TSG101 interaction with HRS mediates endosomal trafficking and receptor down-regulation

Quan Lu[†], Lila Weiqiao Hope[†‡§], Michael Brasch[¶‖], Christoph Reinhard[**], and Stanley N. Cohen[†§††‡‡]

Departments of [†]Genetics and [††]Medicine, [‡]Program in Cancer Biology, Stanford University School of Medicine, Stanford, CA 94305-5120; [¶]Invitrogen, Incorporated, Gaithersburg, MD 20878; and [**]Chiron Corporation, Emeryville, CA 94608

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Down-regulation of mitogenic signaling in mammalian cells relies in part on endosomal trafficking of activated receptors into lysosomes, where the receptors are degraded. These events are mediated by ubiquitination of the endosomal cargo and its consequent sorting into multivesicular bodies that form at the surfaces of late endosomes. Tumor susceptibility gene 101 (*tsg101*) recently was found to be centrally involved in this process. Here we report that TSG101 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), an early endosomal protein, and that disruption of this interaction impedes endosomal trafficking and endocytosis-mediated degradation of mitogenic receptors. TSG101/HRS interaction occurs between a ubiquitin-binding domain of TSG101 and two distinct proline-rich regions of HRS, and is modulated by a C-terminal TSG101 sequence that resembles a motif targeted in HRS. Mutational perturbation of TSG101/HRS interaction prevented delivery of epidermal growth factor receptor (EGFR) to late endosomes, resulted in the cellular accumulation of ubiquitinated EGFR in early endosomes, and inhibited ligand-induced down-regulation of EGFR. Our results reveal the TSG101 interaction with HRS as a crucial step in endocytic down-regulation of mitogenic signaling and suggest a role for this interaction in linking the functions of early and late endosomes.

epidermal growth factor receptor | endocytosis | multivesicular body | endosomes

Growth factors initiate mitogenic signaling in eukaryotic cells by activating cognate cell surface receptors, enabling eventual transduction of the signal to the cell nucleus. Although the growth-promoting effects of mitogens are required for a variety of cell functions (for reviews, see refs. 1 and 2), prolonged signaling can lead to developmental defects or tumorigenesis (for review, see ref. 3). Down-regulation of mitogenic signaling is accomplished at least in part by the trafficking of receptors to lysosomes for degradation, preventing their cycling back to the cell surface (for reviews, see refs. 4–6). This down-regulation is mediated by structurally and functionally distinct organelles termed early and late endosomes. Activated mitogenic receptors enter cells by internalization of clathrin-coated pits and commonly acquire a ubiquitin tag en route to early endosomes (7). The delivery of early endosomal cargo to late endosomes is associated with invagination of activated receptors into the lumen of structures known as multivesicular bodies (MVBs), where receptors designated for degradation are sorted. Fusion of MVBs with lysosomes deposits the receptors into the lysosome lumen, enabling their degradation by lysosomal proteases and the consequent attenuation of mitogenic signaling (6).

Tumor susceptibility gene 101 (*Tsg101*) recently was found to have a central role in the sorting of endocytosed proteins. *Tsg101*, which is essential for both embryonic development and normal cell growth (8, 9), initially was discovered by its ability to neoplastically transform mouse 3T3 fibroblasts reversibly when either deficient or overexpressed (10). Subsequently, the TSG101 protein was implicated in a variety of cellular functions, including transcriptional regulation (11–13), cell growth and cycling (14, 15), modulation of the MDM2/p53 feedback control loop (16), and the release of HIV-1 and Ebola viruses from cells (17, 18). The subcellular location of the TSG101 protein, whose concentration is maintained within a narrow range posttranslationally by a highly conserved C-terminal region (19), is cell cycle-dependent, and during interphase, TSG101 prominently colocalizes with perinuclear proteins of the Golgi apparatus (20). TSG101 is a homolog of ubiquitin conjugase (E2) enzymes, but because its N-terminal ubiquitin-conjugating E2 variant (UEV) domain lacks a cysteine residue required for thioester bond formation with ubiquitin, it is inactive as a ubiquitin conjugase (21, 22). Nevertheless, the TSG101 protein can bind to ubiquitin (23) and can affect ubiquitin-dependent proteolysis (16).

