Mechanisms of Signal Transduction:

Suppressors of Cytokine Signaling 4 and 5 Regulate Epidermal Growth Factor Receptor Signaling

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Suppressors of cytokine signaling (SOCS) are Src homology-2-containing proteins originally identified as negative regulators of cytokine signaling. Accumulating evidence indicates a role for SOCS proteins in the regulation of additional signaling pathways including receptor tyrosine kinases. Notably, SOCS36E, the *Drosophila* ortholog of mammalian SOCS5, was recently implicated as a negative regulator of the *Drosophila* ortholog of EGFR. In this study, we aimed at characterizing the role of SOCS5 in the negative regulation of EGFR. Here we show that the expression of SOCS5 and its closest homolog SOCS4 is elevated in cells following treatment with EGF, similar to several negative feedback regulators of EGFR whose expression is up-regulated upon receptor activation. The expression of SOCS5 led to a marked reduction in EGFR expression levels by promoting EGFR degradation. The reduction in EGFR levels and EGF-induced signaling in SOCS5-expressing cells requires both the Src homology-2 and SOCS box domains of SOCS5. Interestingly, EGFR is degraded by SOCS5 prior to EGF treatment in a ligand- and c-Cbl-independent manner. SOCS5 can associate with EGFR and can also bind the ElonginBC protein complex via its SOCS box, which may recruit an E3 ubiquitin ligase to promote EGFR degradation. Thus, we have characterized a novel function for SOCS5 in regulating EGFR and discuss its potential role in controlling EGFR homeostasis.

Signal transduction through receptor tyrosine kinases (RTKs)[1] of the epidermal growth factor receptor (EGFR/ErbB) family occurs subsequent to binding of extracellular ligands and plays a critical role in the development and maintenance of adult tissues. A balance of both positive and negative signals is critical for proper cell function, and its deregulation is often implicated in the development of human diseases (1). Removal of activated EGFR-ligand complexes from the cell surface by endocytosis and their subsequent degradation in the lysosome is the main process for the attenuation of signaling processes initiated by activated receptors (2). ErbB receptors that show decreased down-regulation following ligand stimulation have been shown to possess increased and prolonged signaling capacity (3, 4).

A key component in the negative regulation of EGFR signaling is the E3 ubiquitin ligase c-Cbl. Following EGF stimulation, EGFR is phosphorylated and c-Cbl binds to a phosphorylated tyrosine residue of the receptor (5–7). c-Cbl can also bind the receptor indirectly through the adaptor protein Grb2 (3). Subsequently, c-Cbl itself is phosphorylated and, as a result, can mediate the ubiquitylation of the EGFR and promote receptor degradation (5). c-Cbl can associate with and ubiquitylate the receptor at the plasma membrane (8); however, c-Cbl remains associated with the EGFR throughout the endocytic pathway, resulting in prolonged ubiquitylation of the receptor and thus its enhanced degradation (9, 10). Indeed, ubiquitylation of the EGFR is sufficient to serve as a signal for internalization and recent reports demonstrate that attachment in-frame of ubiquitin to EGFR results in constitutive internalization and enhanced degradation of the chimeric protein (11, 12).

In addition to c-Cbl, other proteins take part in the negative regulation of RTKs. Among these proteins are the suppressors of cytokine signaling (SOCS), a family of proteins initially identified as negative regulators of cytokine signaling. The SOCS family consists of eight intracellular proteins (SOCS1–7 and the cytokine-induced Src homology-2 (SH2) protein). SOCS proteins contain a central SH2 domain, an amino-terminal domain of variable length and divergent sequence, and a carboxyl-terminal 40 amino acid sequence known as the SOCS box (SB) (reviewed in Ref. 13). In addition to the SOCS family of proteins, ~40 additional proteins have been identified that contain a SB and protein-protein interaction motifs other than SH2 domains (14). SOCS proteins utilize various mechanisms to attenuate signaling. First, they can compete with STATs for binding sites on activated cytokine receptors. Second, SOCS proteins can bind JAKs to inhibit their tyrosine kinase activity. Third, SOCS proteins can utilize their SOCS box to recruit an E3 ubiquitin ligase protein complex, which targets SOCS-associated proteins for proteasomal degradation (13). For example, following stimulation with cytokines, SOCS1 can undergo SH2 domain-mediated binding to phosphorylated tyrosine residues

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[1] The abbreviations used are: RTK, receptor tyrosine kinase; CHO, Chinese hamster ovary; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; DER, *Drosophila* EGFR; FGFR, fibroblast growth factor receptor; JAK, Janus kinase; NGFR, nerve growth factor receptor; GFP, green fluorescent protein; SOCS, suppressor of cytokine signaling; SB, SOCS box; SH2, Src homology 2; STAT, signal transducers and activator of transcription; mAb, monoclonal antibody.

of activated JAK2. When bound to JAK2, SOCS1 can recruit ElonginC through the SOCS box and ElonginC binds ElonginB, a protein that contains a ubiquitin-like domain (15). The ElonginBC complex interacts with a Cullin family member, which binds to a small ring finger protein, Roc/Rbx1 (16). Together, the SOCS-ElonginBC-Cullin-Roc complex has E3 ubiquitin ligase activity with the substrate recognition function fulfilled by the SOCS protein and the E3 ubiquitin ligase activity fulfilled by Roc1. In the presence of the ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2), the E3 complex can transfer polyubiquitin chains to its substrate JAK2, resulting in JAK degradation by the proteasome (14, 17).

Accumulating evidence indicates a role for SOCS proteins in the regulation of signaling pathways other than cytokine receptors. Recent reports suggest a regulatory role for SOCS proteins in focal adhesion kinase, insulin receptor, and EGFR signaling (18–21). Expression of SOCS proteins, including SOCS1 and SOCS3, is enhanced by EGF treatment, and SOCS3 is also phosphorylated following EGF treatment (20, 21). SOCS3 phosphorylation is required for its association with the Ras inhibitor p120RasGAP, and this association ablates the inhibitory role of p120RasGAP and promotes activation of the Ras/ERK cascade. SOCS1 and SOCS3 physically associate with EGFR and reduce EGF-mediated STAT1 tyrosine phosphorylation (21). Additional signaling molecules, such as the insulin receptor substrate proteins and the guanine nucleotide exchange factor Vav1, are also regulated by SOCS family members that mediate the degradation of these substrates (22, 23).

SOCS genes have also been identified in *Drosophila melanogaster* as well as in *Caenorhabditis elegans*. Worms lack a JAK ortholog, again suggesting a role for SOCS proteins in the regulation of signaling pathways other than cytokine signal transduction (14). One of the three predicted *Drosophila* SOCS family members, SOCS36E, has been implicated as a regulator of DER, the *Drosophila* EGFR ortholog. SOCS36E is most similar to mammalian SOCS5 in its SH2 and SOCS box domains, although its amino terminus is unique and shows no significant homology to vertebrate SOCS proteins. The expression of SOCS36E in the *Drosophila* embryo results in phenotypes in the adult fly that are consistent with defects in JAK/STAT or EGFR signaling and are exacerbated in flies heterozygous for either *D-jak* (hopscotch), *D-stat* (stat92e), or DER (24).

In an attempt to further characterize the outcome and regulation of EGFR signaling, microarray analysis was performed to identify genes whose expression is altered upon stimulation of cells with EGF. We detected increased expression of several genes implicated in the regulation of EGFR signaling, such as various mitogen-activated protein kinase phosphatases and the transmembrane protein LRIG1 (25). In addition, we observed up-regulation of SOCS5 as well as its closest homolog, SOCS4, upon treatment with EGF, which led us to study the role of SOCS proteins in the regulation of EGFR signaling. Here we show that SOCS5 functions to enhance the degradation of EGFR in a ligand- and c-Cbl-independent manner. Both the SH2 and SB domains of SOCS5 are required for EGFR degradation. Furthermore, we show that, in an SH2 domain and SB-dependent manner, enhanced SOCS5 expression results in the translocation of EGFR to intracellular vesicles and in the attenuation of EGF-induced signaling. These results are discussed in the context of an emerging picture regarding the homeostatic machinery that regulates RTK levels.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise indicated, materials were purchased from Sigma. A mixture of ^{35}S-labeled amino acids was purchased from Amersham Biosciences. Lipofectamine was purchased from Invitrogen (San Diego, CA). JET-PEI was supplied by Poly-Transfection (Illkirch, France). Anti-hemagglutinin rat monoclonal antibody (mAb) 3F10 was purchased from Roche Applied Science. The mAbs SG565, specific for EGFR, and L87, specific for ErbB2, have been described previously (5, 26). For EGFR immunoblot analysis, an anti-EGFR mAb (Alexis, San Diego, CA) was used. Murine anti-Myc and anti-phosphotyrosine (pY20) mAbs, rabbit antibodies specific for ErbB-4, and anti-SOCS3 and SOCS5 goat antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies specific for p75-NGFR and fibroblast growth factor receptor (FGFR) were kind gifts from M. Fainzilber (Weizmann Institute of Science, Rehovot, Israel) and D. Ron (Technion, Haifa, Israel), respectively.

