Molecular Biology of the Cell
Vol. 15, 4337–4346, September 2004

The Growth-Regulatory Protein HCRP1/hVps37A Is a Subunit of Mammalian ESCRT-I and Mediates Receptor Down-Regulation

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Submitted March 24, 2004; Revised June 7, 2004; Accepted June 23, 2004
Monitoring Editor: Jean Gruenberg

The biogenesis of multivesicular bodies and endosomal sorting of membrane cargo are driven forward by the *e*ndosomal *s*orting *c*omplexes *r*equired for *t*ransport, ESCRT-I, -II, and -III. ESCRT-I is characterized in yeast as a complex consisting of Vps23, Vps28, and Vps37. Whereas mammalian homologues of Vps23 and Vps28 (named Tsg101 and hVps28, respectively) have been identified and characterized, a mammalian counterpart of Vps37 has not yet been identified. Here, we show that a regulator of proliferation, *h*epatocellular *c*arcinoma *r*elated *p*rotein *1* (HCRP1), interacts with Tsg101, hVps28, and their upstream regulator Hrs. The ability of HCRP1 (which we assign the alternative name hVps37A) to interact with Tsg101 is conferred by its mod(r) domain and is shared with hVps37B and hVps37C, two other mod(r) domain-containing proteins. HCRP1 cofractionates with Tsg101 and hVps28 by size exclusion chromatography and colocalizes with hVps28 on LAMP1-positive endosomes. Whereas depletion of Tsg101 by siRNA reduces cellular levels of both hVps28 and HCRP1, depletion of HCRP1 has no effect on Tsg101 or hVps28. Nevertheless, HCRP1 depletion strongly retards epidermal growth factor (EGF) receptor degradation. Together, these results indicate that HCRP1 is a subunit of mammalian ESCRT-I and that its function is essential for lysosomal sorting of EGF receptors.

INTRODUCTION

Growth factor receptors play crucial roles in cellular proliferation, survival, migration, and differentiation. Proper down-regulation of mitogenic receptors is thought to be important to control their cell surface expression and thereby the transduction of signals delivered in response to growth factors (Waterman and Yarden, 2001). This process requires agonist stimulation, internalization into endosomes, and degradation in lysosomes. The receptors are sorted within the endosomal system and relocalized to the internal vesicles of late endosomes or multivesicular bodies (MVBs), which then fuse with lysosomes. Intraluminal vesicles are then degraded by lipases, and associated proteins are degraded by proteases (Mullins and Bonifacino, 2001; Katzmann *et al*., 2002). The molecular mechanism for selection of receptors destined for down-regulation seems to be conserved through evolution from yeast to mammals. It involves conjugation of ubiquitin onto the receptor (Dupre *et al*., 2001; Hicke, 2001), and retention of the receptor from the recycling pathway by ubiquitin-binding proteins localized on sorting endosomes, such as yeast Vps27 and its mammalian homologue *h*epatocyte growth factor *r*egulated tyrosine kinase *s*ubstrate Hrs (Bilodeau *et al*., 2002; Bishop *et al*., 2002; Raiborg *et al*., 2002; Shih *et al*., 2002). In addition, Vps27 and Hrs function as docking sites for the endosomal sorting complex required for transport (ESCRT)-I (Bache *et al*., 2003; Katzmann *et al*., 2003). These events initiate the MVB sorting reaction at the membrane of the endosome.

In a yeast genetic screen for vacuolar protein sorting (*vps*) mutants, the so-called class E *vps* mutants, and the ESCRT-I, -II, and -III complexes, which function sequentially in protein sorting and MVB formation, were identified (Katzmann *et al*., 2001; Babst *et al*., 2002a,b). ESCRT-I, a 350-kDa protein complex consisting of the class E Vps proteins Vps23, Vps28, and Vps37, is required for binding and sorting of ubiquitinated MVB cargoes, an interaction that seems to occur via the Vps23 subunit (Katzmann *et al*., 2001). The putative mammalian Vps23 homologue tumor susceptibility gene 101 (Tsg101) (Babst *et al*., 2000) binds ubiquitin and is required for the MVB pathway (Bishop and Woodman, 2001; Bishop *et al*., 2002; Pornillos *et al*., 2002). The human Vps28 homologue hVps28 (Bishop and Woodman, 2001) also seems to play a role in receptor down-regulation (Bishop *et al*., 2002). To date, no mammalian homologue of Vps37 is known. Deletion of Vps37 results in a class E Vps phenotype, but analyzes of the Vps37 primary sequence failed to identify any motifs that offered insight into the function of Vps37 in yeast (Katzmann *et al*., 2001).