Mouse fibroblast SL6 cells (10), which have a decreased cellular level of TSG101, were found to be defective in delivering receptors and their ligands into late endosomal compartments (24), and subsequent studies have implicated TSG101 specifically in the sorting of ubiquitinated receptors into MVBs (23, 25). In human cells, depletion of TSG101 by short interfering RNA (siRNA) results in the accumulation of the epidermal growth factor receptor (EGFR) adjacent to vacuolated membranes presumed to be endosomal compartments and the inhibition of EGFR degradation (23). Mutations in Vps23, the yeast ortholog of TSG101, lead to missorting of plasma membrane proteins (26) and defective biosynthetic transport of integral membrane protein cargo into yeast vacuoles, the fungal counterparts of lysosomes (24, 25). Consistent with the ability of TSG101 to interact with ubiquitin, a yeast protein complex [ESCRT-1 (*e*ndosomal *s*orting *c*omplex *r*equired for *t*ransport)] containing Vps23 binds to ubiquitin, and this binding is required for the sorting of ubiquitinated cargo (25). It has been proposed that a mammalian cell analog of ESCRT-1 recognizes ubiquitinated receptors and guides invagination of the late endosomal membrane around them to form MVBs (6).

Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) is another key component of the mechanism that attenuates signaling from cell surface receptors (for review, see ref. 27). It associates primarily with the cytosolic surface of early endosomes, and knockout of the *Hrs* gene in mice leads to enlargement of these endosomes (24). In baby hamster kidney cells, HRS has been involved in sorting ubiquitinated proteins into clathrin-coated microdomains of early endosomes (28), and in *Drosophila*, ablation of *Hrs* expression results in failure to degrade activated receptor tyrosine kinases and leads to enhanced tyrosine kinase signaling (29). Like TSG101, HRS interacts with ubiquitin, and mutation of its ubiquitin-interaction motif destroys its endocytic sorting function(s) (28, 30, 31).

Here we report that TSG101 interacts with the early endosomal protein HRS. We define the domains that mediate this

Abbreviations: MVBs, multivesicular bodies; EGFR, epidermal growth factor receptor; UEV, ubiquitin-conjugating E2 variant; siRNA, short interfering RNA; IP, immunoprecipitation; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; ESCRT-1, endosomal sorting complex required for transport.

[§]Present address: Cooley Godward LLP, Palo Alto, CA 94304.

[‖]Present address: Atto Bioscience, Rockville, MD 20850.

[‡‡]To whom correspondence should be addressed. E-mail: sncohen@stanford.edu.

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interaction and show that mammalian cells expressing TSG101 and HRS proteins having compromised interaction ability exhibit defective trafficking of activated EGFR from early endosomes to late endosomes.

Materials and Methods

Yeast Two-Hybrid Screen and Assay. The ProQuest yeast two-hybrid system (Invitrogen) was used. pDBLeu-*Tsg101* and the a mouse cDNA library (in pDC86 vector) were introduced into yeast Mav203 cells. Positive clones were isolated on the basis of three selectable markers: HIS3, URA3, and LacZ. Deletion constructs of *Tsg101* and *Hrs* were made by PCR and subcloning into either pDBLeu or pPC86 (or pEXP-AD502). Interaction was indicated by activation of URA3 and by a liquid β-galactosidase assay (17).

Plasmid Constructs. Expression vector pLLEXP1 (16) was used. pLLEXP-HA-TSG contains N-terminally HA-tagged TSG101. pLLEXP-Tsg*-Flag constructs were made by subcloning an "siRNA-resistant" *Tsg101* fragment from pIRES-GFP-TSG*-F vector (a gift from W. I. Sundquist, University of Utah, Salt Lake City; ref. 17). pLLEXP-mHRS contains full-length mouse *Hrs* cDNA with a C-terminal Flag tag. Mutant derivatives of the constructs were made by site-directed mutagenesis (QuikChange kit; Stratagene). pEYFP-C1-TSG was made by subcloning mouse *Tsg101* into pEYFP-C1 (CLONTECH); by subcloning human *Hrs* cDNA pECFP-Hrs was cloned into pECFP-C1. pCDNA3-EGFR is a gift from H. Band (Harvard Medical School, Boston).

Mammalian Cell Culture and Transfection. NIH 3T3, HeLa and 293T cells were maintained in DMEM supplemented with antibiotics and 10% FBS (Invitrogen). Plasmid DNA transfections were performed by using either Lipofectamine (Invitrogen) in NIH 3T3 and 293T cells, or Effectene (Qiagen, Valencia, CA) in HeLa cells. Oligofectamine (Invitrogen) was used for transfections of RNA oligonucleotides.

Oligonucleotides. A 2′-*O*-methyl RNA/phosphorothioate DNA modified chimeric antisense oligo (TSG-AS) was synthesized against the *Tsg101* protein-coding sequence (nucleotides 410–434; 5′-aggacgagagaagactggaggttca; ribonucleotides are underlined). A control chimeric oligo that has the reversed sequence (TSG-RC; 5′-acttggaggtcagaagagagcagga) was similarly synthesized. *Hrs*-siRNA was directed against the human *Hrs* coding region nucleotides 195–216 (5′-aagtggaggtaaacgtccgta) and was synthesized by using a siRNA construction kit (Ambion, Austin, TX).