Cell Culture and Transfection—CHO cells were cultured in Dulbecco's modified Eagle's/Ham's F-12 medium supplemented with a penicillin-streptomycin mixture (100 units/ml), 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum. HEK-293T, HeLa, and COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with a penicillin-streptomycin mixture (100 units/ml), 1 mM sodium pyruvate, and 10% serum. Transfections were performed using Lipofectamine for CHO cells, JET-PEI for COS cells, and the calcium-phosphate method for HEK-293T and HeLa cells. The total amount of DNA in each transfection was normalized with pcDNA3 or pEF empty vectors. For inducible expression of SOCS5, HEK-293-EcR cells stably expressing the pVgRXR vector were co-transfected with pInd-SOCS5 along with a puromycin resistance-encoding vector. 24 h after transfection, cells were incubated in puromycin-containing medium (1 μg/ml). Muristerone (2 μM) was then added to the cells for the indicated time intervals for induction of SOCS5 expression.

Lysate Preparation, Immunoprecipitation, and Immunoblotting Analyses—48 h after transfection, cells were washed briefly with ice-cold saline and lysed (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 30 mM β-glycerol phosphate, 0.2 mM Na_3VO_4, and a protease inhibitor mixture). For direct electrophoretic analysis, gel sample buffer was added to cleared cell lysates. For EGFR immunoprecipitation, lysates were incubated with anti-EGFR mAbs precoupled to anti-mouse IgG-agarose beads. FLAG-tagged proteins were immunoprecipitated using anti-FLAG-agarose beads, and SOCS5 was immunoprecipitated with anti-SOCS5 goat antibodies precoupled to protein G-Sepharose beads. The immunoprecipitates were washed three times with HNTG (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol), resolved by SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane prior to Western blot analysis.

Quantitative Real-time PCR—cDNA was synthesized from total RNA isolated from HeLa cells using 250 ng of random primers, 3 μg of total RNA, 1 μl of 10 mM dNTP, 4 μl of 5× first-strand buffer, 2 μl of 0.1 M dithiothreitol, and 1 μl of ribonuclease inhibitor RNaseOUT (40 units/μl) and 1 μl (200 units) of Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the protocol recommended by Invitrogen. The following primers were used: *Socs2* (forward, 5′-GCTCGGTCAGACAGGATGGT-3′; reverse, 5′-TTGGCTTCATTAACAGTCATACTTCC-3′); *Socs4* (forward, 5′-CACTCTTCAGGGCTTCCGTC-3′; reverse, 5′-AGGCTAAATCTGATCGAGGTGG-3′); and *Socs5* (forward, 5′-ATCTGGAGACAGCCATACCCA-3′; reverse, 5′-CAAATCAGGCACGAGGCAGT-3′). Quantification of cDNA targets was performed on ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA), utilizing the SDS 2.1 software. All of the reactions were run in duplicates, and transcripts were detected using SYBR Green I according to manufacturer instructions and normalized to β-actin as an internal control. Optimal reaction conditions for amplification of the target genes were performed according to manufacturer's (Applied Biosystems) recommendation.

STAT3 Transcription Assays—Cells grown in 100-mm plates were transfected with GFP- and SOCS-encoding vectors along with a STAT3-luciferase plasmid containing a STAT3 response element fused to a luciferase reporter gene. 24 h later, the cells were split into 24-well plates. 12 h later, the cells were serum-starved for 12 h and then treated with EGF (20 ng/ml) for 5.5 h or left untreated. The luciferase reporter assay was performed using a Promega luciferase assay system (Madison, WI). Light intensity was measured using a luminometer, and the results were normalized to GFP expression levels.

Metabolic Labeling of Cellular Proteins—36 h after transfection, CHO cells were rinsed twice and incubated for 12 h in cysteine- and methionine-free medium supplemented with 10% dialyzed serum and a mixture of ^{35}S-labeled amino acids (pulse). The labeling medium was replaced with fresh medium supplemented with non-radioactive amino acids, and the cells were incubated at 37 °C for various time intervals prior to extraction in lysis buffer (chase). Cell lysates were cleared by

centrifugation and subjected to immunoprecipitation followed by electrophoresis, signal amplification, and autoradiography.

Immunofluorescence—Transfected HeLa cells grown on coverslips were washed twice in phosphate-buffered saline, fixed for 15 min (3% paraformaldehyde in phosphate-buffered saline), and then washed twice in phosphate-buffered saline. Thereafter, the cells were mounted in Mowiol and analyzed using a confocal Zeiss microscope with a 63X/1.4 plane Apochromat objective attached to a Bio-Rad Radiance 2000 laser-scanning system.

Construction of Expression Vectors—pcDNA3-based expression vectors encoding EGFR, ErbB-2, ErbB-4, and the Y1045F point mutant of EGFR were described previously (5). pEF vectors encoding FLAG-SOCS4, FLAG-SOCS2, FLAG-SOCS3, FLAG-SOCS5, and FLAG-SOCS6 were a kind gift from Dr. Paulo De Sepulveda (INSERM, Marseille, France). SOCS5 expression vectors containing point mutations, namely R406K, ΔSB (L469stop), and LC-to-PF (L484P,C488F), were generated by site-directed mutagenesis using the pEF-FLAG-SOCS5 vector. pInd-FLAG-SOCS5 was generated by PCR amplification of FLAG-SOCS5 from the pEF vector. The resulting PCR product was digested with HindIII and XhoI and cloned into pInd. The following plasmids were kind gifts. STAT3-response element luciferase reporter plasmid was from A. Gertler (the Hebrew University's Faculty of Agriculture, Rehovot, Israel), pcDNA3-FGFR1 was from D. Ron (the Technion, Haifa, Israel), pcDNA3-p75-NGFR was from M. Fainzilber (The Weizmann Institute, Rehovot, Israel), hemagglutinin-ubiquitin was from D. Bohmann (the European Molecular Biology Laboratory, Heidelberg, Germany), pCEFL/Myc-Cbl was from S. Lipkowitz (National Institutes of Health, Bethesda, MD), and pEF-FLAG-ElonginB and pEF-Myc-ElonginC were from N. Nicola (Walter and Eliza Hall Institute, Melbourne, Australia).

RESULTS

The Expression of SOCS5 Is Enhanced by EGF—The expression of multiple negative regulators of signaling through RTKs is induced upon growth factor stimulation. This reflects evolutionary conserved feedback loops aimed at homeostatic regulation (27). Notably, a recent report by Cacalano *et al.* (20) showed that treatment of cells with growth factors such as EGF and platelet-derived growth factor induces a rapid elevation in SOCS3 expression. Thus, we examined the effects of EGF treatment on the expression of SOCS family members. In a microarray analysis in which RNA from EGF-treated cells was used, an elevation in both *Socs3* and *Socs5* mRNA levels was detected 20–40 min following EGF stimulation. Soon after, *Socs3* and *Socs5* mRNA levels returned to basal levels (Fig. 1*A*). No expression or change in expression was detected for other SOCS proteins included in the microarrays, specifically *Socs1*, *Socs7*, *Cis*, and *Socs2* (Fig. 1*A* and data not shown).

To confirm the results obtained in the microarray, the expression of several SOCS family members was also analyzed by quantitative real-time PCR. The expression of *Socs5* and *Socs2* as well as *Socs4*, which was not present on the microarray chip, was analyzed using RNA from EGF-treated cells (Fig. 1*B*). We detected an 8-fold increase in the expression of *Socs5* following EGF stimulation. The higher induction detected by RT-PCR is attributable to the relatively high sensitivity of detection as compared with the microarray analysis. Interestingly, the expression of *Socs4* was also increased in a pattern similar to that of *Socs5*. Although the expression of *Socs2* was not altered upon EGF treatment in the microarray analysis using real-time PCR, a 2-fold increase was observed following 60 min of stimulation with EGF.

Analysis of SOCS5 expression at the protein level revealed detectable expression prior to EGF stimulation; however, its expression increased following stimulation as did the expression of SOCS3 (Fig. 1*C*). Taken together, the results presented in Fig. 1 indicate that activation of EGFR results in elevated expression of SOCS3 as well as SOCS4 and SOCS5.