In the present work, we have identified a human counterpart of Vps37, which we term hVps37A. We demonstrate that it interacts with Tsg101 and hVps28 and that it is

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04–03–0250. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04–03–0250.

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Abbreviations used: EGF, epidermal growth factor; ESCRT, endosomal sorting complex required for transport; HCRP1, hepatocellular carcinoma related protein 1; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; MBP, maltose-binding protein; mod(r), modifier of rudimentary; MVB, multivesicular body; siRNA, small interfering RNA; Tsg101, tumor susceptibility gene 101; UEV, ubiquitin E2 variant sequence; vps, vacuolar protein sorting.

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important for efficient degradation of the EGF receptor. Interestingly, this protein is identical to a protein called HCRP1, encoded by a novel gene found to be important for growth inhibition in hepatocellular carcinoma cells (Xu *et al*., 2003). This indicates that HCRP1/hVps37A functions as a subunit of the mammalian ESCRT-I complex and provides a new molecular link between endocytosis, receptor down-regulation, and proliferation/cancer.

MATERIALS AND METHODS

Database Searches

The putative human Vps class E protein hVps37A was identified in BLASTp searches (www.ncbi.nlm.nih.govast) by using the yeast Vps37/Srn2 class E protein (NC_001144) as starting point. BLOSUM62 searches identified a hypothetical protein in rat (XP_214353) as the closest possible Vps37 homologue in mammals (score 32). We found this protein to be highly similar to the recently identified human HCRP1 (AAK54349, score 563). Based on the presence of a mod(r) domain in HCRP1/hVps37A (PFAM protein search, www.sanger.ac.uk/Software/Pfam/index.shtml), we identified three other human protein sequences in the database: FLJ20847 (hereafter named *hVps37C*), FLJ12750 (hVps37B), and Wbscr24 (*W*illiam-*B*euren *S*yndrome *c*ritical *r*egion *24*, *hVps37D*). A partial coding sequence of FLJ20847, called CAD38936 (containing residues 149–523 of FLJ20847), was used in the cloning procedure.

Plasmid Constructs and Small Interfering RNA (siRNA) Oligonucleotides

The DNAs encoding human Tsg101 (Li and Cohen, 1996), hVps28 (Bishop and Woodman, 2001), and HCRP1 (Xu *et al*., 2003) were amplified by polymerase chain reaction (PCR) from a marathon-Ready HeLa cDNA (BD Biosciences Clontech, Palo Alto, CA) and verified by sequencing. Hrs was generated by PCR with mouse Hrs (Komada and Kitamura, 1995) as the template. For use in the two-hybrid system, constructs were cloned into pLexA/pBTM116 (Vojtek *et al*., 1993) as bait and pGAD GH (BD Biosciences Clontech) as prey. For expression as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli*, HCRP1 and hVps28 were cloned into pGEX-6P-1 (Amersham Biosciences, Pollards Wood, United Kingdom), and for expression of maltose-binding protein (MBP) fusion proteins they were cloned into pMAL-c2 (New England Biolabs, Beverly, MA). For in vitro translation, hVps28 and Tsg101 were cloned into pGEM-myc4 (Simonsen *et al*., 1998), whereas Hrs and HCRP1 were cloned behind the myc-epitope of pcDNA3-myc (Raiborg *et al*., 2001). For bacterial expression of GST-HCRP1 deletion proteins, the regions (1–148) and (208–397) were amplified by PCR and cloned into pGEX-6P-1. GST-hVps37C (146–330) and GST-hVps37B (1–168) were cloned similarly by PCR amplification of the respective mod(r)-containing regions of CAD38936 and FLJ12750. For expression in mammalian cells, hVps28 was cloned into pcDNA3 (Invitrogen, Carlsbad, CA), and HCRP1 into pEGFP-C-1 (BD Biosciences Clontech). For RNA interference against Tsg101, we used the same oligonucleotides as described previously (Bishop *et al*., 2002), and for HCRP1 we used oligonucleotides with sense sequence 5′-CAACAAGUCAUACCA-CAGCTT-3′ and antisense sequence 5′-GCUGUGGUAUGACUUGUUGTT-3′, corresponding to nucleotide 548–566 in *HCRP1*, located 5′ to the mod(r) region. A BLAST search confirmed that this sequence was unique to HCRP1. As negative control, we used scrambled sequences of the same nucleotides: sense control 5′-CAGAUACCACCAAUCAGACTT-3′ and antisense control 5′-GUCUGAUUGGUGGUAUCUGTT-3′.

