Suppressors of T-cell Receptor Signaling Sts-1 and Sts-2 Bind to Cbl and Inhibit Endocytosis of Receptor Tyrosine Kinases*

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Suppressors of T-cell Receptor Signaling Sts-1 and Sts-2 Bind to Cbl and Inhibit Endocytosis of Receptor Tyrosine Kinases*

The ubiquitin (Ub) ligase Cbl plays a critical role in attenuation of receptor tyrosine kinase (RTK) signaling by inducing ubiquitination of RTKs and promoting their sorting for endosomal degradation. Herein, we describe the identification of two novel Cbl-interacting proteins, p70 and Clip4 (recently assigned the names Sts-1 and Sts-2, respectively), that inhibit endocytosis of epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor. Sts-1 and Sts-2 contain SH3 domains that interacted with Cbl,Ub-associated domains, which bound directly to mono-Ub or to the EGFR/Ub chimera as well as phosphoglycerate mutase domains that mediated oligomerization of Sts-1/2. Ligand-induced recruitment of Sts-1/2 into activated EGFR complexes led to inhibition of receptor internalization, reduction in the number of EGFR-containing endocytic vesicles, and subsequent block of receptor degradation followed by prolonged activation of mitogenic signaling pathways. On the other hand, interference with Sts-1/Sts-2 functions diminished ligand-induced receptor degradation, cell proliferation, and oncogenic transformation in cultured fibroblasts. We suggest that Sts-1 and Sts-2 represent a novel class of Ub-binding proteins that regulate RTK endocytosis and control growth factor-induced cellular functions.

Controlled degradation of activated RTKs1 represents one of the main mechanisms by which cells negatively regulate receptor signaling and maintain normal cellular homeostasis (1). Growth factor binding to RTKs leads to their internalization and delivery to endosomes, where receptors undergo sorting for either recycling back to the plasma membrane or trafficking to the lysosome for destruction (1, 2). Recruitment of receptors into specialized membrane domains and trafficking of receptors and their ligands within endocytic compartments are regulated by reversible protein modifications and multiprotein interactions (2–5). Among these changes, ubiquitination seems to play a crucial role in directional targeting of activated receptors for degradation in lysosomes (1, 2). It was proposed that the Ub-ligase Cbl, which associates with receptors after ligand-induced activation, mediates monoubiquitination of activated EGF and PDGF receptors on multiple sites (6, 7). These Ub-moieties are thought to be recognized by proteins possessing Ub-binding modules that contribute to sorting of Ub-coupled cargo in the endosome (8). For example, the yeast homologues of epsin and Eps15 have proven to be essential for Ste2p receptor internalization, and clathrin-associated epsin-Eps-15 complexes might bind ubiquitinated receptors via their ubiquitin-interacting motifs (UIM), thus driving receptor internalization (9, 10). Moreover, epsin and Eps15 are found on endosomal membranes complexed with other ubiquitin receptors, STAM2 and Hrs, which enables these proteins to act in concert to sort ubiquitinated proteins into the multivesicular body of the late endosome (11). Inclusion of the ubiquitin-modified EGF receptors into the multivesicular body and their subsequent sorting for lysosomal degradation is additionally dependent on the Ub-binding ubiquitin conjugating enzyme E2 variant domain-containing protein Tsg101 (12).

How these different Cbl functions are negatively controlled under physiological conditions remains unknown. It has been suggested that Cbl-binding proteins, such as Sprouty 2, might temporally inhibit the capacity of Cbl to interact with and ubiquitinate EGF receptors (13, 14). Here we describe a new mechanism by which Cbl-mediated endocytosis and degradation of RTKs are modulated. We have identified the novel Cbl-interacting proteins Sts-1 and Sts-2, which are recruited to EGFR receptors upon ligand binding. Both proteins potentially regulate interactions between trafficking receptors and the Ub-sorting machinery in the endosome, thus impairing receptor internalization and subsequent sorting for lysosomal degradation. As a result, Sts-1 and Sts-2 stabilize activated receptors and modulate growth factor-induced biological responses. Therefore, we propose that Sts-1/Sts-2 act as negative regulators of Cbl functions and modulators of biological responses elicited by RTKs.

