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# Association with Hrs Is Required for the Early Endosomal Localization, Stability, and Function of STAM

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**Members of the STAM family of proteins, STAM1 and STAM2, are associated with Hrs through their coiled-coil regions. Both Hrs and STAM bind ubiquitin and are involved in endosomal sorting of ubiquitinated cargo proteins for trafficking to the lysosome. Here we examined the biological significance of STAM binding to Hrs. Endogenous STAM1 and STAM2 were mostly localized on the early endosome, suggesting that they are resident endosomal proteins. A STAM2 mutant that lacks the coiled-coil region and does not bind Hrs, in contrast, mislocalized to the cytoplasm. Deletion of a region located N-terminal to the coiled-coil region and conserved among STAM proteins also severely affected Hrs binding and the endosomal localization of STAM2, suggesting that this region is also involved in these activities. Depletion of endogenous Hrs by RNA interference similarly caused the mislocalization of exogenously expressed STAM2 to the cytoplasm. These results indicate that STAM is localized to the early endosome by binding to Hrs on the target membrane. In addition, the expression level of endogenous STAM proteins was drastically reduced in Hrs-depleted cells, suggesting that STAM is stabilized by binding to Hrs. Finally, STAM2 mutants lacking the Hrs-binding activity were defective in causing the enlargement of early endosomes, accumulating ubiquitinated proteins on this aberrant organelle, and inhibiting the degradation of ligand-activated epidermal growth factor receptors, suggesting that the association with Hrs is a prerequisite for STAM function.**

**Key words: class E Vps, endosome, membrane traffic, protein sorting, ubiquitin.**

Abbreviations: CC, coiled-coil; EGF, epidermal growth factor; EGFR, EGF receptor; ESCRT, endosomal sorting complex required for transport; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hrs, hepatocyte growth factor-regulated substrate; MVB, multivesicular body; PI(3)-P, phosphatidylinositol(3)-phosphate; RNAi, RNA interference; RT, reverse transcription; siRNA, small interfering RNA; SSM, STAM-specific motif; STAM, signal-transducing adaptor molecule; UIM, ubiquitin-interacting motif; Vps, vacuolar protein sorting.

Cell surface receptors that are destined for lysosomal degradation, as well as newly synthesized lysosomal hydrolases, are delivered to the lumen of the lysosome *via* the endosomal compartments (*1*). At the endosome, these proteins are incorporated into luminal vesicles that bud inward from the endosomal limiting membrane. The resultant organelle, called the multivesicular body (MVB), then fuses with the lysosome to release the sorted proteins into the lysosomal lumen. Monoubiquitination has been shown to serve as a sorting signal for some lysosome-targeted proteins to be incorporated into the luminal vesicles of the MVB in yeast (*2*). In addition, class E Vps (vacuolar protein sorting) proteins including three protein complexes, ESCRT (endosomal sorting complex required for transport)-I, -II, and -III, have been identified as sorting machinery for the ubiquitinated proteins on the endosomal membrane (*3*). The presence of class E Vps protein orthologs in mammals suggests that the molecular mechanisms of this trafficking route, the MVB pathway, are conserved among eukaryotic cells (*3*).

Members of the STAM (signal-transducing adaptor molecule) family of proteins, STAM1 and STAM2, are composed of the same domain structures, including the VHS (Vps27/Hrs/STAM) domain, ubiquitin-interacting motif (UIM), Src homology 3 domain, and a coiled-coil (CC) region (*4*). Through their CC regions, both STAM1 and STAM2 bind Hrs (hepatocyte growth factor-regulated substrate), a protein that is localized on the cytoplasmic face of the early endosome (*5*, *6*). Hrs and STAM proteins play roles in the sorting of ubiquitinated proteins in the MVB pathway. Vps27 and Hse1 (Hbp/STAM/EAST1), yeast orthologs of Hrs and STAM, respectively, belong to the class E Vps category of proteins, and disruption of either of the genes leads to a defect in the trafficking of ubiquitinated cargo to the lysosome (*7*, *8*). Disruption of the genes for Hrs or STAM1/2 in flies and mice similarly causes a defect in the degradation of ligand-activated receptor tyrosine kinases (*9*, *10*). Also, depletion of Hrs by RNA interference (RNAi) in mammalian cells has been shown to inhibit the degradation of ligand-activated growth factor receptors (*11–13*). Furthermore, both Hrs (*9*, *14–17*) and STAM (*11*, *18*) bind ubiquitin. These proteins possess the UIM, a conserved ~15-amino-

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acid sequence that was originally identified as a polyubiquitin-binding site in the S5a subunit of the 26S proteasome (*19*). The defect in the MVB pathway in yeast cells deficient for Vps27 or Hse1 can not be complemented by expressing these proteins lacking functional UIMs, indicating an essential role for this motif in the functions of these proteins (*7*, *8*). For Hrs and Vps27, the UIMs play a major role in binding ubiquitin (*7*, *8*, *14–17*, *20*). For STAM, in contrast, the VHS domain located just N-terminal to the UIM is also essential (*18*). Both Hrs and STAM are co-localized with ubiquitinated proteins on the early endosome, and overexpression of these proteins causes an accumulation of ubiquitinated proteins, including ligand-activated growth factor receptors, on this organelle (*11*, *14*, *15*, *18*). It has been shown that Hrs and STAM mutants with defective ubiquitin-binding activity do not cause this effect (*18*, *20*). Furthermore, recent studies demonstrate that Hrs/Vps27 interacts through its PSAP/PTVP motif with TSG101/Vps23, a component of the ESCRT-I complex; thereby, Hrs/Vps27 recruits ESCRT-I to the endosomal membrane for further sorting processes and MVB formation (*13*, *21–23*).

For Hrs and STAM to exert functions in the MVB pathway, their localization to the early endosome is likely to be prerequisite. Hrs has a FYVE (Fab1/YOTB/Vac1/EEA1) domain, a conserved RING finger domain that is present in several endosomal proteins and specifically binds phosphatidylinositol(3)-phosphate [PI(3)-P] (*24–26*). Although the mechanism by which Hrs is localized to the early endosome is not completely understood (*27*), the FYVE domain-mediated interaction with PI(3)-P on the early endosomal membrane has been shown to play an essential role. Treatment of cells with wortmannin, an inhibitor of PI 3-kinase which is responsible for the PI(3)-P synthesis, causes the dissociation of Hrs from the early endosome to the cytoplasm (*28*, *29*). In addition, Hrs mutants with a defective FYVE domain are not localized to the early endosome but cluster within aberrant proteinaceous aggregates (*27*). Similarly in yeast cells, the ortholog Vps27 dissociates from the endosome when the FYVE domain is mutated or when PI(3)-P is depleted by inactivating the gene for the PI 3-kinase, Vps34 (*22*). The mechanism behind the localization of STAM, however, has not been extensively studied. In Vps27-deficient yeast cells, Hse1 is localized to the cytoplasm but not to the endosomal compartment (*8*). A more recent study showed that the overexpression of Hrs increases the amount of STAM2 on the early endosome whereas the depletion of endogenous Hrs by RNAi dissociates STAM2 from the organelle in mammalian cells (*11*). These results indicate that Hrs/Vps27 is required for the localization of STAM/Hse1. However, how Hrs/Vps27 regulates the localization has not been experimentally demonstrated.

Here, we show that STAM proteins are localized to the early endosome by binding to Hrs via the CC region, and that a previously uncharacterized conserved region just N-terminal to the CC region is also involved in the interaction with Hrs. We further show that the association with Hrs is also required for the stability and endosomal sorting function of STAM.