Antibodies

Antibodies against a recombinant MBP-hVps28 fusion protein have been described previously (Bache *et al*., 2003). Rabbit antibodies against recombinant MBP-HCRP1 were raised and affinity purified as described previously (Raiborg *et al*., 2001). These antibodies recognized endogenous HCRP1 by Western blotting but not by immunocytochemistry of HeLa cells. Because only a single band of 45 kDa was detected by Western blotting, the antibody is presumably specific for HCRP1, unless hVPS37B-D are expressed at undetectable levels in HeLa cells. A mouse monoclonal antibody (mAb) against Tsg101 was obtained from GeneTex (San Antonio, TX). A mouse mAb against human LAMP-1 was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). A mouse mAb against α-tubulin was from Sigma-Aldrich (St. Louis, MO). Sheep antibodies against epidermal growth factor (EGF) receptor were from Fitzgerald (Concord, MA). Antibodies against Alix were prepared by immunizing a rabbit with recombinant MBP-Alix fusion protein and affinity purifying the antiserum on Affi-Gel beads (Bio-Rad, Hercules, CA) containing immobilized Alix.

Two-Hybrid Methods

The yeast reporter strain L40 (Vojtek *et al*., 1993) was cotransformed (Schiestl and Gietz, 1989) with the indicated pLexA (bait) and pGAD (prey) plasmids, and β-galactosidase activities of transformants were determined as described previously (Guarente, 1983).

Expression of MBP and GST Fusion Proteins in E. coli

MBP-fusion proteins of HCRP1 and hVps28 were produced in *E. coli* BL21 (DE3) cells transformed with the respective pMAL constructs. GST fusion proteins were produced in *E. coli* BL21 (DE3) cells transformed with the respective pGEX constructs. The recombinant MBP fusion proteins were purified on amylose resin (New England Biolabs), and the GST fusion proteins were purified on glutathione-Sepharose (Amersham Biosciences), after lysis of the bacteria in B-PER reagent (Pierce Chemical, Rockford, IL) according to the instructions from the manufacturers.

In Vitro Transcription and Translation

pGEM-Tsg101 and pGEM-hVps28 were linearized with *Sal*I and pcDNA3-HCRP1 with *Xba*I. They were in vitro transcribed with T7 RNA polymerase (Promega, Southampton, United Kingdom) according to the protocol from the manufacturer. The resulting mRNA was translated in the presence of [^{35}S]methionine (Amersham Biosciences) in rabbit reticulocyte lysate (Promega) according to the instructions from the manufacturer. The lysate was dialyzed overnight against assay buffer (20 mM HEPES, pH 7.2, 140 mM NaCl, 1 mM dithiothreitol).

GST Pull-Down Assay

Lysates from transformed *E. coli* BL21 (DE3) cells containing GST or GST fusion protein (2 μg) were bound in Tris buffer (20 mM Tris, 60 mM NaCl, pH 7.5, 0.01% IGEPAL) to 20-μl aliquots of glutathione-Sepharose (Amersham Biosciences) at 4°C for 60 min. The beads were then washed with assay buffer, and 50 μl of in vitro translated Tsg101, hVps28, HCRP1, or Hrs was added with assay buffer containing 0.01% IGEPAL. After rotation at 4°C for 60 min, the beads were washed three times with assay buffer and then analyzed by SDS-PAGE and fluorography. Films were scanned with a ScanJet 6100C (Hewlett Packard, Palo Alto, CA), and images were processed with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Cell Culture and Transfection

HeLa cell cultures were maintained as recommended by American Type Culture Collection (Manassas, VA). For expression in mammalian cells, we used the FuGENE system according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Cells were analyzed 24 h after transfection. Transfections of HeLa cells with siRNA against Tsg101 and HCRP1 were performed as described previously (Elbashir *et al*., 2001). The cells were first transfected with siRNA for 3 d, and then the cells were replated and left for another 3 d before analysis.