SOCS5 Reduces the Expression of EGFR—Various SOCS proteins can mediate the degradation of tyrosine kinases such as JAK and focal adhesion kinase, and as a result terminate downstream signaling events (17, 18, 28). As the data shown in Fig. 1 demonstrate that the expression of several SOCS proteins is elevated following EGF stimulation, we examined whether these proteins would affect EGFR expression levels. These analyses were performed by co-transfection of plasmids encoding EGFR and SOCS into CHO cells, which lack endogenous EGFR. Interestingly, EGFR levels were significantly reduced only in cells co-expressing SOCS5 or its closest homolog, SOCS4, irrespective of EGF treatment and despite the relatively low levels of ectopic SOCS5 expression (Fig. 2*A*). Expression of other SOCS proteins including cytokine-induced SH2 (CIS), SOCS2, SOCS3, and SOCS6 did not alter EGFR expression (Fig. 2*A*). Because EGF stimulation results in the phos-

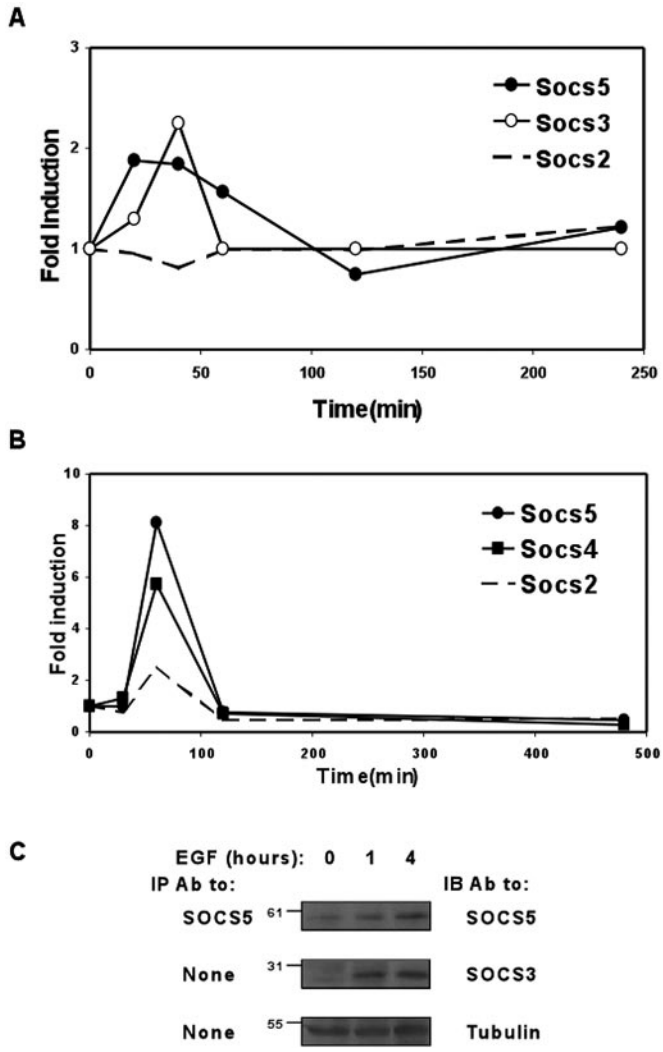

FIG. 1. **The expression of SOCS5 is enhanced by EGF.** *A*, subconfluent HeLa cells (~1.7×10^6/plate) were serum-starved for 24 h followed by treatment with EGF (20 ng/ml) for increasing time periods. Cells were then lysed, and total RNA was prepared followed by reverse transcription with random hexamer primers for preparation of cDNA. Affymetrix Hu133A oligonucleotide arrays were utilized for analysis of the cDNA. Following normalization of the signals for all six DNA arrays, the expression levels of *Socs2* (*dashed line*), *Socs3* (*open circles*), and *Socs5* (*closed circles*) were determined. *B*, subconfluent HeLa cells (~1.7×10^6/plate) were serum-starved for 24 h followed by treatment with EGF (20 ng/ml) for increasing time periods. Cells were then extracted, and total RNA was prepared followed by reverse transcription with random hexamer primers. Real-time PCR was carried out with primers specific for *Socs2* (*dashed line*), *Socs4* (*closed squares*), and *Socs5* (*closed circles*). The level of gene expression was quantified in comparison to a standard curve by serial dilution of the template cDNA. *C*, A431 cells were starved for serum factors for 12 h. The cells were then incubated at 37 °C for the indicated time intervals without or with EGF (100 ng/ml). Thereafter, the cells were lysed and the extracts were subjected to analyses with the indicated antibodies. *Ab*, antibody; *IP*, immunoprecipitation; *IB*, immunoblotting.

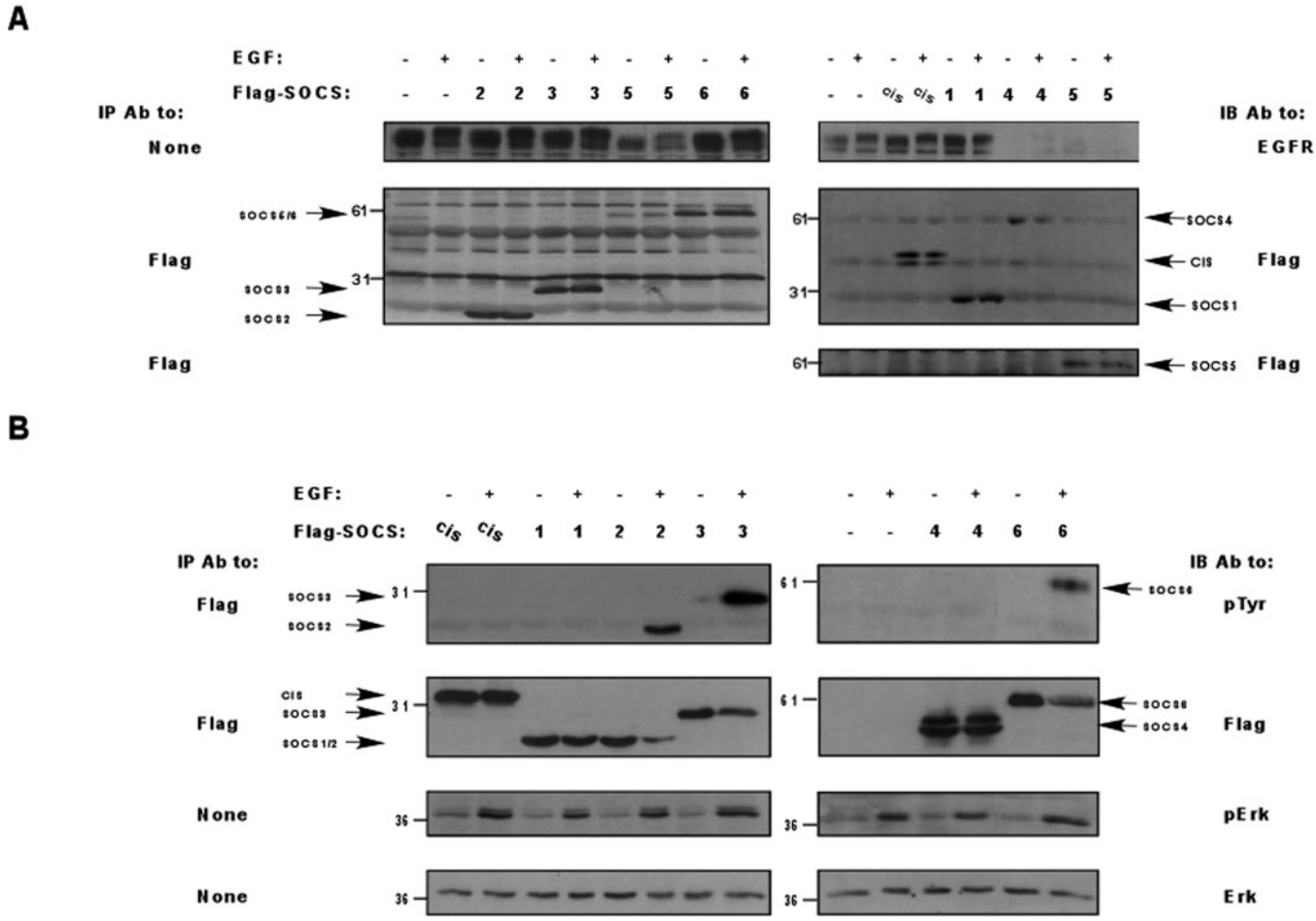

FIG. 2. **SOCS5 reduces the expression of EGFR.** *A*, CHO cells were transfected with an EGFR-encoding plasmid along with plasmids encoding the indicated SOCS proteins. 36 h after transfection, the cells were serum-starved for 12 h and then incubated for 10 min at 37 °C without or with EGF (100 ng/ml). Thereafter, the cells were lysed and the lysates were subjected to analyses with the indicated antibodies. The *arrows* mark the locations of the different SOCS proteins. The *bottom right panel* is a longer exposure of the FLAG immunoblot for the detection of SOCS5. *B*, COS7 cells were transfected with a plasmid encoding each of the indicated SOCS plasmids. 24 h after transfection, the cells were serum-starved for 12 h and incubated for 10 min at 37 °C without or with EGF (100 ng/ml). Thereafter, the cells were lysed and the lysates were subjected to analyses with the indicated antibodies (*pTyr*, phosphotyrosine). *Ab*, antibody; *IP*, immunoprecipitation; *IB*, immunoblotting.

phorylation of SOCS3 (20), we examined the phosphorylation of SOCS family members following EGF stimulation. SOCS2, 3, 5, and 6 all underwent tyrosine phosphorylation following EGF stimulation, but no tyrosine phosphorylation of SOCS4 was detectable (Figs. 2*B* and 6*C*). Notably, the expression of either SOCS4 or SOCS5 leads to a reduction in EGFR expression levels (Fig. 2*A*) but only SOCS5 undergoes ligand-induced phosphorylation, raising the possibility that down-regulation of EGFR requires no EGF-induced phosphorylation of the respective SOCS protein.