## EXPERIMENTAL PROCEDURES

*Expression Constructs and Transfection*—The FLAG-tagged STAM2, HA-tagged STAM2, and HA-tagged Hrs expression vectors, pME-FLAG-STAM2, pME-HA-STAM2, and pmiw-Hrs-HA were constructed as described previously (*18*, *30*). STAM2 deletion constructs lacking the CC region (amino acid residues 333–367) and the STAM-specific motif (amino acid residues 285–310) were obtained by *in vitro* mutagenesis using the QuikChange site-directed mutagenesis system (Stratagene). cDNAs for the truncated STAM2 constructs, $STAM2_{310–462}$ and $STAM2_{285–462}$, were generated by PCR using the STAM2 cDNA as a template, and were cloned into pME-HA (*31*). The nucleotide sequences of the constructs were verified by DNA sequencing. The human epidermal growth factor receptor (EGFR) expression vector, pRc/CMV-hEGFR, was constructed as described (Morino *et al.*, submitted). The FLAG-tagged monoubiquitin expression vector, pcDNA3.1-FLAG-Ub, and the HA-tagged c-Cbl expression vector, pcDNA3-HA-c-Cbl, were provided by Dr. T. Suzuki (Tokyo Metropolitan Institute of Medical Science, Tokyo) and Dr. K. Yokote (Chiba University, Chiba), respectively. The expression vectors were transfected into cells for two days using FuGENE 6 Transfection Reagent (Roche).

*Anti-STAM1 Antibody*—To avoid cross-reactivity to STAM2, a C-terminal region of STAM1 that is not conserved in STAM2 (28% amino acid sequence identity between STAM1 and STAM2) was chosen as an antigen. A cDNA corresponding to amino acid residues 391–547 of mouse STAM1 was fused in-frame to the glutathione S-transferase cDNA in the pGEX vector (Amersham Biosciences). The glutathione S-transferase-STAM1 fusion protein was expressed in *E. coli* strain BL21 and purified using glutathione-Sepharose affinity beads (Amersham Biosciences). Polyclonal anti-STAM1 antibody was raised by immunizing rabbits with the purified fusion protein.

*Immunofluorescence Staining*—Cells were fixed with 4% formaldehyde for 10 min on ice, permeabilized with 0.2% Triton X-100, and stained with rabbit polyclonal anti-STAM1 (1:500 dilution), rabbit polyclonal anti-STAM2 (1:500 dilution; *6*), rabbit polyclonal anti-Hrs (1:2,000 dilution; *32*), mouse monoclonal anti-HA (4 μg/ml, Sigma), mouse monoclonal anti-EEA1 (1 μg/ml, Transduction Laboratories), mouse monoclonal FK2 (10 μg/ml, Affiniti Research Products), mouse monoclonal anti-FLAG (4 μg/ml, Sigma), and rabbit polyclonal anti-FLAG (2 μg/ml, Affinity BioReagents) antibodies by standard procedures. Secondary antibodies used were Alexa488- and Alexa594-conjugated anti-mouse IgG and anti-rabbit IgG antibodies (Molecular Probes). To visualize the localization of endocytosed transferrin, cells were incubated with Alexa594-conjugated transferrin (50 μg/ml, Molecular Probes) for 15 min at 37°C before fixation. Fluorescence images were captured using a confocal microscope. The proportion of cells exhibiting Hrs- and FK2-positive enlarged early endosomes among those overexpressing the STAM2 constructs were semi-quantified as described previously (*18*). The experiments were repeated three times and the means ± SD of the proportions were determined.

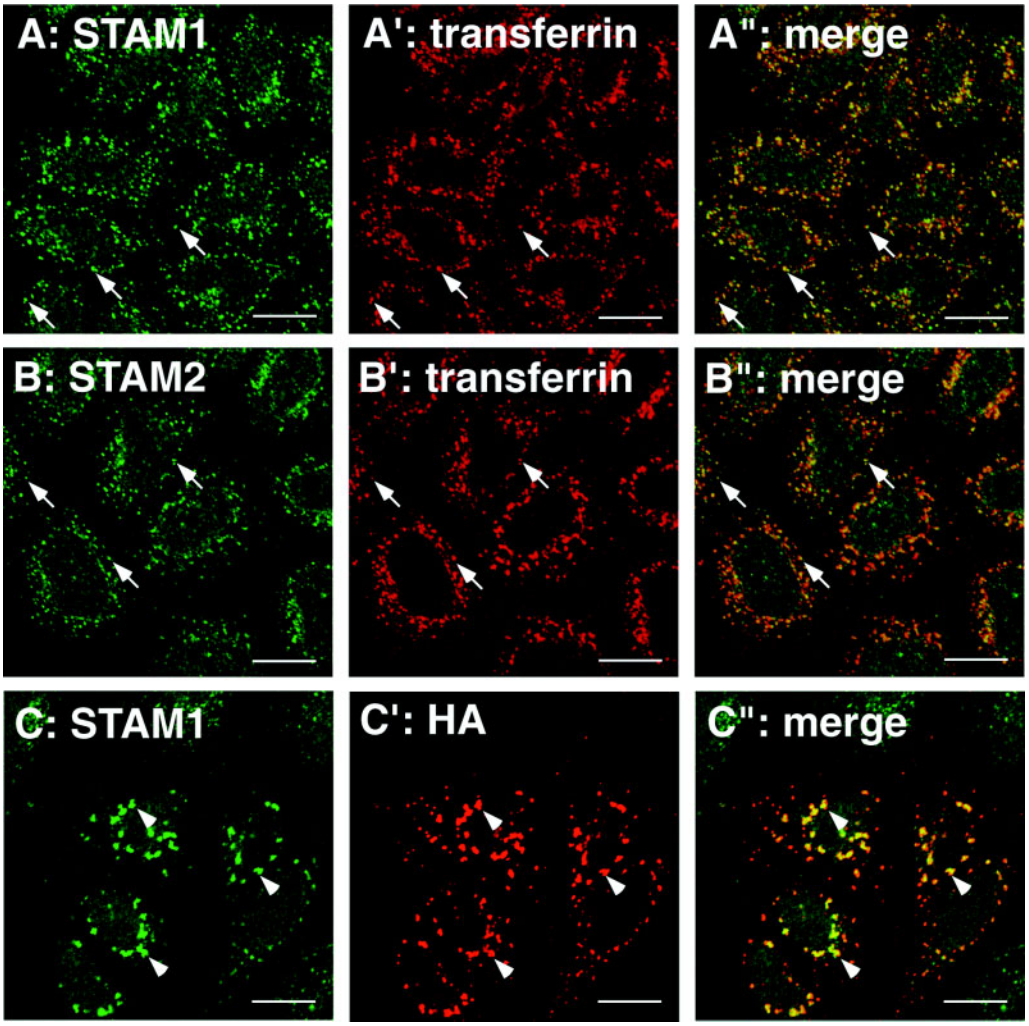


Fig. 1. **Subcellular localization of endogenous STAM proteins.** (A–B″) HeLa cells incubated with Alexa594-conjugated transferrin were fixed and immunostained with anti-STAM1 (A) or anti-STAM2 (B) antibody. A′ and B′ show the localization of endocytosed Alexa594-transferrin. (C–C″) HeLa cells transfected with HA-tagged Hrs were double-stained with anti-STAM1 (C) and anti-HA (C′) antibodies. A″–C″ are merged images. Arrows indicate the co-localization of STAM1 (A–A″) and STAM2 (B–B″) with endocytosed transferrin. Arrowheads in C–C″ indicate enlarged early endosomes in Hrs-overexpressing cells. Bars, 20 μm.