Antibodies, Western Blotting, and Immunoprecipitation (IP). Primary antibodies were anti-TSG101 (C-2, mouse monoclonal, Santa Cruz Biotechnology), anti-HRS (mouse monoclonal, a gift from A. Bean, University of Texas, Houston), anti-EGFR (clone F4, mouse monoclonal, Sigma), anti-EGFR (rabbit polyclonal, Santa Cruz Biotechnology), anti-HA (Sigma), anti-Flag (Sigma), and anti-α-tubulin (Sigma). HRP-conjugated secondary antibodies were goat-anti-mouse IgG and goat-anti-rabbit IgG (Santa Cruz Biotechnology). Western blots and IP were performed essentially as described (32).

Immunofluorescence Microscopy. Immunostaining was performed according to a standard protocol (32). In brief, cells were grown on cover slips, fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with primary and secondary antibodies. Primary antibodies were anti-EGFR (rabbit polyclonal, Santa Cruz Biotechnology) at 1:100, anti-EEA1 (mouse monoclonal, PharMingen) at 1:200, and anti-CD63 (mouse-monoclonal, Chemicon) at 1:200. Secondary antibodies were Alexa-Fluor 594 goat anti-mouse IgG and Alexa-Fluor 488 goat anti-rabbit IgG (Molecular Probes). The cover slips were mounted onto slides with ProLong antifade solution (Molecular Probes), and cells were examined under a deconvolution microscope (Applied Precision, Issaquah, WA).

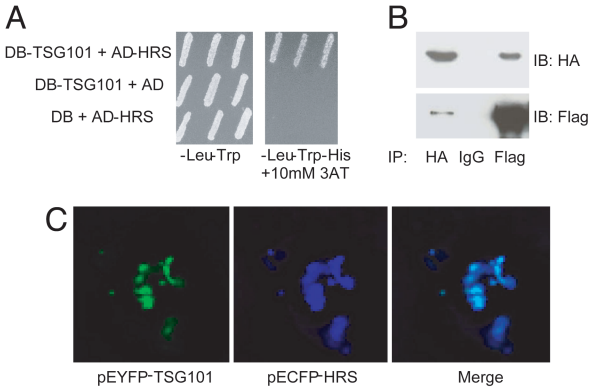

Fig. 1. Physical interaction between TSG101 and HRS. (*A*) TSG101 and HRS interact in a yeast two-hybrid assay. Bait (TSG101) and prey (HRS) two-hybrid constructs were introduced by transformation into yeast strain MaV203, and three random transformants were transferred onto SC-Leu-Trp media (to demonstrate the presence of both plasmids) and onto SC-Leu-Trp-His + 10 mM 3-amino-triazole media (to examine induction of the HIS3 reporter gene). (*B*) TSG101 and HRS coimmunoprecipitate. 293T cells were transfected with constructs containing HA-tagged TSG101 and Flag-tagged HRS. Cell lysates were made 48 h after transfection. TSG101 was immunoprecipitated (IP) with anti-HA antibody, and HRS was immunoprecipitated with anti-Flag antibody conjugated beads. The complexes were immunoblotted with anti-HA or anti-Flag antibodies. Mouse IgG was used in the control IP. (*C*) Partial colocalization of TSG101 and HRS. NIH 3T3 cells were transfected with the pEYFP-TSG101 and pECFP-HRS constructs. Twenty-four hours after transfection, cells were fixed and observed under a deconvolution fluorescence microscope. The images are pseudocolored.

EGFR Down-Regulation Assay. HeLa cells were starved in serum-free medium for 1 h and were either mock-treated as controls or incubated with EGF (Sigma) at a concentration of 150 ng/ml in 37°C for 90 min. Cells were then washed with cold PBS and immediately lysed. Lysates were subjected to SDS/PAGE and Western blotting.

Results

TSG101 Interaction with HRS. During a yeast two-hybrid screen for proteins that may interact with TSG101, we identified ≈20 positive clones, three of which were independent clonal isolates that contained protein-coding sequences of *Hrs*. The interaction identified by yeast two-hybrid analysis (Fig. 1*A*) was confirmed *in vivo* in human cells by IP experiments in which both HA-tagged TSG101 and Flag-tagged HRS were found to be precipitated by either anti-HA antibody or anti-Flag antibody (Fig. 1*B*). Using fluorescence-emitting fusion proteins (EYFP for TSG101; ECFP for HRS), we found that both fusion proteins showed cytoplasmic aggregates present in a punctuate distribution, and that the HRS-ECFP signal overlapped with, but also extended beyond, the signal of TSG101-EYFP (Fig. 1*C*).