Both the SOCS Box and SH2 Domain of SOCS5 Are Required for the Reduction in EGFR Levels—Consistent with the ability of SOCS5 to down-regulate EGFR expression, its *Drosophila* ortholog, SOCS36E, was recently implicated as a negative regulator of DER, the *Drosophila* EGFR (24). Therefore, we investigated the mechanism underlying SOCS5-mediated regulation of EGFR. Because SOCS proteins utilize their SB to recruit an E3 ubiquitin ligase complex and thereby degrade substrate proteins (14, 22, 28), we examined whether the SB of SOCS5 is essential for the reduction in EGFR expression levels. To this end, we constructed a mutant of SOCS5, ΔSB-SOCS5, in which the SB was deleted (Fig. 3*A*). In contrast to WT SOCS5, ΔSB-SOCS5 was unable to reduce EGFR levels (Fig. 3*B*), demonstrating that the SB is required for the decrease in receptor levels. In addition to the SB, SOCS proteins utilize their SH2 domain for the recognition of substrates, which appears to be essential for protein degradation (28). Hence, we modified the SH2 domain by introducing a point mutation (mutant denoted R406K-SOCS5 in Fig. 3*A*), which severely compromises binding to phosphotyrosine residues (29). We next established and utilized an inducible system of HEK-293-EcR cells for R406K-SOCS5 expression. In these cells, the expression of SOCS5 is under the control of an ecdysone-responsive element and can be induced by muristerone. Following 12 or 24 h of treatment with muristerone, cells were lysed and endogenous EGFR was immunoprecipitated. As expected, the wild type form of SOCS5 was expressed only following treatment with muristerone and its expression was accompanied by a marked reduction in the levels of endogenous EGFR (Fig. 3*C*). Although R406K-SOCS5 expression was induced by treatment with muristerone, EGFR levels were not affected (Fig. 3*C*), suggesting that the SH2 domain of SOCS5, similar to SB, is essential for the decrease in receptor levels.

SOCS5 Reduces the Expression of ErbB Family Members but Not Other Cell Surface Receptors—We next assessed the effect of SOCS5 on the levels of additional ErbB/EGFR family members, namely ErbB-2 and ErbB-4. Similar to our finding regarding EGFR/ErbB-1, ErbB-2 and ErbB-4 levels declined in cells co-expressing WT SOCS5 (Fig. 3*D*). However, neither ErbB-2 nor ErbB-4 levels were significantly affected by the expression of the ΔSB-SOCS5 mutant. To determine whether receptors that do not belong to the ErbB family are also affected by

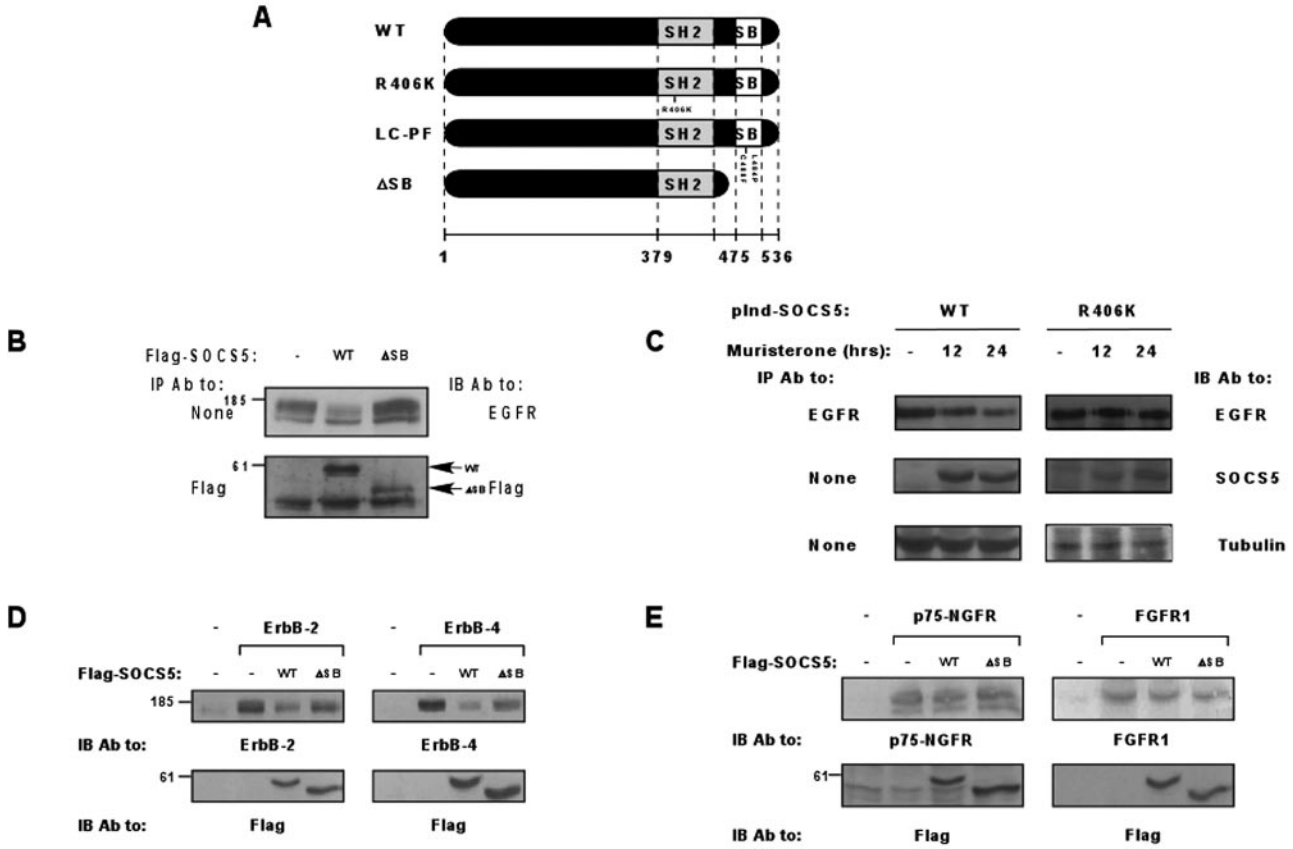

FIG. 3. **SOCS5 reduces the expression of ErbB family members but not other cell surface receptors.** *A*, schematic representation of WT and mutant SOCS5 proteins. *Numbers* below the scheme refer to amino acids. *B*, CHO cells were transfected with an EGFR-encoding vector along with plasmids encoding the indicated SOCS proteins or an empty control vector (−). 48 h after transfection, the cells were lysed and the lysates were subjected to analyses with the indicated antibodies. *C*, HEK-293-EcR cells were transfected with the pInd-WT SOCS5 or pInd-R406K-SOCS5 vectors along with a vector encoding puromycin resistance. 24 h after transfection, the cells were transferred to puromycin-containing medium (1 μg/ml) for 24 h. Muristerone (2 μM) was then added to the medium for the indicated time intervals. Thereafter, the cells were lysed and the lysates were subjected to analyses with the indicated antibodies. *D* and *E*, COS7 cells were transfected with the indicated SOCS plasmids along with vectors encoding one of the following receptors listed in *D* and *E*. *D*, ErbB-2 and ErbB-4. *E*, the low affinity NGFR (p75-NGFR) or the FGFR1. 48 h after transfection, the cells were lysed and the lysates were subjected to immunoblotting (*IB*) with the indicated antibodies. *IP*, immunoprecipitation.

SOCS5, we co-transfected cells with WT or ΔSB-SOCS5 along with the FGFR1, a receptor tyrosine kinase, or the p75 low affinity nerve growth factor receptor (p75-NGFR). The latter has no intrinsic catalytic activity but can form heterodimers with Trk receptor tyrosine kinase (30). Neither FGFR1 nor p75-NGFR levels were altered by the expression of WT or ΔSB-SOCS5 (Fig. 3*E*). Thus, these results demonstrate that SOCS5 can reduce the expression of ErbB receptors, whereas it does not affect the expression of other cell surface receptors such as the FGFR1 or p75-NGFR.

EGFR Is Rapidly Degraded in SOCS5-expressing Cells—To determine whether EGFR levels are reduced in SOCS5-expressing cells because of enhanced receptor degradation, we performed a pulse-chase experiment. As shown in Fig. 4*A*, the turnover of EGFR was significantly enhanced in cells co-expressing SOCS5. Whereas EGFR half-life was ~10 h, SOCS5 expression enhanced the decay rate, reducing the half-life of EGFR to ~6 h (Fig. 4*A*). Interestingly, EGFR expression enhanced the rate of SOCS5 degradation, reducing its half-life from 60 min in control cells to ~25 min (Fig. 4*B*). In EGFR-expressing cells treated with MG132, a proteasome inhibitor, the half-life of SOCS5 was ~2 h. Thus, the results depicted in Fig. 4 show that the reduction in EGFR levels in cells co-expressing SOCS5 is due to enhanced EGFR degradation that is accompanied by enhanced degradation of SOCS5 itself.