*Immunoblotting and Immunoprecipitation*—Cell lysates were prepared by solubilizing cells with lysis buffer [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 50 mM NaF, 1 mM phenylmethyl sulphonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A] for 30 min and collecting the supernatants after centrifugation at 12,000 ×*g* for 15 min. The lysates were used directly for immunoblotting, or immunoprecipitated with anti-Hrs antiserum (2 μl; *32*) or anti-FLAG antibody (1 μg, Sigma). Immunoblot analysis was performed by standard procedures. Primary antibodies used were anti-Hrs (1:200; *32*), anti-STAM1 (1:200), anti-STAM2 (1:200; *6*), anti-FLAG (4 μg/ml, Sigma), anti-HA (0.5 μg/ml, Sigma), and anti-Shc (1 μg/ml, Santa Cruz Biotechnology) antibodies. Secondary antibodies were peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies (Amersham Biosciences). Blots were detected using the ECL reagent (Amersham Biosciences).

*RNAi*—Two complementary oligonucleotides, 5′-GTACAAGGTGGTCCAGGACTTCAAGAGAGTCCTGGACCACCTTGTACTTTTTTGGAA-3′ and 5′-AATTTTCCAAAAAAGTACAAGGTGGTCCAGGACTCTCTTGAAGTCCTGGACCACCTTGTACGGCC-3′, were annealed and cloned into the *Apa*I and *Eco*RI sites of the small interfering RNA (siRNA) expression vector, pSilencer 1.0-U6 (Ambion). This plasmid allows the transcription of the inserted DNA that is to be processed to the siRNA for human Hrs (nucleotide residues 372–390 from the translation initiation codon). This Hrs siRNA expression vector or the control empty vector was transfected into HeLa cells twice at 48 h intervals using FuGENE 6 Transfection Reagent (Roche). To express HA-tagged STAM2 constructs in these cells, the expression vectors were co-transfected with the siRNA expression vector in the second round of transfection.

*Reverse Transcription (RT)-PCR*—Total RNA was isolated from HeLa cells using the ISOGEN RNA extraction kit (Nippon Gene). First strand cDNAs were synthesized from the RNAs (5 μg) using the SuperScript First-Strand Synthesis System (Invitrogen). The cDNAs were ampli-

fied by PCR [27, 30, 34, and 25 cycles for Hrs, STAM1, STAM2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively]. At the number of PCR cycles used, the cDNA amplifications were not saturated. Primers used were 5′-CTCCTGTTGGAGACAGATTG-3′ and 5′-CAGGTACAGGATCTTGTTAC-3′ for Hrs, 5′-GTTGAATACTGCTGAGGACT-3′ and 5′-CTGAGAGCCAATAGCTGGGA-3′ for STAM1, 5′-AAAAGCCACAAATGAGTACAACAC-3′ and 5′-GACAGTCTGGGAGCCTGC-3′ for STAM2, and 5′-AGGTGAAGGTCGGAGTCAAC-3′ and 5′-TACTCCTTGGAGGCCATGTG-3′ for GAPDH.

*Detection of Degradation and Ubiquitination of EGFR*—COS-7 cells were transfected with HA-tagged STAM2 constructs together with EGFR, HA-tagged c-Cbl, and FLAG-tagged ubiquitin for 24 h, serum-starved for another 24 h, then incubated with EGF (100 ng/ml, PeproTech) for 15, 60, or 120 min. For cells that were treated with EGF for 60 or 120 min, EGF was removed from the culture medium 15 min after EGF addition. The cell lysates were immunoprecipitated with anti-EGFR (0.1 μg, MBL) and immunoblotted with the same antibody (1 μg/ml) or anti-FLAG. Total cell lysates were immunoblotted with anti-HA antibody to assess the expression level of the STAM2 constructs and c-Cbl.

## RESULTS

*Endogenous STAM Proteins Are Localized on the Early Endosome*—Transfected STAM1 and STAM2 are localized to Hrs-positive early endosomes when expressed at a low level (*18*). To examine the localization of endogenous STAM proteins, we stained HeLa cells with anti-STAM1 and anti-STAM2 antibodies. Staining with anti-STAM1 showed that the protein is localized to cytoplasmic vesicular structures that are mostly positive for endocytosed fluorescence-labeled transferrin, a marker for the early endosome (Fig. 1A–A″, arrows). Anti-STAM2 antibody exhibited a similar staining pattern, although the signal was fainter than that of anti-STAM1 (Fig. 1B–B″). These results suggest that STAM proteins are resident early endosomal proteins.

We were not able to double-stain cells with anti-STAM and anti-Hrs antibodies as they were all raised in rabbits. However, endogenous STAM1 co-localized completely with transfected HA-tagged Hrs on morphologically aberrant (enlarged) endosomes caused by Hrs overexpression (Fig. 1C–C″, arrowheads), supporting the idea that endogenous STAM1 is associated with Hrs on the early endosome. The same results have also been recently reported for STAM2 (*11*).

*The CC Region and Another STAM-Specific Region Are Required for Hrs Binding of STAM2*—To test whether the association with Hrs is required for the early endosomal localization of STAM proteins, we first determined the regions in STAM2 that are required for stable binding to Hrs. It has been shown that STAM associates with Hrs *via* the CC region (*5*, *6*). Consistent with this, an HA-tagged STAM2 mutant lacking the CC region (Fig. 2A, STAM2ΔCC), when expressed in HeLa cells, was not co-immunoprecipitated with Hrs by anti-Hrs antibody (Fig. 2C). Next, a truncated STAM2 construct comprising amino acid residues 310–462 (Fig. 2A, $STAM2_{310–462}$) was

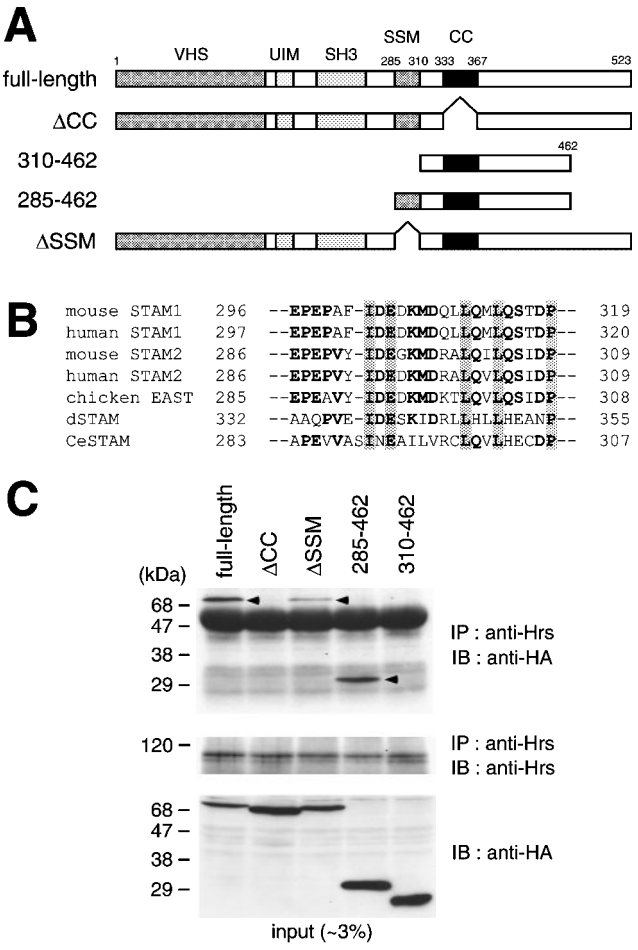


Fig. 2. **Schematic representation and the Hrs-binding activity of STAM2 constructs.** (A) Domain structure of STAM2 and its mutants used in this study. The VHS domain, UIM, Src homology 3 (SH3) domain, SSM, and the CC region are indicated. (B) An alignment of the SSM of mouse and human STAM1 and STAM2, chicken EAST (an ortholog of STAM), *Drosophila* STAM (dSTAM), and *C. elegans* STAM (CeSTAM). Amino acid residues conserved in all seven proteins are boxed; those conserved in five or more proteins are shown in boldface type. (C) The Hrs-binding activity of the STAM2 constructs shown in A. Lysates of HeLa cells transfected with the HA-tagged STAM2 constructs were immunoprecipitated (IP) with anti-Hrs antibody and immunoblotted (IB) with anti-HA (upper panel) or anti-Hrs (middle panel) antibody. Arrowheads in the upper panel indicate the co-precipitated STAM2 constructs. The amounts of STAM2 constructs expressed in HeLa cells were assessed by immunoblotting of the total cell lysates (~3%) with anti-HA antibody (lower panel). Positions of size markers are indicated on the left.

tagged with the HA epitope and expressed in HeLa cells. This region contains the CC region and a subsequent 95-amino acid sequence that is not conserved between STAM1 and STAM2 (31% amino acid sequence identity). However, $STAM2_{310–462}$ was not co-immunoprecipitated with anti-Hrs antibody (Fig. 2C), suggesting that the CC region is not sufficient for Hrs binding.