EXPERIMENTAL PROCEDURES

Materials, Plasmids, Cells, and Antibodies—Two different antibodies recognizing Sts-2 were generated: α-phosphoglycerate mutase (PGM) antibodies raised against GST-fused protein encompassing amino acids 908–1867 of human Sts-2 and α-992 against the peptide RGFPLENYT-DRAESD beginning at amino acid 859. Sts-1-specific antibodies were kindly provided by J. Ihle and N. Carpino. Antibodies recognizing phosphotyrosine (PY99), autophosphorylated EGF receptor (C14, anti-pEGFR), extracellular-regulated kinase (ERK2/C14), pERK (R-4), PDGF-β (P-20), and anti-epsin (R-20) antibodies were from Santa Cruz, mouse anti-HA (12CA5) from Roche, mouse anti-FLAG M5 anti-

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§ The abbreviations used are: RTK, receptor tyrosine kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; UIM, ubiquitin-interacting motif; PGM, phosphoglycerate mutase; GST, glutathione S-transferase; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; Ub, ubiquitin; HEK, human embryonic kidney; CHO, Chinese hamster ovary; Clip, Cbl-interacting protein; UBA, ubiquitin-associated; Sts, suppressor of T-cell receptor signaling; SH3, Src homology 3; EGFR, epidermal growth factor receptor; PDGF, platelet-derived growth factor receptor.

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bodies were from Sigma. Anti-Cbl (RF) and anti-EGFR receptor (RK2 and 10b) antibodies were used as described previously (5, 15). Wheat germ lectin-phosphothreonine was from Amersham Biosciences.

EGF was purchased from Intergen, rhodamine-labeled EGF was purchased from Molecular Probes, and PDGF-BB from Amgen (Thousand Oaks, CA). Constructs for expressing EGF, PDGFR, Cbl, CIN85, and Flag- or HA-tagged Ub have been described previously (5). The expression vectors for FLAG-Eps15, FLAG-epsin and GST-Ub were provided by S. Polo and P. D. Fiore. The construct for FLAG-Hrs was provided by H. Stemmer. Yeast two-hybrid screening was performed as described previously (5) using the Gal4-based Matchmaker two-hybrid system (BD Biosciences Clontech). PC-12, HEK293T, COS-1, Jurkat, and HeLa cells were purchased from American Type Culture Collection. NIH3T3 cells were used as described previously (5). Stable expression of Sts-2 constructs in NIH3T3 cells and of EGFR in CHO cells was obtained by the presence of 1.2 mg/ml or 2.4 mg/ml of G418 in the culture medium, respectively. Where indicated, cells were transfected with Lipofectamine reagent (Invitrogen) following the manufacturer’s instructions. Thirty hours after transfection, the cells were starved for an additional 12 h and stimulated with 50 ng/ml EGF or PDGF-BB for indicated times. Cell lysates were subjected to immunoprecipitation, GST-pull-down assays, and immunoblotting as described previously (5, 15).

Full-size Sts-2 cDNA was cloned from the Jurkat cell line using sequence-specific primers (Table 1). All Sts-2 and Sts-1 constructs were generated by PCR. Constructs of Sts-2 in mammalian expression vector were generated in pCDNA3-FLAG (Invitrogen), and for GST-fusion proteins, expression pGEX-4T1 (Amersham Biosciences) was used as a basic vector. The primers shown in Table 1, all tagged with appropriate restriction sites, were used for subcloning.

Biochemical Assays—For EGFR degradation assays, HEK293T cells were transfected with EGF, Cbl, Flag-Ub, and, where indicated, with Sts-2 and its different mutants, were serum-starved and stimulated with EGF (50 ng/ml) for the indicated times. The cell lysates were subjected to immunoblotting with anti-EGFR (RK2) antibodies to monitor receptor levels and anti-ERK2 antibodies to ensure equal loading in each well. To examine ERK activity, total cell lysates were immunoprecipitated with phosphospecific anti-ERK antibodies. Densitometric analysis of specific protein bands from the immunoblots was performed using the AIDA software, version 3.10.039 (Fujifilm). Likewise, for PDGF receptor degradation assays, HEK293T cells were transfected with PGF-β, Cbl, Flag-Ub and, where indicated, with Sts-2, serum-starved, and stimulated with PDGF-BB (50 ng/ml) for 30 min. The cell lysates were subjected to immunoblotting with anti-PDGFR, anti-ERK2, or anti-β-Actin antibodies.

For Ub-binding assays, cell lysates were incubated with protein A-agarose (Roche) or Ub-agarose (Boston Biochem), GST was conjugated to Sts-2 and its different mutants, were serum-starved and stimulated with EGF (50 ng/ml) at 37 °C for the indicated times. Cells underwent an acid wash with phosphate-buffered saline + 0.1% BSA, pH 3.4, to remove surface EGF, and were incubated for 1 h with 125I-EGF at 4 °C. Surplus 125I-EGF was removed and cells were washed two times with cold internalization medium. Surface-bound 125I-EGF was determined by lysis of cells in 1 M NaOH and analysis in a γ-counter (1470 Wizard; PerkinElmer). Unspecific binding of 125I-EGF was measured by incubation of non-transfected CHO cells with 5 ng/ml 125I-EGF. Average c.p.m. measured from six samples were deducted from the individual c.p.m. of each test sample. Values for EGF-stimulated cells were compared with nonstimulated cells to calculate the percentage of surface remaining receptor. Each time point was measured in triplicate, and each experiment was repeated two times.