EGFR Degradation by SOCS5 Is Independent of c-Cbl—Upon EGF stimulation, EGFR is degraded through a process involving its ubiquitylation by c-Cbl (5, 6). Therefore, we examined the role of c-Cbl in the SOCS5-mediated EGFR degradation process. First, we tested the ability of SOCS5 to reduce the levels of the Y1045F-EGFR mutant in which the c-Cbl binding site, tyrosine 1045, is mutated (3). The initial expression levels of WT and Y1045F-EGFR were not equal, because the Y1045F-EGFR mutant could not undergo direct c-Cbl-mediated ubiquitylation and degradation and hence was more stable than WT EGFR (3). Although the initial levels of WT and Y1045F-EGFR were different, co-expression of SOCS5 comparably reduced the levels of both types of receptors (Fig. 5*A*). Next, we transfected CHO cells with an EGFR-encoding vector along with a combination of SOCS5 and either WT or 70Z-Cbl. 70Z-Cbl is a naturally occurring mutant with an impaired ubiquitin ligase activity due to a deletion of 17 amino acids amino-terminal to the RING finger domain (31). EGFR levels in SOCS5-expressing cells were reduced prior to EGF stimulation. As expected, EGFR levels in cells expressing WT c-Cbl were reduced only subsequent to ligand stimulation but were unaffected in 70Z-Cbl-expressing cells (Fig. 5*B*, compare *lanes 6* and *8*). In cells co-expressing SOCS5 and 70Z-Cbl, EGFR levels were reduced to a similar extent as in cells transfected with SOCS5 and an empty control vector (Fig. 5*B*, compare *lanes 4* and *10*). Thus, the expression of 70Z-Cbl along with SOCS5 did not block SOCS5-mediated EGFR degradation, implying that SOCS5 utilizes an alternative mechanism for EGFR degradation.

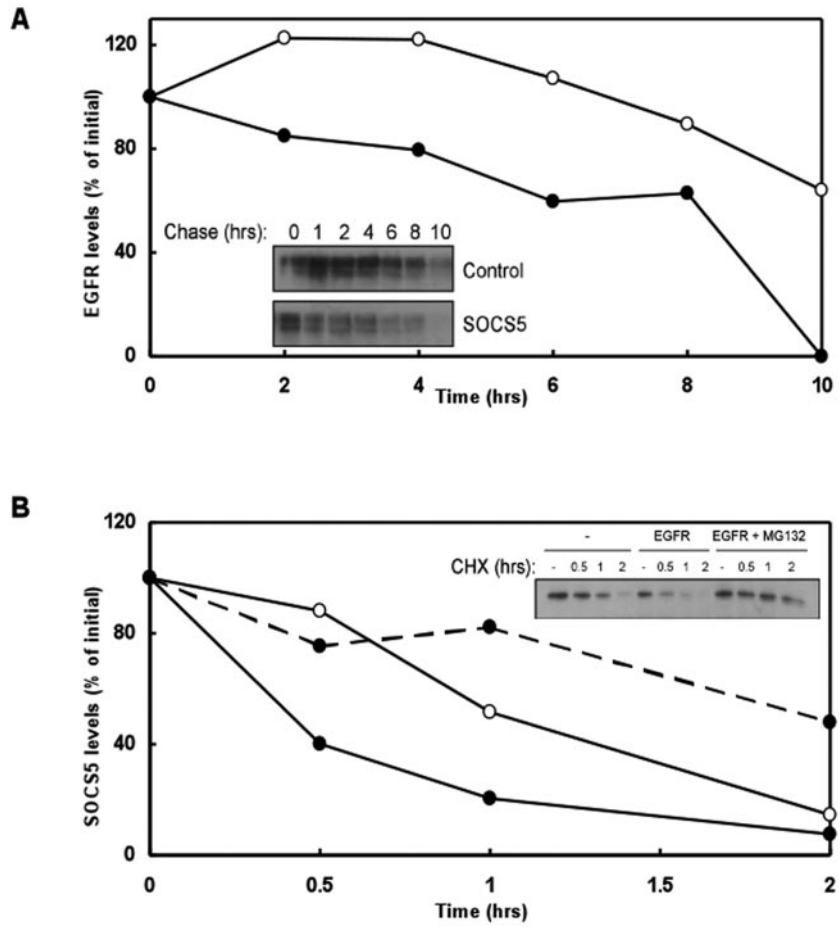

FIG. 4. **EGFR is rapidly degraded in SOCS5-expressing cells.** *A*, CHO cells were transfected with an EGFR-encoding vector along with a vector encoding a FLAG-tagged SOCS5 (wild type; *closed symbols*), or an empty control vector (*open symbols*). 36 h after transfection, the cells were washed and pulse-labeled with ^{35}S-labeled methionine and cysteine for 12 h. The cells were then washed three times and chased for the indicated periods. Thereafter, the cells were lysed and the lysates subjected to IP with an anti-EGFR antibody. The relative intensity of the EGFR bands was quantified using a densitometer. *B*, CHO cells were transfected with a FLAG-SOCS5-encoding plasmid along with an empty control plasmid (*open symbols*) or an EGFR-encoding plasmid (*closed symbols*). 48 h after transfection, the cells were preincubated for 30 min at 37 °C without or with MG132 (10 μM; *closed symbols*, *dashed line*). Cyclohexamide (*CHX*, 10 μg/ml) was then added to the medium for the indicated times. Thereafter, the cells were lysed and the lysates were subjected to analyses with an anti-SOCS5 antibody. The relative intensity of the SOCS5 bands was quantified using a densitometer.

Our results imply that SOCS5- and c-Cbl-mediated degradation of EGFR are independent processes (Fig. 5, *A* and *B*). To confirm this hypothesis, we tested whether R406K-SOCS5, which cannot mediate SOCS5-dependent EGFR degradation (Fig. 3*C*), would disrupt c-Cbl-mediated EGFR degradation. As predicted, c-Cbl accelerated EGFR degradation upon EGF stimulation in both control and R406K-SOCS5-expressing cells (Fig. 5*C*). Thus, c-Cbl and SOCS5 function independently to promote EGFR degradation. EGF-dependent c-Cbl-mediated degradation of EGFR is not affected by the expression of a SOCS5 mutant, and comparably, EGF-independent SOCS5-mediated degradation of EGFR cannot be blocked by the expression of either the 70Z-Cbl mutant or the mutated Y1045F-EGFR.

SOCS5 Can Physically Associate with the EGFR—The enhanced degradation of the EGFR upon expression of SOCS5 may involve physical association between these two proteins. To assess this interaction, EGFR was expressed along with SOCS5 in HEK-293T cells. The cells were treated with MG132, because SOCS5 is a short-lived protein (Fig. 4*B*) and inhibition of proteasomal activity is required for the accumulation of sufficient amounts of SOCS5 for co-immunoprecipitation experiments. Indeed, we demonstrated that SOCS5 physically associates with EGFR as it was detected in EGFR immunocomplexes (Fig. 6*A*). In experiments that were not presented, we also detected this interaction with the endogenous SOCS5 and EGFR. In addition, we also examined the ability of point mutants of either the SH2 or the SB domains of SOCS5 (see below) to bind the EGFR. The SB mutant weakly associated with EGFR, whereas the SH2 mutant did not co-immunoprecipitate with the receptor (data not shown). These results lead us to suggest that the physical interaction of SOCS5 with EGFR is mediated by the SH2 domain of SOCS5 with an additional role of the SB.

The SOCS Box Domain of SOCS5 Is Essential for EGFR Degradation and Mediates the Binding of the ElonginBC Protein Complex—Our results demonstrate that the SH2 domain of SOCS5 is necessary for both association with EGFR and for EGFR degradation (Fig. 3*C* and data not shown). Furthermore, the deletion of the SB of SOCS5 ablates EGFR degradation (Fig. 3*B*). To confirm the requirement for the SB in SOCS5-mediated EGFR degradation, we extended our analyses to another SB-defective mutant of SOCS5 (see Fig. 3*A*). Thus, we tested the function of a double point mutant of SOCS5 in which mutations were introduced in two critical residues, Leu-484 and Cys-488 (mutant denoted LC-to-PF), that mediate the binding of ElonginC to the SB (22). To test whether SOCS5 can recruit the ElonginBC complex via the SOCS box domain, either WT or LC-to-PF SOCS5 proteins were expressed in COS7 cells together with FLAG-ElonginB and Myc-ElonginC. ElonginB functions to stabilize ElonginC (32); therefore, ElonginC expression levels are higher in cells co-expressing ElonginB. In cells co-expressing both ElonginB and ElonginC, an interaction of both these proteins with WT SOCS5 could be detected (Fig. 6*B*). The Elongin proteins did not co-immunoprecipitate with the LC-to-PF SOCS5 mutant, confirming that Leu-484 and Cys-488 of SOCS5 are necessary for the binding of the ElonginBC complex.