N-terminal deletions of HRS extending to the start of the central proline-rich region did not affect HRS ability to bind to TSG101 (Fig. 2*Ab*). However, further deletion of the proline-rich segment or a deletion of the C-terminal end of the HRS protein prevented binding, implying that at least two distinct regions of HRS are necessary for interaction with TSG101 (Fig. 2*Ab*). Deletion analysis performed with TSG101 identified the N-terminal region containing the UEV domain as the site of interaction with HRS (Fig. 2*Bb*). Truncated TSG101 proteins containing only this region interacted with HRS and, in fact,

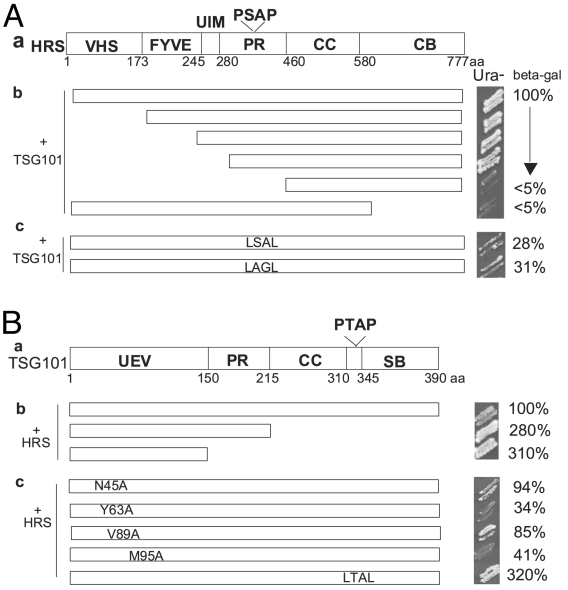

Fig. 2. Identification of structural elements involved in TSG101/HRS interaction. TSG101 (fused to Gal4-activation domain) and HRS (fused to Gal4-DNA-binding domain) constructs were introduced by cotransformation into yeast Mav203 cells. Interactions were indicated by growth on uracil-minus plate and quantitated by liquid β-galactosidase assay. Averages from three independent β-gal assays were used, and the interaction between wild-type TSG101 and wild-type HRS was designated as 100%. (*A*) Interaction between TSG101 and HRS with deletions (*b*) or mutations (*c*). (*a*) The schematic domain structure of Hrs. VHS, Vps27-Hrs-STAM; FYVE, lipid interaction domain; UIM, ubiquitin interaction motif; PR, proline-rich; CC, coiled-coil; CB, clathrin binding. (*B*) Interaction between HRS and TSG101 with deletion (*b*) or mutations (*c*). (*a*) The schematic domain structure of TSG101. PR, proline-rich; CC, coiled-coil; SB, steadiness box.

showed approximately three times the binding signal (i.e., β-galactosidase activity) of the wild-type protein, suggesting that the C-terminal half of TSG101 contains a locus that can modulate interaction of the N-terminal region with HRS.

The UEV domain of TSG101 previously was shown to possess unique affinity to a tetrapeptide motif (PSAP or PTAP; refs. 33, 34). We noticed that HRS contains a PSAP motif (i.e., amino acids proline, serine, alanine, and proline) in a proline-rich region near the center of the molecule, and mutated this sequence to LSAL or LAGL to learn its role in the TSG101/HRS interaction. As seen in Fig. 2*Ac*, mutation of the HRS PSAP sequence reduced interaction with TSG101 by two thirds, as assessed by two-hybrid-induced β-galactosidase activity in yeast strains, but did not entirely eliminate binding, indicating that this motif has an important role in the TSG101/HRS interaction, but also suggesting that additional amino acids near the center of HRS are involved. Further experiments showed that mutations in TSG101 UEV domain amino acids known to be crucial for TSG101 interaction with PTAP/PSAP motifs (i.e., TSG101 Y63A and M95A; ref. 34) reduced interaction to about the same extent as mutations in the HRS PSAP motif, whereas replacement of other amino acids in the UEV domain of TSG101 had little or no effect (Fig. 2*Bc*).

TSG101 itself contains a PTAP motif, which is located in the C-terminal half of the protein between the coiled-coil (10) and steadiness box (19) domains. Because the PTAP motif of TSG101 can interact with the TSG101 UEV domain (Q. Lu and S. N. Cohen, unpublished data), we speculated that intermolecular or intramolecular interaction between the UEV/PTAP motifs on TSG101 may negatively affect interaction of TSG101 with HRS, and consequently may account for our observation that TSG101 proteins lacking the C-terminal half have an enhanced ability to interact with HRS. This notion was supported by the increased two-hybrid signal detected after mutation of the PTAP motif of TSG101 to LTAL (Fig. 2*Bc*).

Effect of TSG101 and HRS Mutations on Endocytic Degradation of EGFR. Both TSG101 and HRS are required for endocytic attenuation of mitogenic signals (24, 29). To investigate a possible role for the TSG101/HRS interaction in this process, we expressed TSG101 or HRS in cells made deficient in the endogenous proteins by antisense oligonucleotides (for TSG101) or by siRNA (for HRS) that targeted endogenous but not adventitious transcripts. The levels of adventitious proteins expressed were comparable to levels of native endogenous proteins. We then tested the effects of perturbation of the interaction between adventitious TSG101 and HRS proteins on ligand-induced EGFR degradation, a prototypical assay for receptor down-regulation (for review, see ref. 35).