Thymidine Incorporation—NIH3T3 cells stably expressing Sts-2 and its indicated mutants or GFP as a control were seeded at a concentration of 5 × 10⁴ cells/well. For 24 h, 4-h pulse of [3H]thymidine was added to the culture medium to a final concentration of 1 µCi/ml. Thereafter, cells were washed with phosphate-buffered saline, fixed in 5% trichloroacetic acid (TCA), rinsed with water, and dehydrated in 70% ethanol. DNA was extracted in 0.1 N NaOH. After addition of scintillation mixture, radioactivity was measured in a β-counter. The data are plotted as average values with standard errors of triplicate repeats for each condition.

Immunofluorescence—COS-1 and CHO-EGFR cells seeded on collagen-coated cover slips were transfected with indicated constructs for 24 h and stained for an additional 12 h. Cells were left untreated or were stimulated with rhodamine-labeled EGF (40 ng/ml) for 30 min at 37 °C and then fixed in 4% paraformaldehyde. After permeabilization and blocking, cells were incubated with primary antibodies and then with Alexa Fluor-conjugated secondary antibodies (DakoCytomation). The preparations were mounted using Fluoromount G (Immunkemi), and the images were taken with a Zeiss AxioPlan 2 microscope.

RESULTS

Cbl Couples Sts-1/Sts-2 Adaptor Molecules to the Activated Receptor Complexes—In our searches for cellular effectors of Cbl, we used the yeast two-hybrid system and identified several Cbl-interacting proteins (Clips), namely Clip1/SH3P2 (16), Clip2/CIN85 (5), Clip3/ArgBP2 (17), Clip4/UBASH3A/STs-1 (18), and Clip5/Nck (19). All these clones encoded one or more SH3 domains that interacted strongly with the proline-rich sequences of Cbl in yeast cells (data not shown). Cloning of the full-size Clip4 cDNA revealed that it is encoded by the UBASH3A gene (18) but represents a spliced variant with an internal deletion of 39 amino