Having established that the LC-to-PF SOCS5 mutant does not bind ElonginBC, we determined the effect of this mutant on EGFR. These experiments were performed in COS7 cells, which express high levels of EGFR, enabling the assessment of the effect of SOCS5 on endogenous EGFR. Both WT and mutant SOCS5 were phosphorylated on tyrosine residues follow-

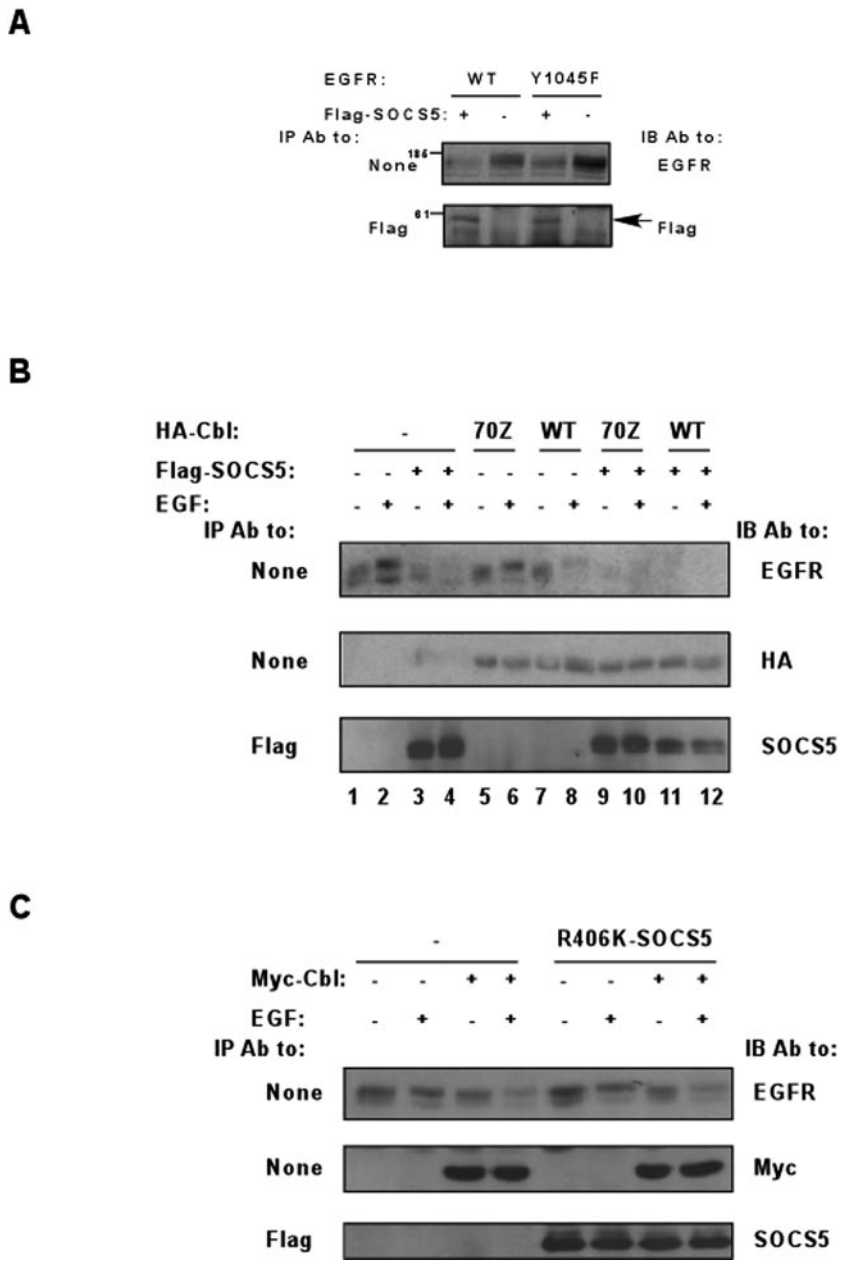

FIG. 5. **SOCS5-induced degradation of EGFR is independent of c-Cbl.** *A*, CHO cells were transfected with plasmids encoding WT or Y1045F-EGFR along with an empty vector or a FLAG-SOCS5 plasmid as indicated. 48 h after transfection, the cells were lysed and the lysates were subjected to analyses with the indicated antibodies. An *arrowhead* marks the location of the SOCS5 band. *B*, CHO cells were transfected with an EGFR-encoding plasmid along with each of the indicated Cbl and SOCS5 plasmids. 36 h after transfection, the cells were serum-starved for 12 h and incubated for 20 min at 37 °C without or with EGF (100 ng/ml). Thereafter, the cells were lysed and the lysates were subjected to analyses with the indicated antibodies. *C*, CHO cells were transfected with an EGFR-encoding plasmid along with each of the indicated SOCS5 and Cbl plasmids. 36 h after transfection, the cells were serum-starved for 12 h and incubated for 15 min at 37 °C without or with EGF (100 ng/ml). Thereafter, the cells were lysed and the lysates were subjected to analyses with the indicated antibodies. *HA*, hemagglutinin; *Ab*, antibody; *IP*, immunoprecipitation; *IB*, immunoblotting.

ing EGF stimulation. However, EGFR levels were decreased only by WT SOCS5 and not by either the R406K or the LC-to-PF SOCS5 mutants (Fig. 6*C*). Thus, similarly to the ΔSB SOCS5 mutant, the LC-to-PF mutant did not mediate EGFR degradation, confirming that the SB is required for this degradation process. Notably, the phosphorylation of SOCS5 was not required for EGFR degradation because it occurred only following EGF stimulation, whereas EGFR degradation precedes EGF stimulation.

The complex recruited by SOCS proteins is composed of ElonginBC, Cullin, and Roc1 (15, 16). Together, this complex has E3 ubiquitin ligase activity. We suspect that the role of the SB domain is to mediate coupling of EGFR with the Elongin-Cullin-Roc E3 ubiquitin ligase complex, resulting in enhanced EGFR degradation. Hence, we analyzed the EGFR ubiquitylation pattern in the presence of SOCS5 and an inhibitor of the 26 S proteasome. As expected, EGFR ubiquitylation was enhanced in SOCS5-expressing cells treated with MG132 (Fig. 6*D*).

EGFR Is Localized in Intracellular Vesicles in SOCS5-expressing Cells—Ligand-induced degradation of EGFR follows its internalization from the plasma membrane and progression into internal vesicles of the endocytic pathway (9, 10). Using confocal microscopy, we determined whether ligand-independent effects of SOCS5 on EGFR degradation are also accompanied by vesicular localization. To this end, we used a fluorescent EGFR fusion protein that retains all of the functions of the wild type receptor (33). HeLa cells were co-transfected with a GFP-EGFR fusion protein and SOCS5-encoding vectors. In control cells, GFP-EGFR was localized mainly to the cell surface (Fig. 7*A*). However, in cells co-expressing SOCS5, GFP-EGFR was absent from the cell surface but was present in intracellular vesicles (Fig. 7*B*). GFP-EGFR localization was not affected by the expression of R406K-SOCS5 and remained localized at the cell surface (Fig. 7*C*), consistent with the inability of this mutant to enhance degradation of EGFR (Figs. 3*C* and 6*C*). Experiments performed using endoglycosidase H and cell-surface biotinylation revealed that EGFR maturation and delivery to the plasma membrane are comparable in SOCS5-expressing and control cells (data not shown), in line with an effect of SOCS5 on EGFR internalization.