STAM has a region comprising ~25 amino acid residues located just N-terminal to the CC region. This region is conserved among STAM proteins in higher eukaryotes (Fig. 2B; *e.g.* 71% identity and 88% similarity between mouse STAM1 and STAM2) but is not homolo-

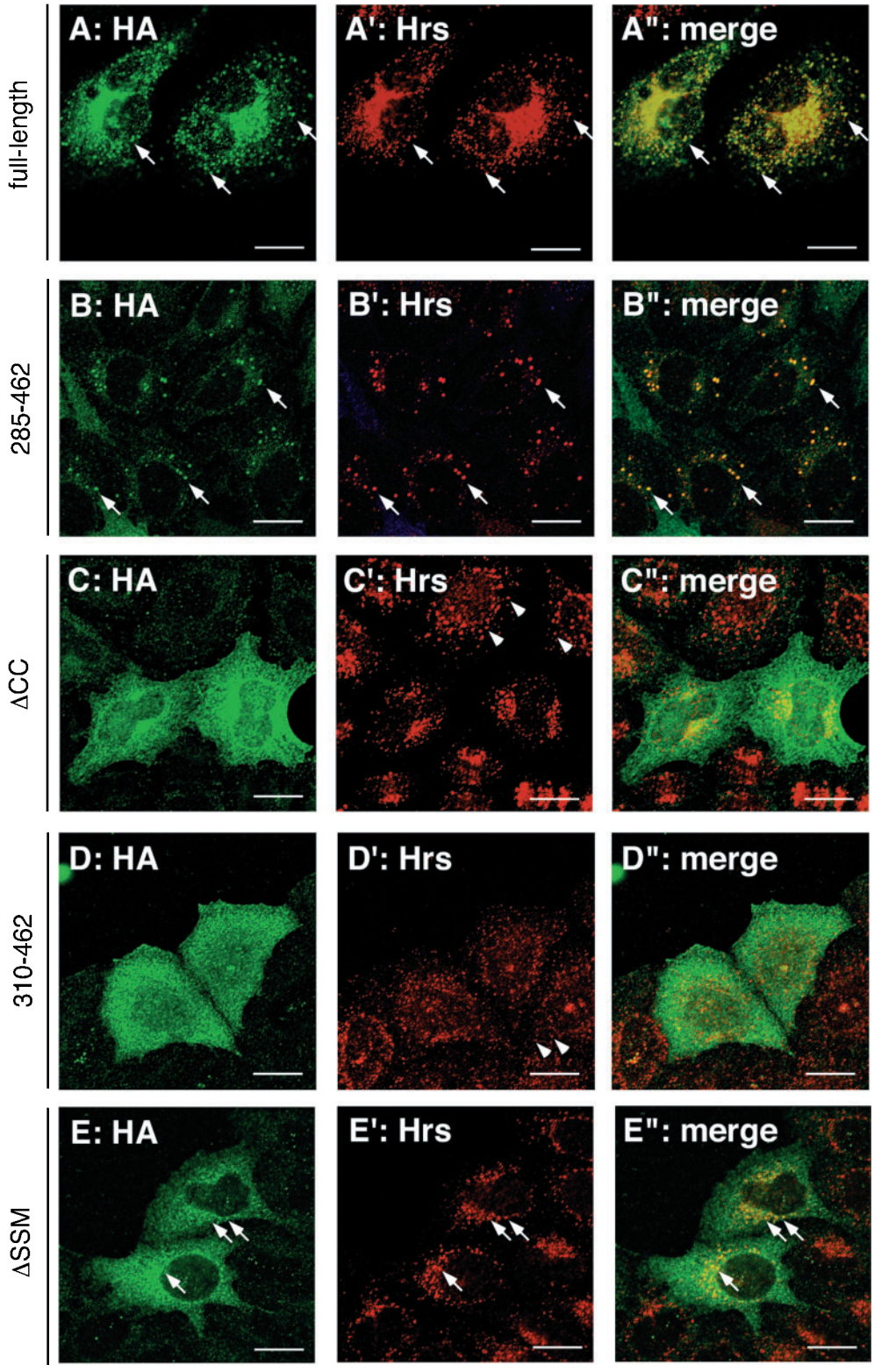


Fig. 3. **Subcellular localization of STAM2 constructs.** HeLa cells were transfected with HA-tagged full-length STAM2 (A–A″), $STAM2_{285–462}$ (B–B″), STAM-2ΔCC (C–C″), $STAM2_{310–462}$ (D–D″), or STAM2ΔSSM (E–E″), and double-stained with anti-HA (A–E) and anti-Hrs (A′–E′) antibodies. A″–E″ are merged images. Arrows in A–A″, B–B″, and E–E″ indicate co-localization of each STAM2 construct with endogenous Hrs. Arrowheads in C′ and D′ indicate normal early endosomes in untransfected cells. Bars, 20 μm.

gous to sequences in any other proteins including the yeast ortholog, Hse1. We therefore refer to this region as the STAM-specific motif (SSM). To examine whether the SSM is involved in Hrs binding, we generated another truncated construct, $STAM2_{285–462}$, containing both the CC region and SSM (Fig. 2A). $STAM2_{285–462}$ co-immunoprecipitated with anti-Hrs antibody as efficiently as full-length STAM2 (Fig. 2C). To examine further whether the SSM is required for Hrs binding, a STAM2 deletion mutant lacking this region was constructed (Fig. 2A, STAM2ΔSSM). Co-immunoprecipitation with anti-Hrs antibody demonstrated that, although not completely