<table>
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**FIG. 1**. Sts-1/Sts-2 are recruited to activated EGF receptors via SH3 domain-mediated binding to Cbl. A, schematic representation of Sts family members indicating defined domains: UBA domain, SH3 domain, PGM domain. B, FLAG-tagged Sts-2 constructs were co-expressed with HA-tagged Cbl in HEK293T cells and lysates were immunoprecipitated (IP) with either anti-HA or anti-FLAG antibodies and immunoblotted (WB) with anti-Cbl or anti-Sts-2 (α-992) antibodies. The numbers indicate the approximate molecular mass in kilodaltons. Mr, relative molecular mass. C, COS-1 cells transfected with indicated Sts-2 constructs were left unstimulated (−) or stimulated with EGF, then immunoprecipitated with anti-Sts-2 (α-PGM) antibodies and blotted with indicated antibodies. D, FLAG-tagged Sts-2 was co-expressed with HA-tagged Cbl-655 or Cbl-480 in HEK293T cells, and lysates were immunoprecipitated (IP) with anti-Sts-2 antibodies and immunoblotted with a mixture of anti-HA/FLAG antibodies. TCL, total cell lysate. E, HA-tagged Cbl, Cbl-655, and Cbl-480 were expressed in HEK293T cells and incubated with GST-fusion proteins containing the SH3 domain of Sts-1. Detection was performed by blotting with anti-HA antibody. F, left, HeLa cells were starved (−) or stimulated with EGF for 10 min, and lysates were subjected to immunoprecipitation with antibodies against Sts-1/Sts-2 (α-PGM), antibodies against EGFR (108), or pre-immune serum, followed by immunoblotting with the indicated antibodies. Right, CHO cells stably expressing EGF receptors were left unstimulated (−) or stimulated with EGF for 10 min. Lysates were subjected to immunoprecipitation with anti-EGFR (108) or anti-Cbl antibodies and blotted with indicated antibodies. G, multiple sequence alignment of Clip4/Sts-2/UBASH3A and p70/Sts-1 family members was performed with the use of ClustalW program (www.ebi.ac.uk/clustalw/). The conserved structural domains are highlighted. The GenBank accession numbers of the family members are as follows: Clip4/Sts-2, identical to AAP80738; UBASH3A human, AAP80738; UBASH3A mouse, mCP91636 (Celera); UBASH3A rat, Ensembl prediction ENSRNOG00000008187; p70/Sts-1 human, NM_032873; Sts-1 mouse, NP_116262; Sts-1 rat, Ensembl prediction ENSRNOG00000008187; D. rerio, Ensembl prediction Ensembl prediction ENSDARG00000008256; D. melanogaster, CG13604 (flybase symbol); C. elegans, T07F12.1 (wormbase transcript).
acid residues between the UBA and SH3 domains (Fig. 1, A and G). Clip4 shares sequence homology with another ubiquitously expressed mammalian protein known as p70 (20) and has orthologues in Drosophila melanogaster and Danio rerio, which all together form a novel family of UBA and SH3 domain-containing proteins (Fig. 1, A and G). Those adaptor proteins have recently been designated as the Suppressor of T-cell receptor signaling (Sts) family (21); therefore, we will refer to p70 as Sts-1 and Clip4 as Sts-2 hereafter. Sts-1 is ubiquitously expressed, whereas Sts-2 is preferentially expressed in hematopoietic cells and tissues (20), although RT-PCR analysis indicates that Sts-2 mRNA is also present at the lower level in placenta (18) and some epithelial cell lines (data not shown). We initially confirmed that the SH3 domains of Sts-1 and Sts-2 bind to the proline-rich region of Cbl in mammalian cells by co-immunoprecipitation from lysates of HEK293T cells expressing Cbl and Sts-2 or their respective mutants (Fig. 1, B–D) and by GST pull-down assays with the SH3 domain of Sts-1 (Fig. 1E). Moreover, with the use of antibodies cross-reacting with Sts-1 and Sts-2, we demonstrated that Cbl readily associates with endogenous Sts-1/Sts-2 in HeLa cells, whereas specific anti-Sts-1 antibodies enabled Cbl/Sts-1 complex detection in CHO-EGFR cells (Fig. 1F). Interactions between Cbl and Sts-1 or Sts-2 were independent of EGF stimulation or EGFR-induced phosphorylation of Cbl (Fig. 1, C and F), whereas the association of Cbl with tyrosine phosphorylated EGFRs was increased upon ligand stimulation (Fig. 1F). Therefore, Sts-1 and Sts-2 were recruited to complexes with activated EGFRs after EGF stimulation (Fig. 1, C and F). In contrast, a mutant form of Sts-2 that contains a non-functional SH3 domain (Sts-2-SH3*, with mutation of tryptophan 279 to alanine) was unable to bind to Cbl and to associate with EGFRs (Fig. 1C), further confirming that Sts-2-Cbl interactions are critical for binding of Sts-2 to activated EGFRs.

In their C-terminal parts, Sts-1 and Sts-2 also contain an evolutionarily conserved domain present in their orthologues in Caenorhabditis elegans and D. melanogaster, thus being named HCD (Human, C. elegans, D. melanogaster) domain (18). This domain is highly homologous to PGM proteins, which are involved in transfer of phosphate groups between the carbon atoms in glycerates (22). However, even though the PGM domains of Sts-1 and Sts-2 share conserved residues with other PGM domains, others and we have not been able to confirm their ability to isomerize phosphoglycerate substrates (Ref. 20 and data not shown). On the other hand, it has been reported previously that phosphoglycerate mutases form dimers or tetramers in mammalian cells (22). Along the lines of these studies, we have observed that Sts-2 or its isolated PGM domain run as doublets in SDS-PAGE when immunoprecipitated from HEK293T cells overexpressing high amounts of proteins (Fig. 2). Therefore, chemical cross-linking performed on the lysates of transfected COS-1 cells revealed that wild type Sts-2 is exclusively present as a dimeric or oligomeric protein (Fig. 2). It is noteworthy that deletion of the PGM domain resulted in a monomeric protein of the molecular mass around 30 kDa, whereas the construct encoding only the isolated PGM domain was efficiently dimerized (Fig. 2), further indicating that the PGM domain is essential for oligomerization of Sts-2 in cells. Together, these results support the notion that Sts-1 and Sts-2 are two structurally related proteins (Fig. 1, A and G) that bind to and regulate Cbl functions in mammalian cells.
UBA Domain-containing Proteins in Receptor Endocytosis