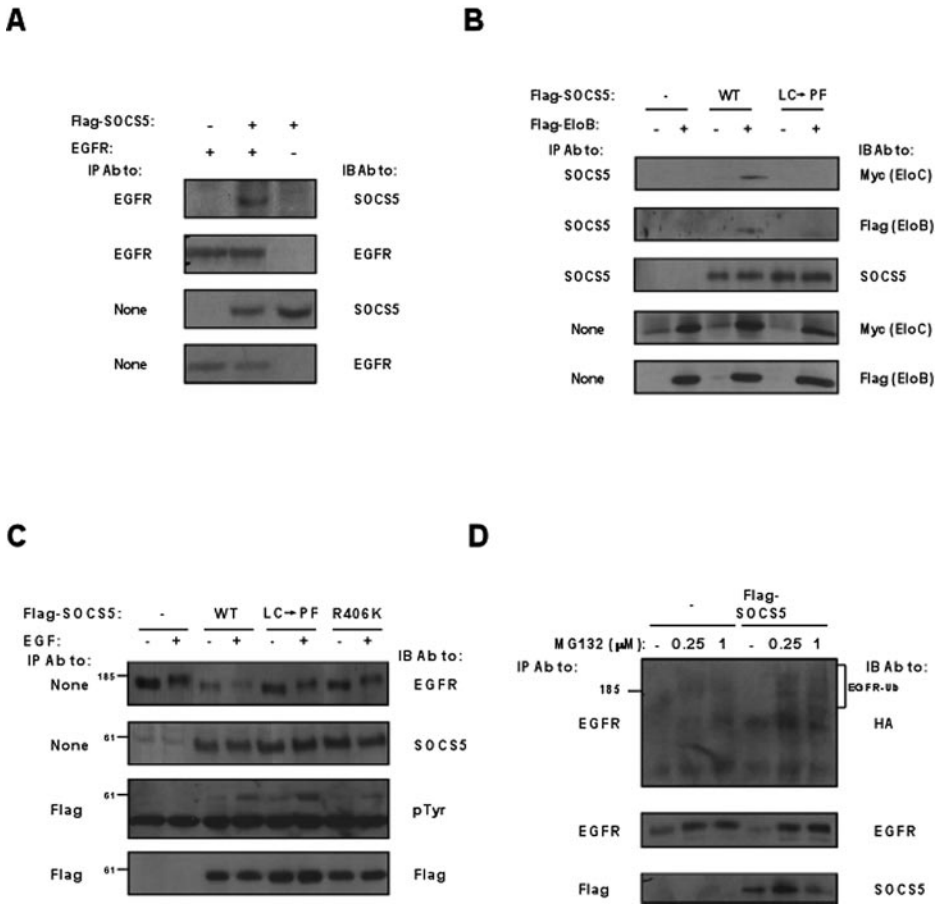

FIG. 6. **Both the SH2 and SB domains are necessary for SOCS5-induced degradation of EGFR.** *A*, HEK-293T cells were transfected with an EGFR-encoding plasmid or an empty control vector along with a SOCS5-encoding plasmid. 48 h after transfection, the cells were incubated for 3 h at 37 °C with MG132 (10 μM). The cells were then lysed and their lysates were subjected to analyses with the indicated antibodies. *B*, COS7 cells were transfected with a plasmid encoding Myc-ElonginC (*EloC*) and the indicated combination of FLAG-SOCS5 and FLAG-ElonginB (*EloB*) expression vectors. 48 h after transfection, the cells were incubated for 2 h at 37 °C with MG132 (10 μM). The cells were then lysed and the lysates were subjected to analyses with the indicated antibodies. *C*, COS7 cells were transfected with a plasmid encoding puromycin resistance along with each of the indicated SOCS5 plasmids. 24 h after transfection, the cells were transferred to puromycin-containing medium (2 μg/ml) for 36 h, serum-starved for 12 h, and incubated for 15 min at 37 °C without or with EGF (100 ng/ml). Thereafter, the cells were lysed and the lysates were subjected to analyses with the indicated antibodies. *D*, CHO cells were transfected with a hemagglutinin (*HA*)-ubiquitin (*Ub*) and EGFR-encoding plasmids along with a SOCS5-encoding plasmid or a control empty vector. 24 h after transfection, the cells were incubated for 17 h at 37 °C with MG132. Thereafter, the cells were lysed and the lysates were subjected to analyses with the indicated antibodies. The location of ubiquitylated EGFR is indicated. *Ab*, antibody; *IP*, immunoprecipitation; *IB*, immunoblotting.

SOCS5 Reduces EGFR-mediated Signaling—Because stimulation of cells with EGF is followed by up-regulation of SOCS5 expression (Fig. 1) and subsequent degradation of EGFR (Fig. 4), we predicted that signaling pathways activated by the EGFR would also be affected. To test our prediction, COS7 cells were transfected with a STAT3-responsive element fused to a luciferase reporter gene along with an empty control vector or a SOCS-encoding plasmid. Following EGF stimulation, STAT3 is activated by phosphorylation mediated directly by EGFR or indirectly by Src kinases (34, 35). While in control cells, EGF stimulation led to a 20-fold increase in luciferase activity, luciferase activity was markedly reduced in both SOCS5 and SOCS4-expressing cells (Fig. 7*D*). The expression of cytokine-induced SH2 (CIS), which does not affect EGFR degradation (Fig. 2*A*, *right panel*), resulted in a slight increase in EGF-induced STAT3 activation (Fig. 7*D*). We then tested the effect of expressing either the R406K or LC-to-PF SOCS5 mutants on EGF-induced STAT3 activation. The expression of both of these SOCS5 mutants did not significantly alter EGF-induced STAT3 activation (Fig. 7*E*). Hence, SOCS5 expression not only reduces EGFR expression levels but also down-regulates EGF-induced STAT3 activation in an SH2- and SB-dependent manner.

DISCUSSION

The amplitude and duration of signaling through cell surface receptors are tightly regulated to induce the appropriate physiological responses. Endocytosis and subsequent receptor degradation constitute the main process for irreversible attenuation of signaling events initiated upon ligand binding to RTKs such as EGFR with a key role played by ubiquitin and the E3 ubiquitin ligase c-Cbl. Furthermore, RTK signaling results in the induction of the expression of multiple additional proteins, which participate in the regulatory process, such as Sprouty and Kekkon1 in *Drosophila* (36). To characterize the regulatory proteins induced following EGF stimulation, we performed a microarray analysis on mRNA extracted from cells stimulated with EGF. We observed that the mRNA levels of several SOCS family members including *socs5* are induced upon EGF treatment and confirmed the increase at both the mRNA and protein levels (Fig. 1). Furthermore, using an insect hormone-inducible system (Fig. 3*C*), we inferred that the newly synthesized SOCS5 molecules mediate the degradation of EGFR in a ligand-independent manner. SOCS5 and its closest homolog, SOCS4, are the only SOCS family members that markedly reduce EGFR levels (Fig. 2*A*) despite the ability of

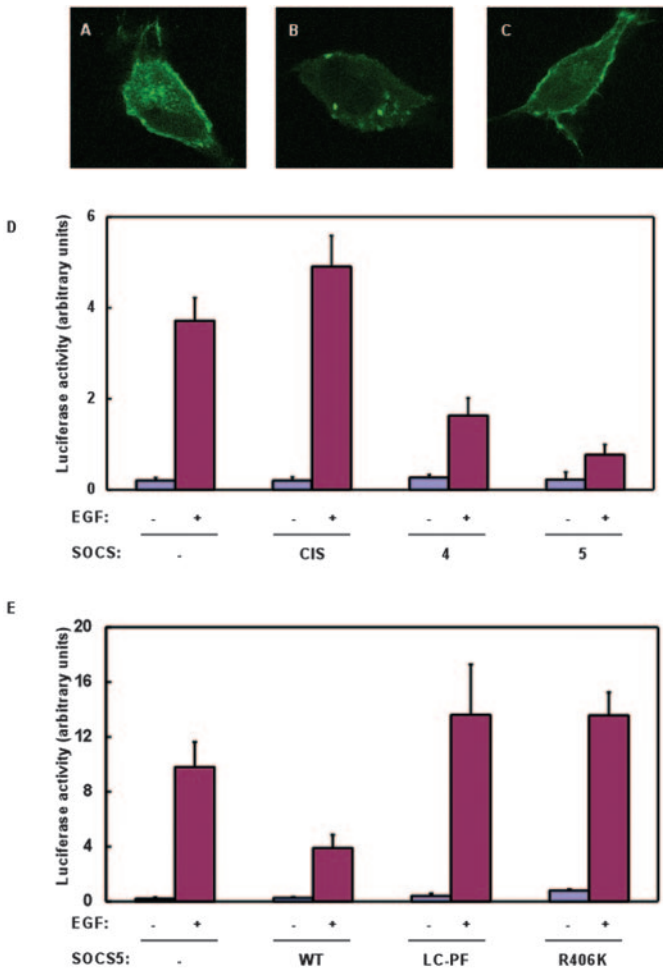

FIG. 7. **SOCS5 suppresses EGFR signaling and translocates the receptor to intracellular vesicles.** HeLa cells were transfected with a GFP-EGFR-encoding vector along with an empty control vector (*A*), WT FLAG-SOCS5 (*B*), or R406K-FLAG-SOCS5 (*C*) encoding vectors. 48 h after transfection, the cells were fixed and the localization of the fluorescent GFP-EGFR was visualized using confocal microscopy. *D* and *E*, COS7 cells were transfected with a STAT3-luciferase reporter gene, a GFP-encoding plasmid, and a control empty vector or a SOCS-encoding plasmid (*D*), or WT or mutant SOCS5-encoding plasmid (*E*). 36 h after transfection, the cells were serum-starved for 12 h. The cells were then incubated for 5.5 h at 37 °C without or with EGF (20 ng/ml). Thereafter, the cells were lysed and the relative luminescence signal was determined. The results represent the mean ± S.D. of triplicates normalized to the GFP signal. *CIS*, cytokine-induced SH2.

EGF to induce the expression and phosphorylation of several additional SOCS proteins (Figs. 1 and 2*B*) (20, 21). The reduced expression level of EGFR in SOCS5-expressing cells is the result of enhanced degradation as demonstrated by metabolic labeling (Fig. 4*A*). In addition to EGFR, SOCS5 also acts to reduce the expression levels of ErbB-2 and ErbB-4, presumably through similar mechanisms, but does not reduce the expression of the unrelated cell surface receptors FGFR or p75-NGFR (Fig. 3, *D* and *E*).