HeLa cells made deficient in TSG101 or HRS consistently showed 80–90% reduction in protein encoded by the targeted gene (Fig. 3 *Aa* and *Ba*). This level of HRS deficiency resulted in a 60% increase in the fraction of EGFR that remained undegraded after EGF induction (Fig. 3*Ab*). Decreased TSG101 resulted in an almost 3-fold increase in the amount of undegraded EGFR (Fig. 3*Bb*). These findings are consistent with previous results showing that deficiency of TSG101 or HRS impedes down-regulation of endocytosed EGFR (23, 36).

An HRS construct expressing wild-type mouse *Hrs* cDNA, which differs in 5 of 21 nucleotides from human *Hrs* in the region targeted by siRNA directed against human transcripts and thus is not subject to inhibition by this siRNA (Fig. 3*Ab*), lowered the percentage of undegraded EGFR protein in HRS-deficient human cells to the control level (Fig. 3*Ab*), indicating that adventitious expression of mouse *Hrs* can reverse the effects of deficiency of the endogenous protein. However, cells expressing HRS mutated in its PSAP motif (LSAL, LAGL, and PLGP substitutions) and consequently defective in the ability to interact with TSG101 showed persistently decreased EGFR degradation (Fig. 3*Ab*).

Analogous experiments using human TSG101 expression constructs containing silent mutations (17) in the region targeted by antisense oligonucleotides provided further evidence for the role of the TSG101/HRS interaction in EGFR degradation. Adventitious expression of wild-type TSG101 protein or of a TSG101 UEV mutant protein that has normal HRS-binding activity (i.e., V89A; Fig. 2*B*) restored EGFR degradation to normal in TSG101-deficient cells (Fig. 3*Bb*). In contrast, mutants Y63A and M95A, which affect TSG101 binding to PTAP motifs (34, 37) and specifically to HRS (Fig. 2*B*), failed to rescue the EGFR degradation defect associated with TSG101 depletion (Fig. 3*B*). A TSG101 UEV region mutant (N45A) that binds normally to PTAP (37) and to HRS (Fig. 2*B*), but shows reduced binding to ubiquitin (37), also failed to reverse the effects of TSG101-deficiency, consistent with the proposal that the ubiquitin-binding ability of TSG101 is necessary for its endocytic function (25). Expression of the N45A or M95A mutant proteins resulted in even more defective down-regulation of EGFR than was observed during antisense inhibition alone, suggesting that these two mutant proteins not only lack the ability to bind to PTAP motif and ubiquitin, as shown previously (37), but also that they may exert a dominant negative effect on EGFR degradation. Expression of a TSG101 protein mutated in its PTAP motif had an intermediate effect on EGFR regulation (Fig. 3*Bb*). Collectively, these results indicate that mutations in either TSG101 or HRS that compromise the TSG101/HRS interaction negatively affect down-regulation of EGFR.