UBA Domains of Sts-1/Sts-2 Bind to Mono-Ub Attached to the EGFR—To further elucidate the role of Sts-1/Sts-2, we analyzed the functional significance of their UBA domains. UBA domains have been demonstrated to mediate Ub binding of several proteins (23, 24). To test whether Sts-1 and Sts-2 are bona fide Ub-receptors, lysates of cells expressing Sts-1 or Sts-2 were incubated with mono-Ub-coupled affinity matrices. Sts-1 and Sts-2 readily bound to mono-Ub linked to agarose (Ub-agarose) and to GST-Ub, but not to control beads (Fig. 3A). Because Cbl also possesses an UBA domain in its C-terminal part, we tested its ubiquitin binding capacity as well. However, no binding of Cbl to GST-Ub was detected (Fig. 3A, right), implying that not all UBA domains can act as Ub-receptors. It is noteworthy that deletion of the UBA domain of Sts-2 (Sts-2-UBA) completely blocked its binding to either GST-Ub or Ub-agarose (Fig. 3B). Furthermore, under identical experimental conditions and using all proteins tagged with the same FLAG epitope, we noted that full-size Sts-2 seems to bind to mono-Ub-coupled matrices more potently than Eps15, epsin, or Hrs (Fig. 3C). These proteins contain UIMs, involved in binding and endocytic sorting of ubiquitinated EGF receptors (9, 25).

We have also observed that Sts-2 is found in complexes with ubiquitinated EGFRs upon EGF stimulation (Fig. 3D), suggesting that the UBA domain of Sts-2 might contribute to recruitment of Sts-1/2 in complexes with activated EGFRs. To test this hypothesis, we analyzed the association of Sts-2 and Eps15 with an EGFR3-UB chimera, consisting of a single Ub fused to the extracellular and transmembrane domain of the EGFR (6). Sts-2 and to a lesser extent Eps15 were found in complexes with the EGFR3-UB chimera but not with the control EGFR3-FLAG protein (Fig. 3E, left). On the other hand, the isolated UBA domain itself did not bind to the EGFR3-UB chimera under identical experimental conditions (data not shown), suggesting that the isolated UBA domain is not sufficient to mediate binding to Ub attached to the EGFR in cells. It is thus possible that dimerization of Sts-2 via its PGM domain (Fig. 2) and recruitment of Sts-2 to Cbl/EGFR complexes (Fig. 1), can increase the avidity of the Sts-2 UBA domain binding to mono-Ub and thus promote interactions between Sts-2 and ubiquitinated receptors in cells.

To further test this possibility, we analyzed the ability of Sts-2 or its mutants to interfere with binding of the endocytic sorting protein epsin with ubiquitinated EGFR complexes in vivo. Expression of Sts-2, but not of the Sts-2 UBA domain, significantly reduced the ligand-dependent association of epsin with ubiquitinated EGFRs (Fig. 3E, middle). In addition, Sts-2-ΔUBA or Sts-2-SH3*, unable to bind to Ub or Cbl, respectively (Figs. 3B and 1C), were also impaired in their ability to block binding of epsin to activated EGFR complexes (Fig. 3E, right). Even though we cannot provide a final proof that the Sts-2 UBA domain indeed associates with ubiquitinated receptors in cells, it is reasonable to assume that Sts-2, once recruited to EGFR complexes via its SH3 domain (Fig. 1), requires the UBA domain to compete against interactions between the endocytic sorting machinery and ubiquitinated EGFR complexes.

Sts-1 and Sts-2 Inhibit Receptor Endocytosis and Degradation—Ubiquitination of RTKs has been recognized as an important signal for receptor down-regulation and degradation (26, 2). To reveal whether Sts-2 binding to EGFRs or other ubiquitinated components of endocytic machinery affects receptor trafficking, we measured the rate of receptor internalization and endocytosis in the presence of Sts-1, Sts-2, or its mutants. Expression of Sts-2 or Sts-1 potently blocked EGFR removal from the cell surface (Fig. 4A), whereas expression of Sts-2-UBA, and to a lesser extent Sts-2-SH3*, had no such inhibitory effect and even slightly accelerated the rate of receptor internalization (Fig. 4A). Consistent with the previous results, Sts-2 efficiently blocked formation of EGFR-containing vesicles in COS-1 cells (Fig. 4B), whereas EGFR endocytosis was unaffected in cells transfected with Sts-2-ΔUBA or Sts-2-SH3* (Fig. 4B). Furthermore, expression of Sts-2 or Sts-1 in HEK293T cells strongly reduced ligand-dependent degradation of EGFRs (Fig. 4C). We also noted that Sts-2 did not affect binding of Cbl to receptors or Cbl-mediated ubiquitination of EGFRs (Fig. 3D). Moreover, the capacity of Sts-2 to inhibit EGFR degradation was abolished when either its SH3 or UBA domain was rendered non-functional (Fig. 4D). It was interesting that deletion of the PGM domain resulted in a slight decrease in the ability of Sts-2 to stabilize receptors (Fig. 4D), thus supporting the view that PGM-mediated oligomerization of Sts-1/Sts-2 might be functionally important. The effects of Sts-2 and its mutants on the EGFR levels were also reflected in the ligand-induced phosphorylation of receptors (Fig. 4E) and activation of the ERK pathway. Expression of Sts-2 enhanced EGF-induced activation of ERK, whereas Sts-2-UBA and Sts-2-SH3* decreased ERK activation to the level of control transfected cells (Fig. 4E, right). These data are also compatible with a model in which binding of Sts-1/Sts-2 to Cbl and to ubiquitinated EGFRs inhibits ligand-induced EGFR endocytosis and degradation, leading to accumulation of activated receptors.