Confocal Fluorescence Microscopy

Transfected or nontransfected HeLa cells grown on coverslips were permeabilized with 0.05% saponin, fixed with 3% paraformaldehyde, and stained for fluorescence microscopy as described previously (Simonsen *et al*., 1998). Coverslips were examined using an LSM 510 META microscope (Carl Zeiss, Jena, Germany) equipped with a Neo-Fluar 100×/1.45 oil immersion objective. Image processing was done with Adobe Photoshop, version 7.0.

Preparation of Membrane and Cytosolic Fractions of HeLa Cells Treated with siRNA against Tsg101 and HCRP1

HeLa cells were transfected with siRNAs against Tsg101 (Bishop *et al*., 2002) or HCRP1 (for sequences, see "Plasmid Constructs and siRNA Oligonucleotides" under *Materials and Methods*), or scrambled RNA duplexes of the same nucleotides as control. The cells were washed three times with ice-cold phosphate-buffered saline, and homogenized in homogenization buffer (10 mM HEPES, 3 mM imidazole, pH 7.2, 250 mM sucrose, mammalian protease inhibitor cocktail; Sigma-Aldrich) by repeated passages through a 22-gauge needle at 4°C. Membrane particulate and cytosolic fractions were prepared from postnuclear supernatants by ultracentrifugation for 20 min at 65,000 rpm in a TLA-100 rotor, using a TableTop ultracentrifuge (Beckman Coulter, Fullerton, CA). The fractions were analyzed by SDS-PAGE and immunoblotted using rabbit antibodies against HCRP1 and hVps28, and mouse antibodies against Tsg101.

Size Exclusion Chromatography

HeLa cells treated with siRNA against HCRP1 or not were lysed for 15 min in lysis buffer (125 mM KAc, 25 mM HEPES, 2.5 mM MgAc, 5 mM EGTA, 0.25% NP-40, 1 mM dithiothreitol, pH 7.2). Lysate from 20×10^6 cells was cleared on a 0.22-μm ultrafree-MC centrifugal filter device (Millipore, Billerica, MA) and applied to a Superdex 200 column connected to an ÄKTA Explorer system (Amersham Biosciences). Samples and standards were run according to the manufacturer's instructions (Amersham Biosciences). Eighteen fractions of 1 ml were collected, protein was precipitated with 5% trichloroacetic acid, and