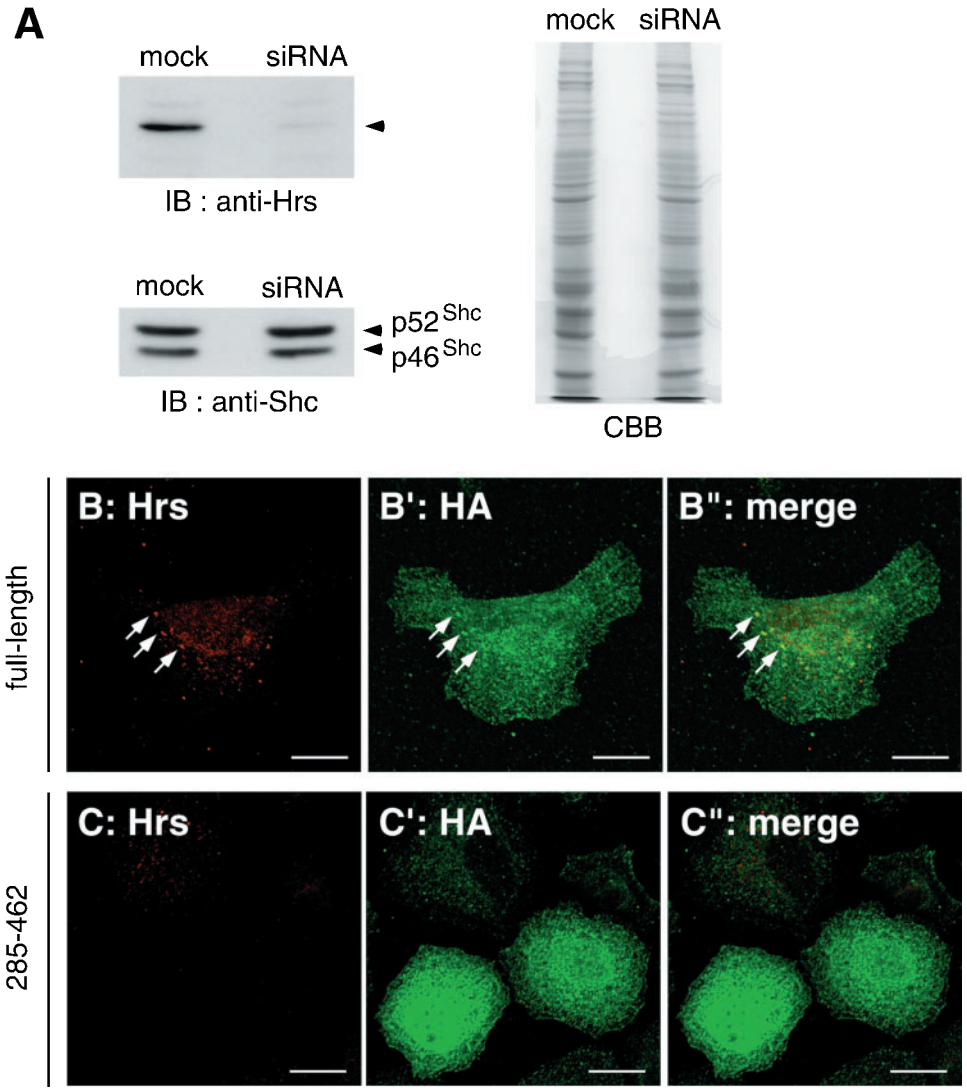


Fig. 4. **Effect of Hrs depletion on the localization of STAM2.** (A) Lysates of HeLa cells transfected with the empty vector (mock) or Hrs siRNA expression vector (siRNA) were immunoblotted (IB) with anti-Hrs and anti-Shc antibodies. The lysates were also stained with Coomassie Brilliant Blue (CBB). (B–C″) HeLa cells were transfected with the Hrs siRNA expression vector together with HA-tagged full-length STAM2 (B–B″) or $STAM2_{285–462}$ (C–C″), and double-stained with anti-Hrs (B and C) and anti-HA (B′ and C′) antibodies. B″ and C″ are merged images. Arrows in B-B″ indicate early endosomes with residual Hrs. Bars, 20 μm.

abolished, the Hrs binding of this mutant is severely affected (Fig. 2C). These results suggest that the SSM is also required for stable association with Hrs, although it is not as critical as the CC region.

*The CC Region and SSM Are Required for the Localization of STAM2*—We next examined the subcellular localization of the STAM2 mutants used in Fig. 2 by staining transfected HeLa cells with anti-HA and anti-Hrs antibodies. As reported previously (*18*), full-length STAM2 co-localized with endogenous Hrs when expressed at a low level (Fig. 3A–A″). $STAM2_{285–462}$, which contains both the CC region and SSM and efficiently binds Hrs, was also completely co-localized with Hrs on cytoplasmic punctate structures, suggesting that it is localized to the early endosome (Fig. 3B–B″). In these cells, however, the Hrs-positive organelles were larger and much fewer than those in untransfected cells (Fig. 3C′, D′, arrowheads). The reason for this phenomenon is currently unknown.

In contrast, the mutants that do not bind Hrs, STAM2ΔCC and $STAM2_{310–462}$, were completely cytoplasmic even when expressed at a low level (Fig. 3C–C″, D–D″). In addition, STAM2ΔSSM, whose Hrs binding is severely affected, was also mostly cytoplasmic (Fig. 3E–E″), although some co-localization with Hrs was faintly detectable (Fig. 3E–E″, arrows). The correlation between Hrs binding and the endosomal localization of these STAM2 mutants suggests that STAM proteins are localized to the early endosome through interaction with Hrs.

*Hrs Is Required for the Early Endosomal Localization of STAM2*—Deletion of the CC region or SSM from STAM proteins might also affect the interaction with molecules other than Hrs. Indeed, the binding of STAM to Jak2/3 tyrosine kinases is mediated through the immunoreceptor tyrosine-based activation motif (ITAM) that overlaps considerably with the CC region (*33*, *34*). To test whether the interaction with Hrs is responsible for the localization of STAM proteins, we next examined whether depletion of endogenous Hrs affects the localization of STAM2. To deplete Hrs, HeLa cells were transfected with a vector expressing an Hrs siRNA under the control of RNA polymerase III. Immunoblot analysis showed that transfection of this vector led to a significant reduction in the Hrs protein level as compared to the mock transfection control (Fig. 4A). Reprobing the immunoblot membrane with anti-Shc antibody, which was chosen as an internal standard, as well as Coomassie Bril-

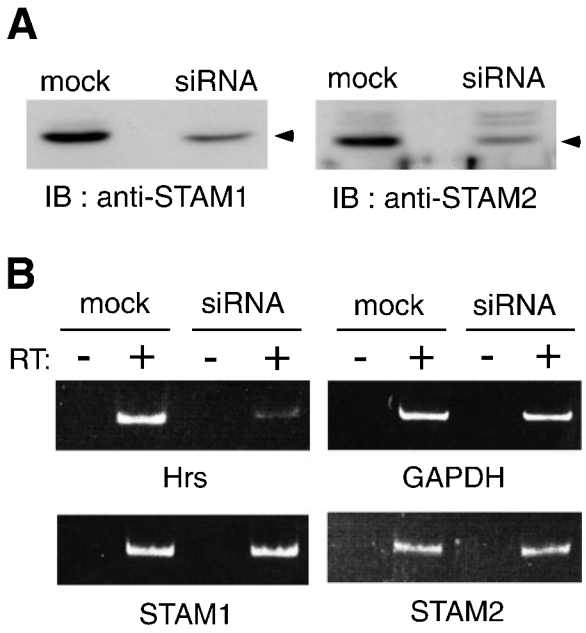


Fig. 5. **Effect of Hrs depletion on the stability of STAM proteins.** (A) Lysates of HeLa cells transfected with the empty vector (mock) or Hrs siRNA expression vector (siRNA) were immunoblotted with anti-STAM1 and anti-STAM2 antibodies. (B) Total RNAs were isolated from cells transfected with the empty vector (mock) or Hrs siRNA expression vector (siRNA), and the expression of mRNAs for Hrs, STAM1, STAM2, and GAPDH were examined by RT-PCR in the presence (+) or absence (–) of RT.

liant Blue staining of the same lysates, showed that the same amounts of cellular proteins were loaded on the membrane (Fig. 4A). Double immunofluorescence staining with anti-Hrs and anti-EEA1 antibodies showed that more than 50% of the siRNA-transfected cells exhibited a nearly undetectable level of anti-Hrs staining, whereas the expression and localization of EEA1 remained apparently normal (not shown).

To test the effect of Hrs depletion on the localization of exogenously expressed STAM, HA-tagged full-length STAM2 and $STAM2_{285–462}$ were co-transfected with the Hrs siRNA expression vector into HeLa cells. The cells were double-stained with anti-Hrs and anti-HA antibodies. Fig. 4B shows a cell in which Hrs was mostly depleted (compare with cells in Fig. 3A′–E′), whereas the cells in Fig. 4C were completely depleted of Hrs. In these cells, full-length STAM2 (Fig. 4B′) and $STAM2_{285–462}$ (Fig. 4C′) were not localized to the early endosome but were scattered throughout the cytoplasm, although partial colocalization of full-length STAM2 with residual Hrs was detectable (Fig. 4B–B″, arrows). The localization of these proteins was not affected by mock transfection (not shown), suggesting that STAM proteins are recruited to the early endosome by the interaction with Hrs.