Last, we wanted to confirm the functional importance of endogenous Sts-1 and Sts-2 proteins in modulating the kinetics of RTK endocytosis in a more physiological context. We therefore decided to down-modulate the expression of Sts-1/Sts-2 levels in HeLa cells by the use of specific small interfering RNAs. Although we managed to efficiently target Sts-2 with two distinct small interfering RNA sequences, our repeated efforts have failed to identify a small interfering RNA that could block expression of Sts-1 in either transfected 293T cells or HeLa cells. As a result, we noticed no change in the rate of ligand-mediated EGF receptor degradation in Sts-2-small interfering RNA-targeted HeLa cells because of the residual expression of Sts-1 (not shown).

Sts-1/Sts-2 Regulate PDGFR Endocytosis and PDGF-induced Cellular Responses—Finally, we tested whether Sts-1/Sts-2 effect was specific for the EGFR or if it was implicated in regulation of other RTKs as well. Therefore, we further analyzed the effect of Sts-1/Sts-2 on endocytosis and biological functions mediated by PDGF receptors, which are known to be...
down-regulated in a Cbl- and Ub-dependent manner (15, 27).

We initially confirmed that the overexpression of Sts-2 blocked the degradation of PDGF receptors in HEK293T cells, leading to their increased phosphorylation and enhanced signaling via the ERK pathway (Fig. 5A). To study the effect of Sts-2 on cellular responses of endogenous PDGF receptors, we established clones of NIH3T3 fibroblasts stably expressing Sts-2, Clip-SH3*, and Sts-2-ΔUBA and monitored their proliferation rate or the ability to form colonies in soft agar. Overexpression of Sts-2 led to a slight increase in PDGF-induced cell prolifer-
ation measured by \(^{3}H\)thymidine incorporation into DNA, compared with clones transfected with control vector (Fig. 5B). On the contrary, the presence of Sts-2-SH3\(^{+}\) or Sts-2-\(\Delta\)UBA, reduced the proliferation index in these cells (Fig. 5B). This might indicate that Sts-2-\(\Delta\)UBA and Sts-2-SH3\(^{+}\) act as dominant interfering mutants on the stability of PDGFRs and receptor-triggered signaling pathways by inhibiting functions of endogenous Sts-1/Sts-2/Cbl/PDGFR complexes. The dominant interfering effect of Sts-2-\(\Delta\)UBA on PDGF-induced response correlated with decreased levels of PDGFRs in these cells (Fig. 5B, right). We next compared the ability of Sts-2 and Sts-2-\(\Delta\)UBA to affect PDGF-induced colony formation in a soft agar assay. Non-stimulated cells did not form colonies in agar after 2 weeks (Fig. 5C). On the other hand, PDGF promoted formation of numerous colonies (Fig. 5C), which were not increased in the presence of Sts-2 but were significantly reduced in cells expressing Sts-2-\(\Delta\)UBA (Fig. 5C). These findings indicate the role of Sts-2 in ligand-dependent regulation of both physiolog-