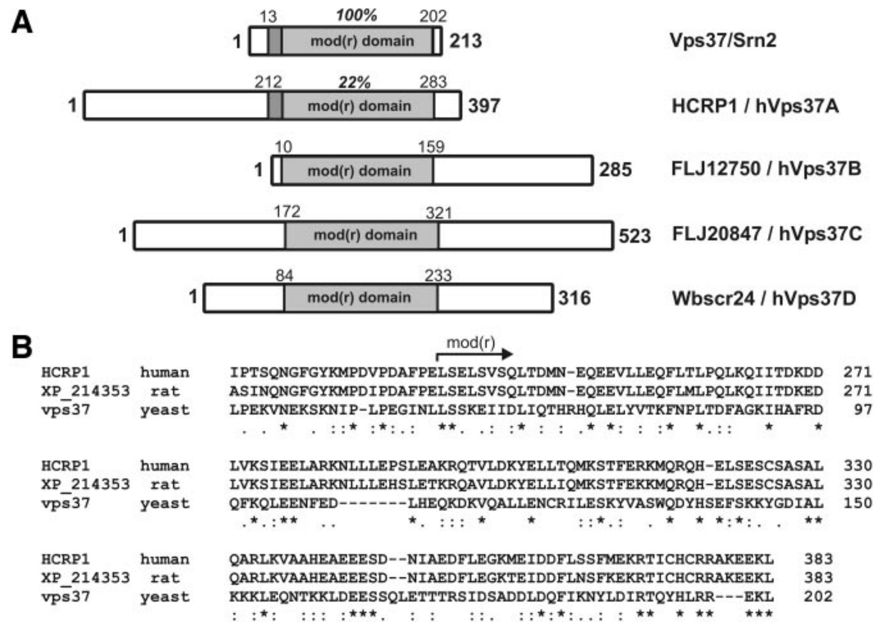

Figure 1. HCRP1 is a possible mammalian counterpart of yeast Vps37. (A) Domain structure of Vps37 (NP_013220) and HCRP1 (AAK54349) is shown. The regions that are most conserved between these proteins are shaded, with the calculated sequence identity indicated. These conserved regions contain a modifier of rudimentary [mod(r)] domain (pfam: PF07200) (light gray), which is found in several eukaryotic proteins. These include three additional human protein products, FLJ20847 (hVps37C), FLJ12750 (hVps37B), and Wbscr24 (hVps37D), which are shown in the figure. The mod(r) domain of CAD38936 (the partial sequence of FLJ20847) includes the residues (26–175) (see *Materials and Methods*). (B) Alignment of the conserved region between Vps37 and HCRP1 is shown. The rat counterpart (XP_214353) of HCRP1 also is included. The starting point of the mod(r) domain is indicated.

analyzed by SDS-PAGE and subsequent immunoblotting by using antibodies against HCRP1, hVps28, and Tsg101.

Down-Regulation of EGF Receptor in Cells Depleted of HCRP1

HeLa cells were transfected with siRNAs against HCRP1 or a scrambled RNA duplex of the same nucleotides as control After 48 h, the cells were replated into 3 × 5-cm dishes with siRNA-treated cells, and 3 × 5-cm dishes with mock-treated cells, and left for another 48 h. The medium was then supplemented with 100 ng/ml EGF and 10 μg/ml cycloheximide for 0, 3, and 6 h before the cells were lysed in lysis buffer. The lysates were analyzed by SDS-PAGE and subsequent immunoblotting by using antibodies against the EGF receptor HCRP1 and tubulin to verify equal loadings. The bands were quantified using ImageQuant 5.0 (Amersham Biosciences).

RESULTS

HCRP1: A Possible Mammalian Counterpart of Vps37

Many of the human homologues of yeast Vps class E proteins have now been identified. In the ESCRT-I complex, mammalian counterparts of Vps23 and Vps28 (named Tsg101 and hVps28, respectively) have been identified and characterized. However, a mammalian counterpart of Vps37 has not yet been recognized. To identify possible human Vps37 proteins, a BLASTp search (www.ncbi.nlm.nih.gov/blast) with the yeast Vps37/Srn2 sequence was performed. The search yielded an unknown protein product, recently identified as HCRP1 (Xu *et al.*, 2003), as a possible human Vps37 candidate (for details, see *Materials and Methods*). HCRP1 has 22% sequence identity (41% sequence similarity) with yeast Vps37 in the C-terminal part (Figure 1, A and B), and in addition, it shows 18% identity with Tsg101 throughout the sequence (Xu *et al.*, 2003). We also found possible HCRP1 counterparts in other mammals. Furthermore, database searches indicated that the conserved region in yeast and HCRP1 contains a modifier of rudimentary [mod(r)] domain (Begley *et al.*, 1995). Thus far, this domain has unknown function, but it is conserved in several eukaryotic proteins, including three hypothetical human protein products. Based on the limited sequence similarity between HCRP1 and Vps37, it was not possible to predict whether the two proteins share similar functions, but the similarity was sufficient that we were encouraged to investigate this topic further.