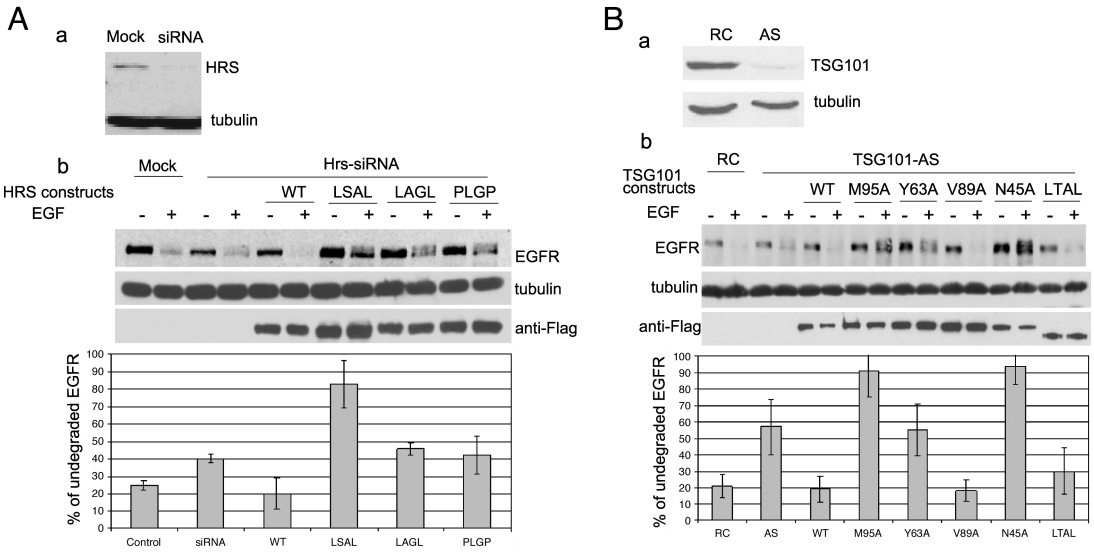

Fig. 3. Assay of ligand-induced EGF receptor degradation. (*A*) Effects of HRS mutations on EGFR degradation. HeLa cells (in a six-well plate) were made deficient in HRS by transfection of 50 nM Hrs-siRNA (*a*) for 24 h. The cells were then transfected with Flag-tagged HRS expression plasmids (siRNA-resistant) (50 ng per well) or control DNA (pBi-Luc) along with pCDNA3-EGFR (200 ng per well). Twenty-four hours after plasmid transfection the cells was starved in Opti-MEM medium for 1 h and then either mock-treated or induced with 150 ng/ml EGF for 90 min. Proteins from cell lysates were separated on SDS-8% polyacrylamide gel, electrotransferred, and immunoblotted with anti-EGFR, antitubulin, and anti-Flag antibodies. EGFR signals were quantitated by x-ray film densitometry (normalized against tubulin signals). Data from three independent experiments were averaged, and percentages of undegraded EGFR upon EGF induction were plotted. (*B*) Effects of TSG101 mutations on EGFR degradation. HeLa cells were made deficient in TSG101 by transfection of 100 nM TSG101 antisense (AS) RNA oligonucleotide (*a*). Cells were then transfected with various TSG101 expression constructs (resistant to antisense inhibition) (200 ng per well) or control DNA along with pCDNA3-EGFR (200 ng per well). Assays were performed as in *A*.

Ubiquitinated EGFR in TSG101/HRS Interaction Mutants Failed to Progress from Early Endosomes to Late Endosomes. Endocytic down-regulation of EGFR-mediated mitogenic signaling is a complex process involving both ubiquitination of the receptor and its trafficking through multiple organelles. To identify the step or steps affected by the TSG101/HRS interaction, we asked whether EGFR ubiquitination was affected by mutations that perturb the interaction (Fig. 4). Although ubiquitinated EGFR was barely detectable in naïve HeLa cells or in HeLa cells expressing adventitious wild-type TSG101 or HRS proteins that replaced endogeneous proteins (lanes 3 and 5), we observed accumulation of ubiquitinated EGFR during adventitious expression of TSG101 or HRS mutants defective in the ability to interact with each other (lanes 4 and 6). These results indicate that ubiquitination of endocytosed EGFR is itself not dependent on TSG101/HRS interaction and suggest instead that disruption of the interaction leads to defective degradation of ubiquitinated EGFR.

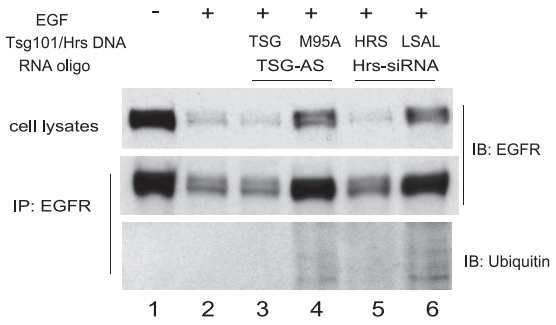

Fig. 4. Effects of TSG101/HRS interaction on accumulation of ubiquitinated EGFR. HeLa cell (in six-well plates) were first mock-treated (lanes 1 and 2), or transfected with 100 nM TSG101 antisense RNA oligonucleotide (lanes 3 and 4) or 50 nM Hrs-siRNA (lanes 5 and 6) for 24 h. The cells were then transfected with various TSG101 or HRS expression constructs (resistant to antisense inhibition) along with pCDNA3-EGFR (200 ng per well). Twenty-four hours after plasmid transfection the cells were starved and induced with EGF (150 ng/ml) for 90 min. A small portion of cell lysates was analyzed by anti-EGFR Western blot, and the remaining lysates were immunoprecipitated (IP) for EGFR. IP samples were Western-blotted with anti-EGFR and antiubiquitin antibodies.