**Fig. 4.** Sts-2 stabilizes activated EGF receptors in a UBA- and SH3-domain dependent manner. A, CHO cells were transfected with EGFR and Cbl together with Sts-2, Sts-2 mutants, Sts-1, or GFP as a control as indicated. Receptor internalization was induced with 50 ng/ml EGF for the indicated times. Thereafter, cell-surface remaining EGFR was determined by incubation with \(^{125}\)I-EGF as described under “Experimental Procedures.” Results were plotted as percentage of cell surface receptor in non-stimulated cells. The levels of different proteins were monitored in TCLs using anti-FLAG antibodies (right). B, COS-1 cells overexpressing indicated FLAG-tagged Sts-2 constructs were stimulated with rhodamine-labeled EGF and subjected to immunofluorescence analysis as described under “Experimental Procedures.” C, HEK293T cells were transfected with EGFR, FLAG-Ub, and, where indicated, with Sts-1 or Sts-2 (right). Cells were stimulated with EGF for indicated periods, and the levels of different proteins were monitored in TCLs using indicated antibodies. D, the experimental procedure was the same as in C, except that either the wild-type or mutant Sts-2 constructs were used for receptor degradation assays. The graph shows quantification of receptor degradation from three independent experiments and the expression levels of the different forms of Sts-2 are displayed in the blot at bottom. E, left, HEK293T cells were transfected with EGFR, FLAG-Ub, Cbl, and, where indicated, Sts-2. Cells were stimulated with EGF for the indicated periods and the levels of different proteins were monitored in TCLs using indicated antibodies. Right, HEK293T cells were transfected with EGFR, ubiquitin, Cbl, and either the control vector or indicated Sts-2 constructs. Cells were left unstimulated or stimulated with EGF for 30 min, and the effects of Sts-2 constructs on MAP kinase pathway activation were monitored with the use of anti-pERK antibodies. The graph shows quantification of densitometric analysis of the immunoblots from three independent experiments. IP, immunoprecipitation; WB, Western blotting.
ical and pathological cellular responses mediated by different growth factor receptors.

**DISCUSSION**

In this report, we have identified the Sts-1/Sts-2 family of adaptor proteins as novel Cbl- and Ub-binding proteins that negatively regulate RTKs endocytosis and degradation. We also provide detailed analysis of the molecular basis for the Sts-1/Sts-2 functions. To stabilize receptors, the Sts proteins require the presence of both an intact SH3, as well as an UBA domain (Figs. 4 and 5). The SH3 domains are common protein-protein interaction motifs, whereas UBA domains have been found in various cellular proteins connected to the Ub-proteasome, deubiquitination, or nucleotide repair system (28), as well as in the yeast homologue of the endocytic sorting protein Eps15-Ede1 (29). The SH3 domains recruit Sts-1/Sts-2 to Cbl and thus indirectly to the activated receptors (Fig. 1, C and F). Therefore, our data suggest that Sts proteins need to be in complexes with receptors to modulate their functions. In turn, the requirement of a UBA domain for receptor stabilization by Sts proteins implies that Ub binding is a prerequisite for the described functions. It is remarkable that Sts-1 and Sts-2 potently bind to mono-Ub, potentially even more strongly than any of the UIM-containing proteins, including epsin, Eps15, or Hrs (Fig. 3C) and may compete for recruitment of epsin in complexes with activated EGFRs (Fig. 3E). Finally, the evolutionarily conserved PGM domain is responsible for Sts dimerization in cells (Fig. 2) and is necessary for the full capacity of Sts-2 to block EGFR degradation (Fig. 4D). It is thus tempting to speculate that the recruitment of Sts-1/Sts-2 to Cbl/EGFR complexes via their SH3 domains (Fig. 1) and the dimerization of Sts-1/Sts-2 via the PGM domains (Fig. 2) could enable the UBA domains to bind with higher avidity to multiply monoubiquitinated EGFRs (Figs. 1 and 2).

We provide the first example of a the involvement of a UBA domain-containing protein in control of receptor endocytosis in mammalian cells. This expands the repertoire of Ub-binding proteins that regulate vesicular transport, as the previous studies have pointed to a critical role of the UIM, the Cue homologous (CUE), and ubiquitin conjugating enzyme E2 variant domains in sorting of ubiquitinated cargo in the endosome (9, 8, 25). Recent reports demonstrated that several UIM and CUE domain-containing proteins are endowed with a dual function of binding ubiquitin and being monoubiquitinated (30, 31). In line with those studies, we observed that both Sts-1 and Sts-2, but not their mutants devoid of the UBA domains, were indeed monoubiquitinated in mammalian cells. Studies in progress are aimed at defining functional significance of monoubiquitination of Sts-1/Sts-2 in vivo.