HCRP1 Colocalizes with hVps28 on LAMP1-positive Late Endosomes

To address whether HCRP1 is part of the ESCRT-I complex, we first asked whether this protein, like hVps28 and Tsg101 (Bishop and Woodman, 2001; Bache *et al.*, 2003; Katzmann *et al.*, 2003), localizes to endosomes. Exogenously expressed HCRP1 has previously been reported to predominantly localize to the nuclei in human hepatocellular carcinoma (SMMC-7721) and fetal kidney cells (293T) (Xu *et al.*, 2003), but the possible membrane localization of this protein has not been studied in detail. Therefore, to facilitate visualization of membrane-bound HCRP1 in HeLa cells, we permeabilized the cells before fixation. Initially, we expressed green fluorescent protein (GFP)-tagged HCRP1 alone. Although we could indeed detect some GFP-HCRP1 within the nucleus, we also noticed extensive aggresome formation (unpublished data). Reasoning that the solubilization and localization of HCRP1 might depend on its association with other ESCRT-I subunits, we expressed GFP-HCRP1 and hVps28 together in HeLa cells and double labeled for confocal immunofluorescence microscopy with antibodies against hVps28 and the late endosome/lysosome marker LAMP1. Under these conditions, we observed extensive colocalization between HCRP1 and hVps28 (Figure 2D). Although we could not rule out the possibility that some of these structures might represent aggresomes, a number of the double-positive structures also colocalized with LAMP1, which generally does not colocalize with aggresomes (Figure 2, C and F; unpublished data). Likewise, we also could observe a partial colocalization between HCRP1-hVps28 and another late-endosome marker, CD63 (unpublished data). We conclude that HCRP1 and hVps28 colocalize on LAMP1-positive late endosomes and that the presence of hVps28 augments the targeting of HCRP1 to these organelles.

HCRP1 Interacts with Tsg101, hVps28, and Hrs

If HCRP1 is part of ESCRT-I, we expected to find interactions with other subcomponents of the complex. For a bio-