Previous studies have shown that HRS functions primarily in early endosomes whereas TSG101 acts in late endosomes to promote the formation of MVBs. We speculated that interaction between TSG101 and HRS may be important for the trafficking of receptor cargo from early endosomes to late endosomes. If so, we reasoned that interference with the interaction potentially could lead to the retention of EGFR in early endosomes. To test this notion, we examined EGFR localization by using two-color immunofluorescence microscopy, with EEA1, an early endosomal marker, or CD63, a marker for late endosomes and/or lysosomes as a costain. As seen in Fig. 5*A*, most of the EGFR was located at the cell surface in control HeLa cells, as is characteristic of the membrane-bound unactivated receptor (35). After activation of the receptor by addition of EGF, the EGFR signal was translocated from the membrane and its overall intensity weakened significantly (Fig. 5*B*), consistent with the expected EGF-induced internalization of, and degradation of, EGFR. However, in cells made deficient in wild-type TSG101 but expressing the TSG101 M95A mutant protein, the signal from internalized EGFR remained strong and immunofluorescence analysis showed a punctuate distribution throughout the cytoplasm (Fig. 5*C*). In these cells, EGFR colocalized with the early endosomal marker EEA1 (yellow merged signal), but not with the late endosomal and lysosomal marker CD63 (indicated by separation of red and green signals). Similarly in cells expressing the HRS-LSAL mutant protein, EGFR was internalized but continued to show a strong signal that colocalized with EEA1 but not with CD63. These results argue that mutations in either TSG101 or HRS that affect the ability of these proteins to interact lead to retention of EGFR in early endosomes rather

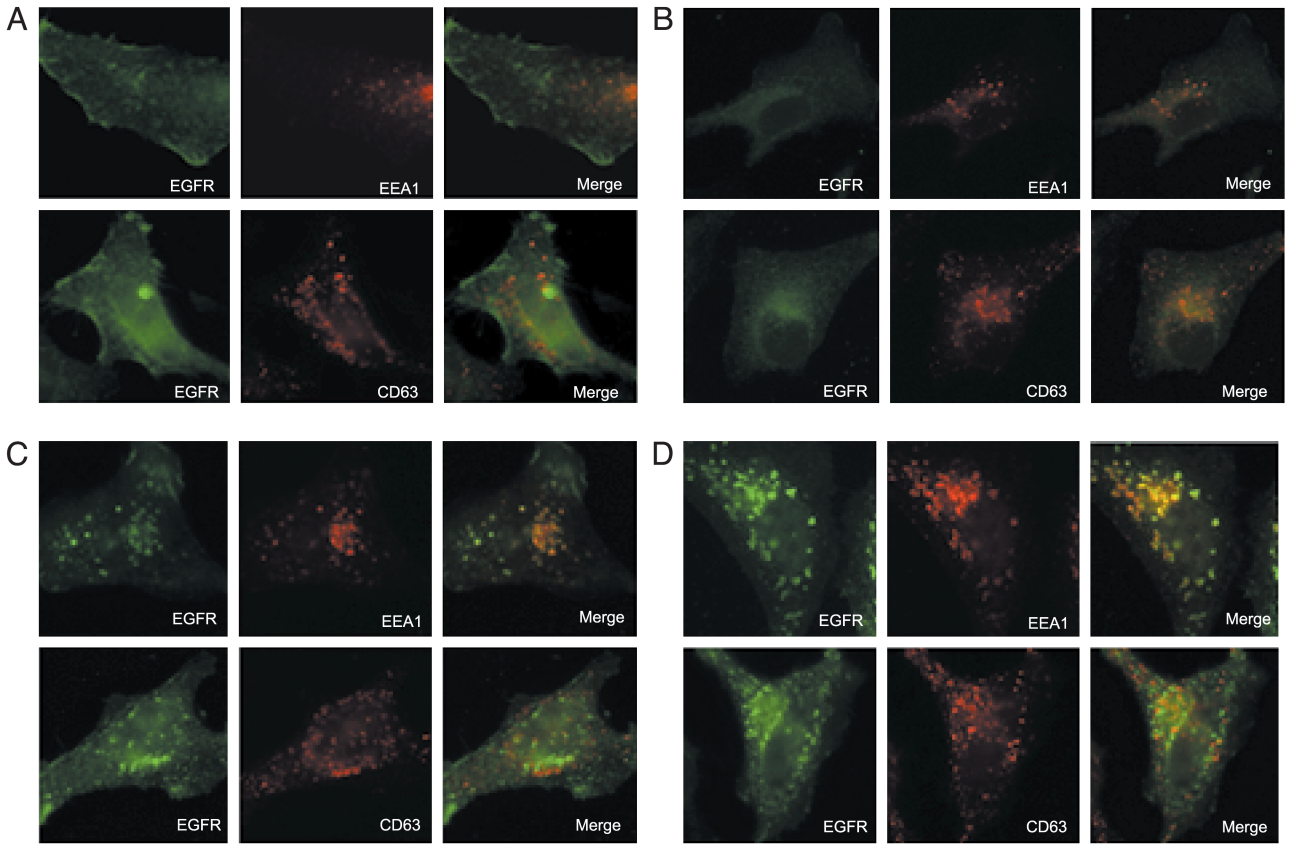

Fig. 5. EGFR colocalization with early and late endosomal markers. (*A* and *B*) Control naïve HeLa cells. (*C*) HeLa cells with mutant TSG101 were made by first transfection of TSG101-antisensed RNA oligo and subsequent transfection of a mutant TSG101 construct (M95A). (*D*) HeLa cells with mutant HRS were made by transfection of HRS siRNA and subsequent transfection of a mutant HRS construct (HRS-LSAL). Cells were either mock-treated (*A*) or induced (*B–D*) with EGF (150 ng/ml) for 60 min. Cells were then fixed, permeabilized, and immunostained for EGFR and EEA1 (or CD63), and imaged under a deconvolution fluorescence microscope as described in *Materials and Methods.*

than the trafficking of receptors to multivesicular late endosomes, a process essential for degradation of receptors and attenuation of mitogenic signaling.