*Hrs Is Required for the Stability of STAM Proteins*—We next examined the expression of endogenous STAM proteins in Hrs-depleted cells. The lysates of mock- and Hrs siRNA-transfected cells shown in Fig. 4A were immunoblotted with anti-STAM1 and anti-STAM2 antibodies to compare the relative amounts of these proteins. As shown in Fig. 5A, the amounts of both STAM1 and STAM2 were drastically reduced in the Hrs-depleted cells. Immunofluorescence staining with anti-STAM1 and anti-STAM2 antibodies also resulted in undetectable signals in more than 50% of the siRNA-transfected cells (not shown), confirming the results obtained by immunoblotting.

To examine whether the expression of the Hrs siRNA affects the mRNA levels of STAM1 and STAM2, we compared the levels using RT-PCR. As expected, the Hrs mRNA was significantly reduced in Hrs siRNA-transfected cells (Fig. 5B). In contrast, the mRNA levels of STAM1 and STAM2, as well as that of GAPDH used as an internal control, were unchanged between mock- and the siRNA-transfected cells (Fig. 5B). These results suggest that STAM proteins can not exist stably without forming a complex with Hrs.

*The CC Region Is Required for the Aberration of Endosomal Morphogy and Function Caused by STAM2 Overexpression*—Overexpression of STAM proteins causes the enlargement of the early endosome and the accumulation of ubiquitinated proteins on this aberrant organelle (*18*). To test whether the association with Hrs is required for STAM function, we examined the effect of overexpressing STAM2ΔCC and STAM2ΔSSM on these phenotypes.

To examine their effect on the morphology of the early endosome, we transfected FLAG-tagged full-length STAM2, STAM2ΔCC, or STAM2ΔSSM into COS-7 cells, and double-stained the cells with anti-FLAG and anti-Hrs antibodies. As shown in Fig. 6, A and C, STAM2ΔSSM was as effective as the full-length protein in causing the enlargement of Hrs-positive early endosomes. STAM2ΔCC, in contrast, did not affect the morphology (not shown) or produced only a slight enlargement of the early endosome (Fig. 6B). To semi-quantify the effect, approximately 100 cells that were strongly positive for anti-FLAG staining were randomly chosen under the fluorescence microscope, and the cells exhibiting enlarged early endosomes among the FLAG-positive cells were counted. As shown in Fig. 6G, the proportion of STAM2ΔCC-overexpressing cells exhibiting slightly enlarged endosomes was approximately one-third that of full-length STAM2- and STAM2ΔSSM-overexpressing cells that showed enlarged endosomes.

Next, to examine their effect on the accumulation of ubiquitinated proteins, COS-7 cells transfected with the FLAG-tagged STAM2 constructs were double-stained with anti-FLAG antibody and FK2, an antibody that recognizes the ubiquitin moiety of ubiquitinated proteins (*35*). Whereas bright FK2 staining of the early endosome was observed in cells overexpressing STAM2ΔSSM as well as the full-length protein (Fig. 6, D and F), it was rarely found in STAM2ΔCC-overexpressing cells (Fig. 6E). Semi-quantification of the effect by counting the cells exhibiting FK2-positive early endosomes among all FLAG-positive cells showed that the effect of STAM2ΔCC was also approximately one-third that of the full-length protein in this assay (Fig. 6H). STAM2ΔSSM was almost as effective as the full-length protein. To confirm this quantification further, COS-7 cells were transfected with FLAG-tagged ubiquitin together with HA-tagged full-length STAM2, STAM2ΔCC, or STAM2ΔSSM, and intracellular ubiquitinated proteins were detected by immunoprecipitation and subsequent immunoblotting of their