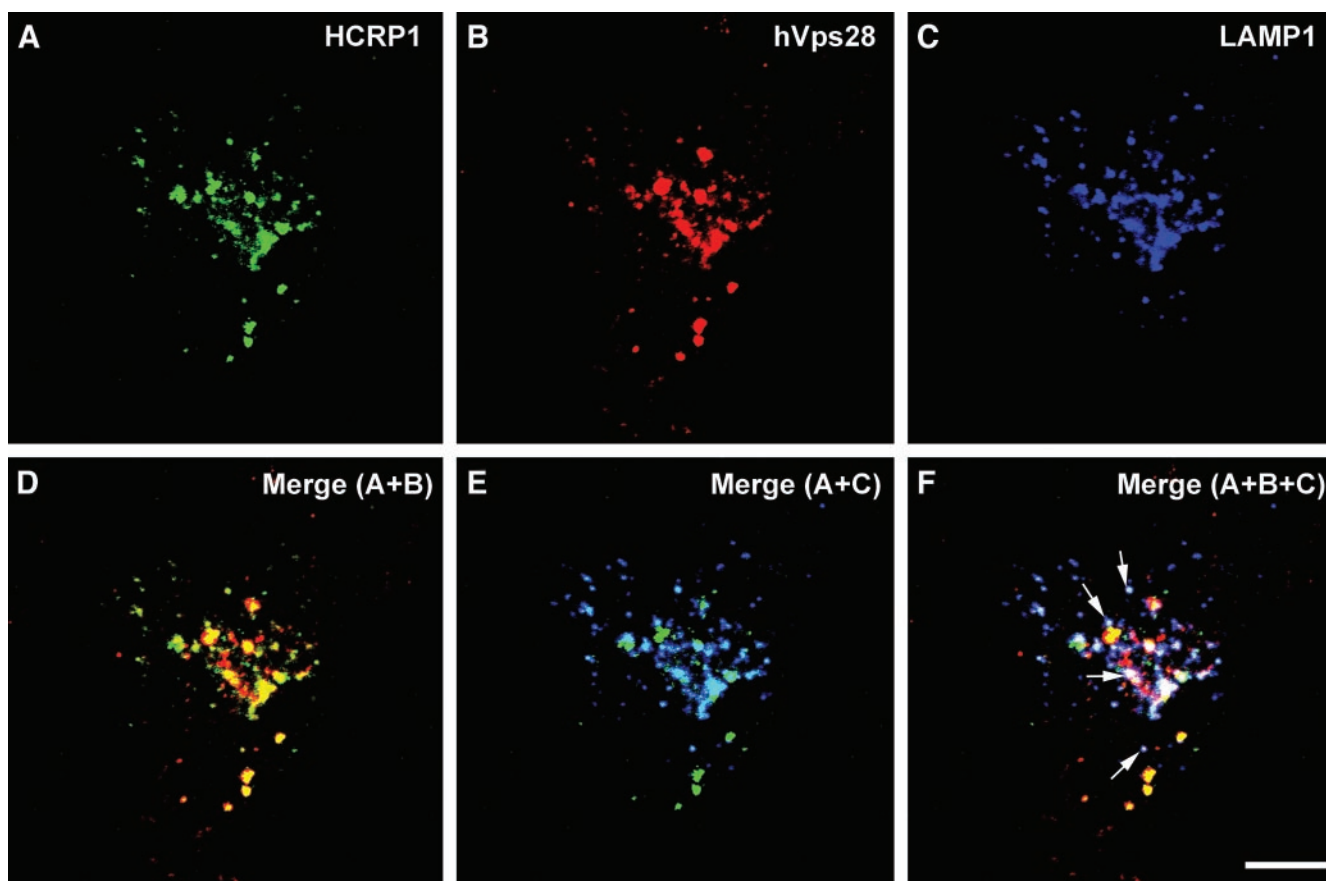

Figure 2. HCRP1 and hVps28 colocalize on LAMP-1–containing endosomes. HeLa cells were cotransfected with hVps28 and GFP-HCRP1 for 24 h and permeabilized before fixation. They were labeled with anti-hVps28 (B) and anti-LAMP-1 (C). Colocalization between HCRP1 and hVps28 is shown in yellow (D), between LAMP-1 and HCRP1 in turquoise (E), and between all three molecules in white (F). Examples of profiles positive for all three molecules are indicated by arrows. Bar, 5 μm.

chemical investigation of this, we prepared GST fusion proteins of HCRP1 and hVps28. The proteins were immobilized onto glutathione-Sepharose beads and incubated with in vitro translated ^{35}S-labeled proteins or a HeLa cell lysate. We found that GST-HCRP1 was able to pull down both Tsg101 and hVps28 from cell lysate (Figure 3A). Endogenous hVps28 also coimmunoprecipitated with endogenous HCRP1 from cell lysate (Figure 3B). Moreover, GST-hVps28 interacted with in vitro translated HCRP1 (Figure 3C), and GST-HCRP pulled down in vitro-translated Tsg101 (Figure 3D). These results show that HCRP1 interacts with hVps28 and Tsg101 in vitro and that the interaction between HCRP1 and hVps28 can be confirmed in vivo. We were not able to investigate coimmunoprecipitation between HCRP1 and Tsg101 because they comigrate with the immunoglobulin heavy chain in SDS-PAGE. However, the interaction between HCRP1 and hVps28 was further confirmed in the yeast two-hybrid system (Figure 3E).

The fact that Hrs recruits ESCRT-I to endosomes via a transient interaction with Tsg101 (Bache *et al*., 2003; Lu *et al*., 2003; Pornillos *et al*., 2003) prompted us to ask whether Hrs also would interact with HCRP1. We observed a weak interaction between immobilized GST-HCRP1 and in vitro-translated Hrs (Figure 3D). This interaction also was shown in the yeast two-hybrid system (Figure 3E). Due to strong reporter transactivation of Tsg101 and HCRP1 LexA fusions, we were not able to investigate their interaction in the two-hybrid system. However, as judged from the GST pull-down experiments, HCRP1 seems to interact much stronger with Tsg101 than with hVps28.