Discussion

The endosomal trafficking of proteins is essential for many biological processes, including the down-regulation of mitogenic signaling. TSG101 and HRS are known to be functionally important in the trafficking process. The results reported here indicate that these two proteins interact, and that mutations disrupting the TSG101/HRS interaction impair endosomal sorting of EGFR, and specifically the transport from early endosomes to late endosomes and lysosomes for eventual degradation.

HRS functions in the sorting of endocytosed ubiquitinated proteins to early endosomes, whereas TSG101 has been implicated in the sorting of ubiquitinated cargo to MVBs in late endosomes. Both TSG101 and HRS can bind to ubiquitin, and importantly, their ubiquitin-binding activities are required for their endocytic functions (25, 28). Our results lead us to suggest that interaction between HRS and TSG101 facilitates the trafficking of cargo-bearing early endosomes to late endosomes capable of incorporating the cargo in MVBs. In this scenario, HRS associated with both ubiquitinated EGFR and the membranes of early endosomes may recruit TSG101 to the proximity of receptors, where TSG101 can bind to the ubiquitin-tagged endosomal cargo and mediate subsequent trafficking. Disruption of the interaction thus would prevent the efficient recruitment of TSG101 to receptors, result in the retention of EGFR in early endosomes, as our data suggest, and ultimately inhibit receptor degradation.

Previous studies have shown that both HRS and TSG101 interact with a variety of other proteins implicated in endosomal trafficking. TSG101 interacts with hVps28 (38), and its yeast ortholog also interacts with Vps37 in the late endosomal complex, ESCRT-1 (25). HRS was found in a separate 550-kDa complex (39), and proteins shown to interact with HRS include STAM (signal transducing adaptor molecule) (40), SNAP-25 (an essential component of membrane fusion machinery) (41), sorting nexin 1 (39), eps15 (42), and most recently clathrin (43). TSG101/HRS interaction may coordinate functions of the disparate macromolecular complexes of early and late endosomes. Only a fraction of the cellular pools of TSG101 and HRS were observed to interact in our IP and colocalization experiments (Fig. 1), indicating that the complexes containing these proteins do not reside entirely at the same subcellular locations. Potentially, TSG101/HRS interaction may occur only at sites of sorting of early endosomal cargo into the MVBs of late endosomes.

We observed that both TSG101 and HRS contain a tetrapeptide motif (PTAP in TSG; PSAP in HRS) that initially was identified in an HIV GAG protein late domain that interacts with the UEV domain of TSG101 (17, 33). TSG101 interaction with this motif of GAG (17, 33) and with a similar motif in the matrix protein of Ebola virus (18) is required for normal viral budding to the cell surface, a process that is topologically

equivalent to the removal of mitogenic receptors from the cytoplasm (44). Our results indicate a bipartite role for PSAP/PTAP motifs in modulating the TSG101/HRS interaction: the PSAP motif of HRS is implicated in HRS interaction with TSG101, whereas TSG101's own PTAP motif, which may have a role in intramolecular or intermolecular TSG101 interaction, is inhibitory to the TSG101/HRS interaction. We suggest that the specific cellular functions shown here to be affected by interaction of the TSG101 UEV domain with PTAP/PSAP motifs have been targeted during the evolution of viral release proteins that use endocytic machinery for the viral budding process.

Prolonged mitogenic signaling in cells made deficient in TSG101 by antisense strategies (refs. 23 and 24; also Fig. 3*B* in this study) may account in part for the observed growth-promoting effects of reduced TSG101 in cells growing in culture (10, 15). However, excessive production of TSG101 also can promote abnormal cell growth (10), consistent with evidence for a cellular imperative to maintain TSG101 levels within a narrow range (19). Aberrant splicing of, partial deletions in, or overproduction of TSG101 protein have been reported to occur in human cancers (45–48). Consistent with a crucial role of the intracellular level of TSG101 in determining its actions is evidence that cells that totally lack TSG101 cannot be propagated (8, 9), whereas a lesser deficiency of TSG101 allows cell survival while still yielding prominent effects on endosomal trafficking and other biological processes (10, 17, 23, 24).

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