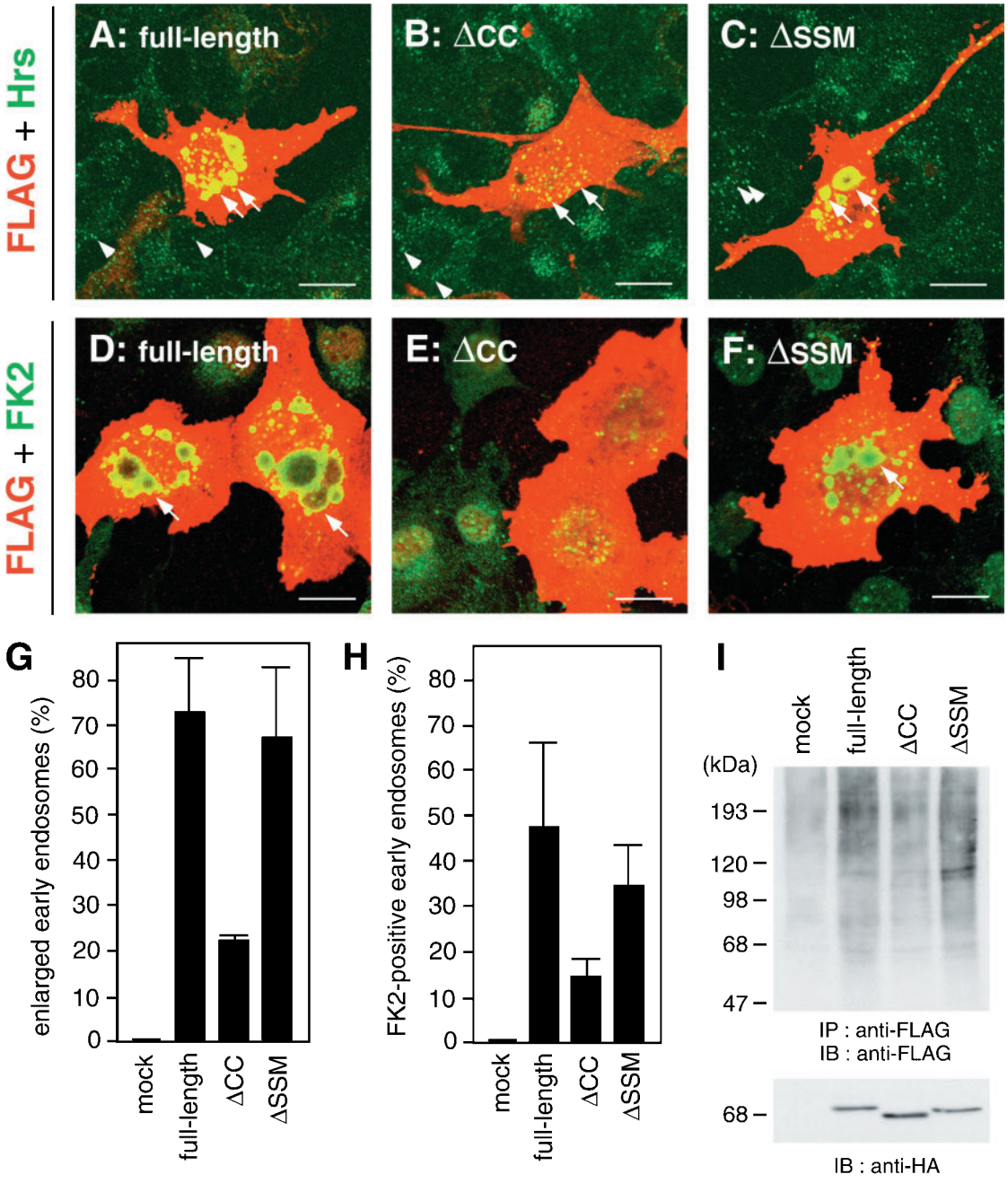


Fig. 6. **Effect of STAM2ΔCC and STAM2ΔSSM on the morphology and function of the early endosome.** (A–F) COS-7 cells were transfected with FLAG-tagged full-length STAM2 (A and D), STAM2ΔCC (B and E), or STAM2ΔSSM (C and F), and immunostained with anti-FLAG antibody (red) together with anti-Hrs (A–C) or FK2 (D–F) antibody (green). Arrows indicate Hrs- or FK2-positive early endosomes in cells overexpressing the STAM2 constructs. Arrowheads in A–C indicate normal early endosomes in untransfected cells. Bars, 20 μm. (G, H) Proportion of cells exhibiting Hrs-positive (G) and FK2-positive (H) enlarged early endosomes among those overexpressing the indicated STAM2 constructs. Mean ± SD of three independent experiments are shown. (I) COS-7 cells were co-transfected with FLAG-tagged ubiquitin and the indicated HA-tagged STAM2 constructs. Ubiquitinated proteins in the cells were detected by immunoprecipitation (IP) and subsequent immunoblotting (IB) of their lysates with anti-FLAG antibody (upper panel). Expression of each STAM2 construct was assessed by immunoblotting of the total cell lysates with anti-HA antidody (lower panel).

lysates with anti-FLAG antibody. Whereas the overexpression of STAM2ΔSSM increased the amount of ubiquitinated proteins to a level similar to that observed with the full-length protein, STAM2ΔCC only slightly increased the level compared with mock-transfected cells (Fig. 6I). Taken together, these results suggest that the association with Hrs on the early endosome is prerequisite for STAM function. As far as assessed by these criteria, however, the residual Hrs binding by STAM2ΔSSM appeared to be nearly sufficient for the function.

*The CC Region and SSM Are Required for the Inhibition of Ligand-Activated EGFR Degradation Caused by STAM2 Overexpression*—Overexpression of STAM2 (*18*) as well as Hrs (*15*, *36*, *37*) has been shown to inhibit the

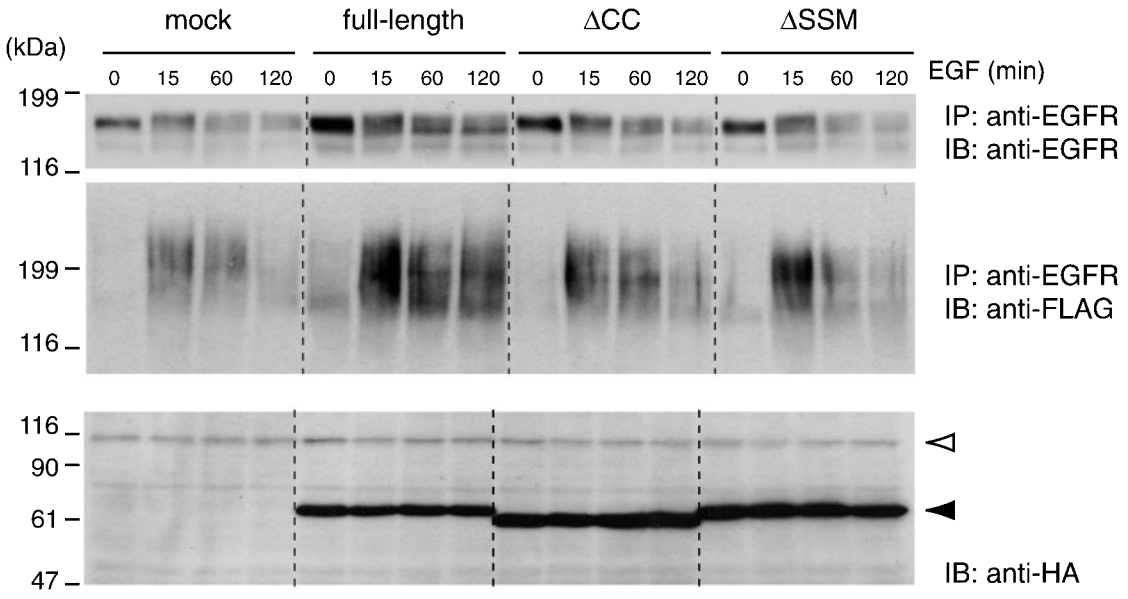


Fig. 7. **Effect of STAM2ΔCC and STAM2ΔSSM on the down-regulation of EGFR.** COS-7 cells were transfected with the indicated HA-tagged STAM2 constructs together with EGFR, FLAG-ubiquitin, and HA-tagged c-Cbl. The cells were serum-starved, stimulated with EGF for the indicated periods, and their lysates were immunoprecipitated (IP) with anti-EGFR. The immunoprecipitates were immunoblotted (IB) with anti-EGFR (upper panel) or anti-FLAG (middle panel) antibody. The expression levels of the STAM2 constructs and c-Cbl were assessed by immunoblotting of the total cell lysates with anti-HA antibody (lower panel). Closed and open triangles indicate the STAM2 constructs and c-Cbl, respectively.

lysosomal trafficking and degradation of ligand-activated growth factor receptors. To test whether the association with Hrs is required for this inhibitory effect of STAM, we examined the effect of STAM2ΔCC and STAM2ΔSSM on the degradation of activated and ubiquitinated EGFR. COS-7 cells were transfected with the HA-tagged STAM2 constructs together with EGFR, FLAG-tagged ubiquitin, and HA-tagged c-Cbl. After serum starvation, the cells were stimulated with EGF for 15, 60, or 120 min, and EGFR was immunoprecipitated with anti-EGFR. The amount of EGFR and its ubiquitination level were examined by immunoblotting of the immunoprecipitates with anti-EGFR and anti-FLAG antibodies, respectively. In mock-transfected cells, EGFR was ubiquitinated by 15 min of EGF stimulation and largely degraded by 120 min (Fig. 7, upper and middle panels). In cells overexpressing full-length STAM2, however, a considerable amount of ubiquitinated EGFR was still present even after 120 min of stimulation (Fig. 7, upper and middle panels). In contrast, the time course of EGFR degradation in cells overexpressing STAMΔCC or STAMΔSSM was similar to that in mock-transfected cells (Fig. 7, upper and middle panels). As assessed by immunoblotting of the total cell lysates with anti-HA antibody, the expression levels of the STAM2 constructs and c-Cbl were similar in each transfectant (Fig. 7, lower panel). These results suggest that Hrs binding is required for the STAM function in lysosomal trafficking of ligand-activated EGFR.

## DISCUSSION

*Subcellular Localization of Hrs and STAM*—Are Hrs and STAM resident endosomal proteins or do they shuttle between the organelle and cytoplasm? These proteins belong to the class E Vps category of proteins. The class E Vps proteins, originally identified as those that are required for membrane trafficking via the endosome in yeast (*38*), play essential roles in the sorting of ubiquitinated cargo proteins for trafficking to the lysosome (*3*). A subset of class E Vps proteins form three complexes, ESCRT-I, -II, and -III, on the early endosomal membrane (*3*). It has been shown that the ESCRT complexes shuttle between the endosomal membrane and cytoplasm, and their dissociation from the endosome requires the ATP-hydrolyzing activity of Vps4, an AAA-type ATPase that also belongs to the class E Vps category (*39–41*). An ATPase-defective mutant of SKD1/mVps4, a mammalian ortholog of Vps4, also acts dominant-negatively to inhibit the dissociation of class E Vps protein orthologs, TSG101 (mVps23) and mVps28, from the endosomal membrane in mammalian cells (*42*).

