Tyr1148, and Tyr1173) have been previously considered as autophosphorylation sites. Our phosphopeptide competition data indicate that additional autophosphorylation tyrosine residues may exist in the C-tail. The fact that the phosphopeptide Tyr1145 residue removed both Stat1 and Stat3 from STAT-DNA complexes suggests that this residue within EGFR may be such a candidate to serve as a STAT-docking site. To various levels, phosphopeptides representing Tyr1086 and Tyr1068 removed Stat3 from the STAT-DNA complexes. Both Tyr1068 and Tyr1086 contain the YXXQ sequence, which agrees with the notion that YXXQ is a Stat3-preferential binding sequence in gp130 (8). However, YXXQ may not be the unique module for Stat3 docking; for instance, Src lacks a YXXQ module but still recruits Stat3 for activation (21). Our peptide competition assay suggests that Tyr992, Tyr1045, and Tyr1173 are also potential docking sites for Stat3, although YXXQ is not presented. These results are consistent with the findings that transmembrane receptors often contain redundant tyrosine residues with which an SH2 domain protein interacts (33).

Greenlund et al. (25) showed that a phosphopeptide mimicking a Stat1 interaction site present in the IFN-γ receptor could completely inhibit Stat1 activation at concentrations as low as 83 μM. The concentration of our peptides derived from the EGFR was required to be 3–4-fold higher in order to disrupt STAT-DNA complex formation as observed by EMSA. Phosphopeptides representing Tyr1086 and Tyr1068 contain the YXXQ sequence, which agrees with the notion that YXXQ is a Stat3-preferential binding sequence in gp130 (8). However, YXXQ may not be the unique module for Stat3 docking; for instance, Src lacks a YXXQ module but still recruits Stat3 for activation (21). Our peptide competition assay suggests that Tyr992, Tyr1045, and Tyr1173 are also potential docking sites for Stat3, although YXXQ is not presented. These results are consistent with the findings that transmembrane receptors often contain redundant tyrosine residues with which an SH2 domain protein interacts (33).

**FIG. 6.** SOCS1 and SOCS3 proteins induce ubiquitination-dependent EGFR degradation and inhibit STAT activation by EGF. A, in 293 cells EGFR, ubiquitin, and SOCS1 or SOCS3 were cotransfected as indicated. After EGF treatment for 6 h, the whole cell lysates were prepared and subjected to Western blot with anti-EGFR antibody (top panel). In the bottom panel the same experiments were performed, except the cells received both lactacystin (LCN) and EGF treatment for 6 h prior to harvest. B, whole lysates prepared from 293 transfectants (EGFR cotransfected with empty vector, SOCS1, or SOCS3 as indicated) were subjected to Western blot with anti-phospho-Stat1, anti-Stat1, anti-phospho-Stat3, and anti-Stat3 respectively. C, in 293 cells, SOCS1 or SOCS3 was cotransfected with different forms of EGFR as indicated. Whole lysates were then analyzed in the Western blot with anti-phospho-Stat1 and anti-Stat1 antibodies. D, the 2xM67SIE-luciferase reporter was transfected together with EGF in the presence or absence of the expression vector for SOCS1 and SOCS3. 24 h post-transfection, cells were treated with EGF for 6 h before harvest. Results are shown as relative-fold induction and represent the mean ± S.E. from triplicate assays.
modes at different receptors. Importantly, STAT1 carrying a mutation critical to the SH2 domain, Arg602Gln, can still bind to EGFR. This is consistent with a recent report that inactivation of the Stat5a SH2 domain did not disrupt association with EGFR (34). The unexpected finding that Stat1 carrying a DNA binding domain mutation loses the ability to interact with EGFR supports the notion that this domain may be important not only for the STAT-DNA interaction but also for the STAT-EGFR interaction. Therefore, STAT and EGFR interaction occurs within multiple domains.

The negative domain (Tyr1101-Glu1172) within the C-tail for STAT activation contains two tyrosine residues, Tyr1114 and Tyr1148. Previously it was reported that c-Cbl associated with EGFR prior to recruitment into clathrin-coated pits and remained associated throughout the clathrin-mediated endocytic pathway (35). Nevertheless, our data show here that by binding to the negative domain within the C-tail, SOCS proteins might stimulate the proteosomal degradation of the EGFR complex by recruiting the ubiquitin ligase machinery through its SOCS box. Because Stat1 activation by EGF was extremely sensitive to the EGFR expression level in the cell, reduced EGFR protein level may consequently affect STAT activation without influencing MAP kinase pathways. STAT has been shown previously to be ubiquitinated prior to degradation (36), and although our work demonstrates EGFR degradation as a result of SOCS1/3 overexpression, we do not exclude the possibility that SOCS may directly influence the STAT ubiquitination process. Hence, it is also possible that by binding to the putative inhibitory domain identified between Tyr1101 and Tyr1114 within the C-tail, SOCS proteins may either induce degradation of EGFR-associated STAT or block EGFR from further recruitment and activation of further STAT proteins.

Stat1 activation often leads to cell growth arrest and/or apoptosis (15, 16). It may be important for EGFR to recruit Stat1 in order to balance or restrict Stat1 and/or other proliferative or survival signaling pathways by the same receptor that may influence the cell growth arrest and/or apoptosis (15, 16). It may be important for EGFR to recruit Stat1 in order to balance or restrict Stat1 and/or other proliferative or survival signaling pathways by the same receptor that may influence the cell growth arrest and/or apoptosis (15, 16).

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Identification of Both Positive and Negative Domains within the Epidermal Growth Factor Receptor COOH-terminal Region for Signal Transducer and Activator of Transcription (STAT) Activation

Ling Xia, Lijuan Wang, Alicia S. Chung, Stanimir S. Ivanov, Mike Y. Ling, Ana M. Dragoi, Adam Platt, Tona M. Gilmer, Xin-Yuan Fu and Y. Eugene Chin

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