SOCS5 has been shown to be induced by interleukin 6 (37), and SOCS5 expression can inhibit interleukin 4, interleukin 6, and leukemia inhibitory factor-induced signaling (38, 39). Our data extend these observations to ErbB RTKs and are consistent with studies of SOCS36E, the *Drosophila* ortholog of mammalian SOCS5. Both DER, the *Drosophila* EGFR ortholog, and the JAK-STAT signaling cascades were affected by the overexpression of SOCS36E (24). SOCS5 may be part of a negative feedback loop in which its expression is up-regulated upon EGF stimulation to temper EGF-induced signaling through receptor degradation. In addition, several cytokines utilize ErbB receptors to activate the mitogen-activated protein kinase signaling cascade (40, 41). Thus, both EGFR and cytokines induce the expression of SOCS5, which may then regulate signaling processes that are activated directly by the EGFR or indirectly as part of a cross-talk between cytokine and growth factor signaling components. SOCS5 knockout mice were recently generated (42) and showed no discernable phenotype in the lymphocyte parameters examined. Because lymphocytes do not express EGFR, it will be of interest to determine whether EGFR-dependent processes are altered in SOCS5-deficient animals.

In examining the requirements for SOCS5-mediated receptor degradation, we analyzed SOCS5 phosphorylation and found that, similar to other SOCS proteins, SOCS5 is phosphorylated by growth factors including EGF and neuregulins (Fig. 6*C* and data not shown). However, SOCS5 phosphorylation does not appear to be necessary for EGFR degradation because its phosphorylation occurs subsequent to treatment with EGF, whereas EGFR degradation is ligand-independent. EGFR-phosphorylated SOCS5 can associate with the adaptor protein Grb2 (data not shown), implying that SOCS5 may also have additional roles in regulating EGF-mediated signaling. In this context, it is of interest to note that tyrosine phosphorylation of SOCS3 by growth factors and cytokines does not serve for negative regulation of signaling but for its association with the Ras inhibitor p120RasGAP, which results in promoting the activation of the ERK cascade (20). As the results presented in Fig. 2*B* show that SOCS2 and SOCS6 are also phosphorylated following EGF treatment, we suggest that these proteins may also participate in the regulation of EGFR signaling in a manner distinct from SOCS5-mediated regulation of receptor expression levels. Likewise, although SOCS4 mediates decreases in EGFR levels similar to SOCS5, it is not phosphorylated upon treatment with EGF and may thereby function differently.

EGFR degradation by SOCS5 requires both functional SH2 and SB domains (Figs. 3*B* and 6*C*). Furthermore, SOCS5 can bind both the EGFR and the ElonginBC complex (Fig. 6). The function of the SOCS5 SB domain is likely in recruiting the ElonginBC complex, as the mutagenesis of SB residues critical for ElonginBC binding disrupts the ability of SOCS5 to mediate EGFR degradation (Fig. 6*C*). In turn, the ElonginBC complex has been shown to bind Cullin proteins and a RING finger protein, Roc/Rbx1, which has E3 ubiquitin ligase activity (14). Thus, the ElonginBC-containing protein complex recruited by the SB domain of SOCS5 may mediate the elevation in EGFR ubiquitylation (Fig. 6*D*), marking it for degradation. In addition to EGFR degradation, this protein complex may be responsible for the rapid degradation of SOCS5 itself (Fig. 4*B*). Other SOCS proteins were also shown to be short-lived and postulated to undergo degradation by an ElonginBC-containing E3 ubiquitin ligase complex (15). EGFR ubiquitylation was only detectable upon inhibition of proteasomal activity, which may be due to rapid degradation of receptors upon ubiquitylation. Alternatively, the complex recruited by SOCS5 may mediate EGFR degradation through additional mechanisms not involving its ubiquitylation. For example, ElonginB contains a ubiquitin-like domain, which in other proteins has been shown to undergo direct interactions with the 26 S proteasome (43).

The mechanistic basis underlying the requirement for the SH2 domain of SOCS5 for EGFR degradation is less clear. EGFR is degraded prior to ligand stimulation, conditions in which the receptor is not tyrosine-phosphorylated to create potential docking sites for SH2 domain-containing proteins such as SOCS5. Nonetheless, our results suggest that the SH2 domain of SOCS5 is necessary for both its interaction with the EGFR and EGFR degradation (Figs. 3*C* and 6*C* and data not

shown). Although the intrinsic tyrosine kinase activity of the receptor is not required for the degradation process (data not shown), EGFR may be transphosphorylated by kinases such as Src, ErbB-2, or Jak to create a binding site for the SH2 domain of SOCS5. Indeed, a kinase-inactive EGFR mutant can still be phosphorylated by JAK to create docking sites for the SH2 domains of signaling molecules such as Grb2 (41). This scenario predicts that SOCS5 may regulate signaling through EGFR transactivated by cellular stimuli in addition to EGF such as cytokines. Alternatively, SOCS5 may bind EGFR indirectly through SH2 domain-mediated interactions with a tyrosine-phosphorylated adaptor protein that binds EGFR.

EGF-induced degradation of EGFR involves ligand-induced ubiquitylation of the receptor by c-Cbl. EGFR degradation upon overexpression of SOCS5 is both ligand-independent and does not require c-Cbl (Fig. 5). Furthermore, EGF-induced endocytosis involves the internalization of EGFR from the cell surface into early endosomes and subsequently to a late endosomal compartment (9, 10). The results of this study show that, in SOCS5-expressing cells, EGFR is localized in intracellular vesicles (Figs. 7, *A–C*). The nature of the intracellular vesicles in which the receptor is found in SOCS5-expressing cells remains to be characterized and may represent early or late endosomal vesicles similar to the trafficking pattern during ligand-induced endocytosis or an as yet uncharacterized cellular compartment. The co-localization of SOCS5 with EGFR in this vesicular compartment could not be determined due to difficulties in visualizing SOCS5 by immunofluorescence, probably because of its short half-life and low steady-state levels. Finally, we show that EGF-mediated signaling was also altered by SOCS5 expression because activation of STAT3 was markedly reduced in SOCS5-expressing cells (Fig. 7, *D* and *E*). EGF-induced STAT3 activation was unaffected by the expression of both the SB and SH2 mutants of SOCS5, again indicating that these two domains are required for the regulation of EGFR. The decreases in EGFR-mediated signaling may be attributed to the reduction in levels of the receptor. In addition, the intracellular localization of EGFR in SOCS5-expressing cells may result in the receptor being unavailable for EGF binding.

The cellular compartment in which EGFR is degraded in SOCS5-expressing cells is yet to be determined. Previously characterized substrates for SOCS-mediated degradation are intracellular proteins such as JAK and Vav (17, 23). Degradation of intracellular proteins is mediated primarily by the 26 S proteasome, and indeed, treatment of cells with proteasome inhibitors blocked SOCS1-mediated degradation of JAK and Vav. In contrast to intracellular proteins, cell surface receptors such as EGFR are removed from the cell surface and processed through an endocytic pathway culminating in lysosomal degradation (44). Nonetheless, recent evidence suggests a role for proteasomal activity in the EGFR degradation process. Longva *et al.* (45) reported that treatment of cells with either proteasome or lysosome inhibitors inhibited ligand-induced EGFR degradation without affecting the rate of receptor internalization. In cells treated with proteasome inhibitors, EGFR was localized to the outer limiting membrane of the multi-vesicular body and its translocation to inner multi-vesicular body vesicles was prevented. In addition, Dong *et al.* (46) reported an interaction between Rab7, a key protein in the regulation of transport from early to late endosomes, and the proteasome subunit XAPC7. This interaction takes place in endosomes, and moreover, the overexpression of XAPC7 significantly impaired the endosomal transport of EGFR. Thus, there is evidence for a physical link between the endocytic and the intracellular proteasomal degradative pathways. The results of this study suggest that EGFR degradation by SOCS5 can be prevented by treatment with a proteasome inhibitor (Fig. 6*D*). However, as mentioned above, this does not necessarily suggest that SOCS5 directly utilizes the proteasome for EGFR degradation. Interestingly, the SCF E3 ubiquitin ligase complex that is analogous to the complex recruited by SOCS proteins has been shown to mediate both proteasomal and lysosomal degradation as demonstrated by Kumar *et al.* (47) who showed that the interferon-α receptor 1 undergoes SCF^{HOS} complex-mediated degradation in the lysosome.

In conclusion, we suggest the following model describing the role of SOCS5 in the regulation of EGFR. Following stimulation of cells by growth factors or cytokines, SOCS5 expression is elevated. SOCS5 can then undergo SH2 domain-dependent binding to the EGFR, either directly via a tyrosine residue phosphorylated by a cytosolic tyrosine kinase or indirectly via a phosphorylated adaptor protein. SOCS5 can then recruit the ElonginBC complex to the receptor to accelerate EGFR degradation. EGFR degradation is accompanied by its removal from the cell surface, thus preventing ligand binding and EGF-induced signaling. The identity of the proteins involved in the degradation process has yet to be characterized and may include adaptor proteins linking SOCS5 and the EGFR as well as proteins associated with SOCS5 and the ElonginBC complex. Furthermore, questions such as the stimulatory context and the precise molecular mechanism of SOCS5-mediated EGFR degradation remain to be resolved.

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