Whereas the exact mechanism by which Sts-1/Sts-2 inhibit receptor endocytosis and thus lead to increased receptor signaling needs to be further established, we propose several models based on our data. We first disproved the possibility

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that Sts-1/Sts-2 sequester Cbl from the receptors, similarly to
the function of Sprouty 2 (14), thereby delaying their degrada-
tion (Fig. 3D). Instead, the suggested Cbl-mediated association
of Sts UBA domains with multiple monoubiquitins on traffick-
ing receptors could preclude/change dynamics of binding of the
UIM-containing endocytic sorting proteins to multiply mono-
ubiquitinated EGFRs and other ubiquitinated components of the
trafficking machinery, slowing down receptor internalization
and degradation (Fig. 3E). In agreement with this scenario,
cells overexpressing Sts-2 are significantly impaired in the
receptor entry into vesicular endosomal compartment, as well
as in its down-regulation measured by radioactively labeled
ligands (Fig. 4, A and B). An alternative possibility is that
binding of the Sts-2 UBA domain to a first Ub attached to the
EGFR might prevent formation of Ub-chains on receptors and
their aberrant degradation by the proteasome system. In either

**FIG. 5.** Sts-2 overexpression modulates biological responses mediated by endogenous PDGFR. A, HEK293T cells were transfected
with PDGFR-β, FLAG-Ub, and Cbl in the presence or absence of Sts-2. Cells were left unstimulated or stimulated with PDGF-BB (50 ng/ml) for
30 min and the levels of different proteins were monitored on TCLs using indicated antibodies. B, NIH3T3 cells stably expressing Sts-2 or Sts-2
dominant-interfering mutants were monitored for proliferation rate in response to PDGF stimulation as described under “Experimental Proce-
dures.” Data represent the average of three experiments. Right, cell lysates from NIH3T3 cells stably expressing indicated Sts-2 constructs were
subjected to pull-down assay with wheat germ lectin, followed by immunoblotting with anti-PDGFR-β antibodies or to immunoprecipitation
with anti-FLAG antibodies to monitor expression of Sts-2 constructs. The equal loading of samples was monitored on TCLs with the use of anti-ERK2
antibodies. C, NIH3T3 cells stably expressing FLAG-Sts-2 and its indicated mutants or an empty vector were tested for their ability to form
colonies in soft agar as described under “Experimental Procedures.” The data are presented as average number of clones formed with standard
errors for each condition. IP, immunoprecipitation; WB, Western blotting.
UBA Domain-containing Proteins in Receptor Endocytosis

32795

scenario, interactions of the Sts-2 UBA domain with mono-ubiquitinated EGFRs, as well as with other ubiquitinated components engaged in vesicular trafficking, are likely to be transient and interchangeable with the UIM-containing endocytic sorting proteins. Such a relationship could provide a mechanism to fine-tune the kinetics of RTK endocytosis and determine the extent of receptor sorting for degradation.

The molecular details by which Sts-1 and Sts-2 control biological processes in vivo are likely to be functionally redundant and include other pathways. Sts-1 has been initially cloned as a binding partner of a Jak2-derived phosphopeptide; however, the consequence of this interaction remains unknown (20). Recent analyses of mice deficient for either Sts-1 or Sts-2 have revealed no obvious abnormalities, whereas the Sts-1/2 double knock-out mice displayed increased T-cell receptor signaling, enhanced phosphorylation of multiubiquitinated ZAP-70, and were more prone to autoimmune disease (21). Double knock-out Sts-1/2 T cells displayed strikingly increased tyrosine phosphorylation of ZAP-70 and several downstream effectors (21), pointing out distinct functions of Sts-1/2, such as recruitment and/or activation of protein phosphatases. On the other hand, in several in vitro assays, we were unable to detect intrinsic or associated phosphatase activity of Sts proteins (data not shown). The gene-targeting approach also revealed strong functional redundancy of Sts-1 and Sts-2, as single knock-out of either displayed no detectable phenotype (21). Furthermore, a recent study has implicated Sts-2/TUL1 in stabilization of the EGFR as well as in regulation of gene transcription downstream of activated ZAP-70/T cell receptor complexes via Cbl-dependent pathways in T cells (32). It is noteworthy that this study also demonstrated complex formation between endogenous Sts-2 and Cbl in hematopoietic cell lines, such as Jurkat or Ramos cells (32). This highlights a potential importance of Cbl-Sts complexes in regulation of T-cell receptor mediated functions. In addition, we note the presence of the evolutionary conserved PGM domain in the carboxyl terminus of Sts-1/2 that mediates their dimerization (Fig. 2). It is interesting that enhanced monoubiquitination of phosphoglycerate mutase B complexes was detected in colorectal cancer cells, pointing to a link between PGM complexes and the ubiquitin system (33). However, the physiological role of PGM domains of Sts-1/2 in cells still remains to be established. Further progress in understanding of Sts-1/2 functions at the molecular level will very likely provide us with a deeper appreciation of their biological roles in vivo.

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