We and others have shown by subcellular fractionation that substantial amounts of Hrs and STAM2 are recovered in the soluble cytoplasmic fraction (*6*, *11*, *30*). These observations raise two possibilities. One is that a cytoplasmic pool of these proteins exists. The other is that they are dissociated from the membrane during the fractionation procedures due to weak or impaired membrane-binding affinity after cell homogenization. Recently, Bache *et al.* reported that immunofluorescence staining with anti-Hrs and anti-STAM2 antibodies produces a speckeled cytoplasmic pattern that can be removed by permeabilizing cells before fixation (*11*). They further showed that Hrs and STAM2 are accumulated on an aberrant endosomal compartment in cells overexpressing the SKD1/mVps4 mutant lacking ATPase activity, suggesting that Hrs and STAM are mostly localized in the cytoplasm and their membrane association is also regulated by SKD1/mVps4 (*11*). Although the cell lines examined differ between their study (BHK cells) and ours

(HeLa cells), our results obtained by immunofluorescence staining are contradictory to this model. In the present study, we demonstrate that the localization of endogenous STAM1 and STAM2 is restricted to the early endosome (Fig. 1). The localization of transfected STAM1 and STAM2 is also restricted to the Hrs-positive early endosome when they are expressed at a low level (*18*). A STAM2 mutant lacking the CC region, in contrast, is completely cytoplasmic (Fig. 3), excluding the possibility that cytoplasmic proteins are not properly fixed and washed away during the staining procedures under our experimental conditions. In addition, the localization of endogenous as well as transfected Hrs is also restricted to the early endosome (Figs. 1, 3; *30*). These results suggest that the majority of STAM molecules exist in complex with Hrs on the early endosomal membrane. A recent report pointed out that PI(3)-P is quickly degraded following homogenization of yeast cells (*22*). As PI(3)-P plays an essential role in recruiting Hrs to the endosome (*27–29*), it is likely that the recovery of Hrs and STAM in the soluble fraction after cell fractionation is due to the degradation of PI(3)-P and the following dissociation of the Hrs/STAM complex from the endosomal membrane. We, therefore, propose that Hrs and STAM are resident early endosomal proteins. The increase in the amounts of Hrs and STAM2 on the endosome in cells overexpressing the dominant-negative form of SKD1/mVps4 (*11*) might be a secondary effect due to an aberration in endosomal function and morphology, as in these cells, impaired membrane trafficking causes the accumulation of a number of proteins, including the transferrin receptor, low-density lipoprotein receptor, Lamp1, and TGN38, on this organelle (*43–45*).

*Mechanisms of the Early Endosomal Localization of STAM*—STAM2 mutants lacking Hrs-binding activity were not correctly localized to the early endosome (Fig. 3). Depletion of endogenous Hrs also caused a mislocalization of transfected STAM2 to the cytoplasm (Fig. 4; *11*). From these results, we conclude that STAM proteins are recruited to the early endosomal membrane through direct interaction with Hrs. It was previously shown that overexpressed STAM proteins leak into the cytoplasm (*18*) whereas overexpressed Hrs can still be localized on the early endosome (*30*). Our conclusions are consistent with these previous observations as they support the idea that the localization of transfected STAM depends on the limited amount of endogenous Hrs, whereas that of transfected Hrs is regulated by STAM-independent mechanisms.

What are the mechanisms by which STAM binds to Hrs? Co-immunoprecipitation experiments have shown that STAM mutants lacking the CC region do not bind Hrs (Fig. 2; *5*, *6*). In a yeast two-hybrid assay, the CC region of STAM2 alone interacts with Hrs (*6*). In this study, however, we demonstrate by co-immunoprecipitation and immunofluorescence staining that the CC region is insufficient for the stable Hrs binding and early endosomal localization of STAM2 (Figs. 2, 3). We further demonstrate that the SSM, a ~25-amino-acid region located N-terminal to the CC region and conserved among STAM proteins in higher eukaryotes, is also required for these activities. The molecular mechanism by which the SSM regulates these activities is unknown. The sequence within this motif is not homologous to that of any proteins other than STAM in databases. It is possible that the SSM interacts directly with a specific region of Hrs. However, STAM2ΔCC, which contains the SSM, does not bind Hrs (Fig. 2; *5*, *6*). In addition, the Hrs-binding activity and early endosomal localization of STAM2 were not completely abolished by deleting the SSM (Figs. 2, 3). Therefore, it is also possible that the SSM regulates binding indirectly by influencing the function of the CC region. For example, the SSM might play a role in maintaining the conformation of STAM so that the CC region gains access to Hrs.

*STAM Functions as A Complex with Hrs*—The expression levels of endogenous STAM1 and STAM2 were drastically reduced in cells in which Hrs was depleted by RNAi (Fig. 5). This could be due to reduced transcription or stability of the mRNAs, or reduced translation or stability of the proteins. RT-PCR experiments showed that the mRNA levels of STAM1 and STAM2 are unchanged by RNAi treatment (Fig. 5), excluding the possibility that the expression or stability of the mRNAs is affected. It is also unlikely that the absence of Hrs affects the translation machinery of STAM proteins from their mRNAs. Therefore, these results suggest that STAM proteins are destabilized and quickly degraded in the absence of Hrs. Forming a complex with Hrs may increase their stability. Otherwise, localization on the early endosomal membrane stabilizes STAM proteins and they are degraded when solubilized in the cytoplasm. In either case, the instability of STAM proteins in the absence of Hrs suggests that the majority of STAM proteins exist in complex with Hrs and they do not have any function independent of Hrs. Compared to endogenous STAM proteins, transfected STAM2 constructs were detected in the cytoplasm of Hrs-depleted cells (Fig. 4). STAM2ΔCC, which completely lacks Hrs-binding activity, was also detected in the cytoplasm of normal cells (Fig. 3). The reason why these transiently transfected STAM constructs were still detectable without forming a complex with Hrs is probably because their synthesis from the expression vectors overcomes the cellular protein degradation machinery.

Both Hrs and STAM bind ubiquitin, and the complex of these proteins is believed to be a sorting receptor for ubiquitinated cargo proteins for trafficking to the lysosome. In this study, we demonstrate that the overexpression of STAM2ΔCC, which lacks Hrs-binding activity, fails to cause enlargement of the early endosome, an accumulation of ubiquitinated proteins on this organelle, and inhibition of the degradation of ligand-activated EGFR (Figs. 6, 7). These results suggest that forming a complex is essential for these proteins to serve as a sorting receptor, although we can not completely exclude the possibility that the CC region plays other roles (*e.g.* interaction with other molecules) that are also essential for STAM function. In contrast, the effects of STAM2ΔSSM, which retains weak Hrs-binding activity, varied among the assays. Although it was nearly as effective as the full-length protein in accumulating FK2-positive ubiquitinated proteins on aberrant endosomes (Fig. 6), it failed to inhibit EGFR degradation (Fig. 7). At present, the reason for these variable effects of STAM2ΔSSM is unclear.

Very recently, it was also shown in yeast that an Hse1 mutant lacking Vps27-binding activity does not comple-

ment the endosomal sorting defect caused by *hse1* gene deletion, indicating that association with Vps27 is required for Hse1 function (*46*). The idea that Hrs and STAM proteins play roles in a single protein complex is also supported by the fact that the loss-of-function of these proteins results in the same phenotype not only in yeast (the class E *vps* phenotype; *8*) but also in mammals. Both Hrs-knockout mice (*28*) and STAM1- and STAM2-double knockout mice (*47*) die during embryogenesis around embryonic day 10–11 due to a defect in ventral folding morphogenesis. It has been shown that another ubiquitin-binding UIM-containing protein, Eps15, is also associated with Hrs/STAM complexes on the early endosome (*11*, *48*, *49*). Therefore, to understand the sorting mechanisms of ubiquitinated cargos at the endosome, further study is necessary to elucidate how the interaction of each component (Hrs, STAM, and Eps15) with ubiquitinated proteins is coordinated within this sorting complex.

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