HCRP1/hVps37A, hVps37B, and hVps37C Interact with Tsg101 via Their mod(r) Domains

To determine whether binding to Tsg101 is mediated by the N- or C-terminal part of HCRP1, immobilized GST-HCRP1 N terminus (1–148) or C terminus (208–397) was incubated with in vitro translated Tsg101. We found that the N terminus of HCRP1 weakly pulled down Tsg101, suggesting that this part of HCRP1 could contribute to the binding to Tsg101. Much more strikingly, the C terminus containing the mod(r) domain strongly pulled down Tsg101 (Figure 4A), indicating that the mod(r) domain is the main interacting interface with Tsg101. Consistently, the similarity between HCRP1 and Vps37 is mainly confined to the mod(r) domain (Figure 1A). Because mod(r) domains are conserved in several other human proteins (Figure 1A), we wanted to investigate whether the ability to bind Tsg101 is a more general property of mod(r) domains. We therefore prepared GST-tagged mod(r) domains of FLJ20847 and FLJ12750 as well and examined their ability to pull down Tsg101. As shown in Figure 4B, these mod(r) domains strongly pulled down Tsg101 in vitro (Figure 4B). Because mod(r) domain proteins bind Tsg101 and display the highest sequence similarity to yeast Vps37, we therefore call these proteins human Vps37

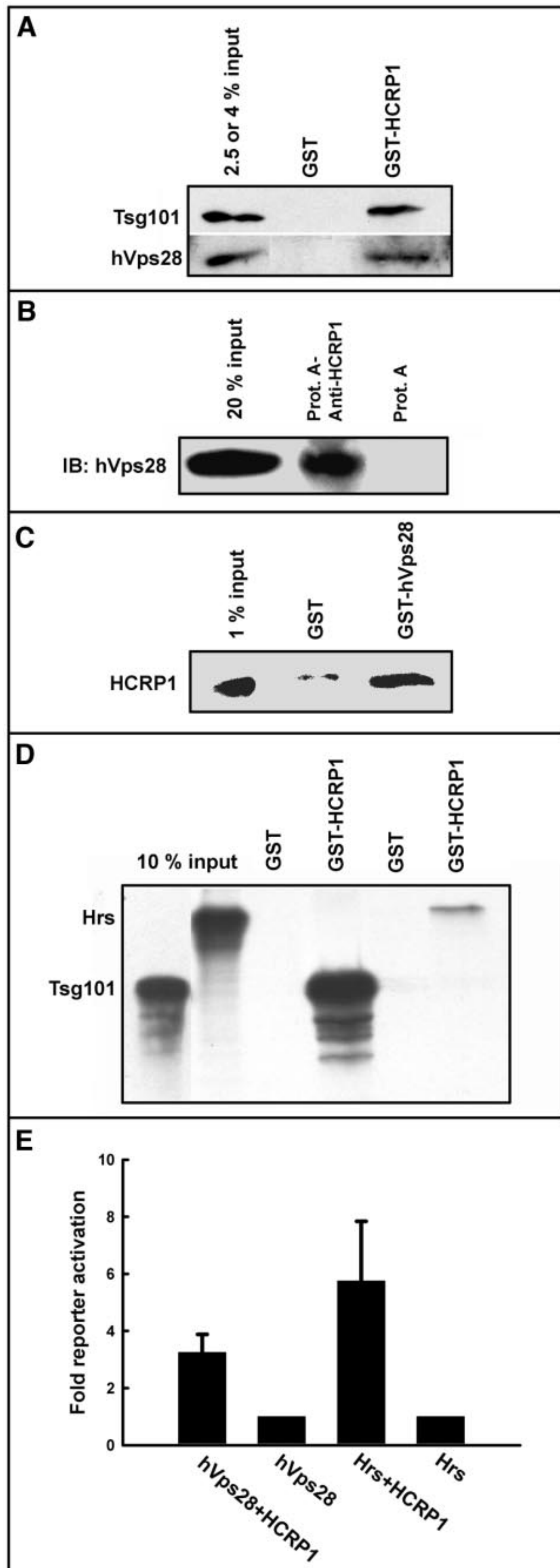

Figure 3.

(hVps37) A-D (Figure 1A). Accordingly, HCRP1 gets the alternative name hVps37A, FLJ12750 is assigned the name hVps37B, FLJ20847 is called hVps37C, and Wbscr24 is called hVps37D. The above-mentioned findings raise the possibility that there may be several alternative Vps37 counterparts in the mammalian ESCRT-I complex. However, the physiological roles of hVps37B-D were not investigated here.

HCRP1 Cofractionates with Tsg101 and hVps28 by Size Exclusion Chromatography

Gel filtration analyses have previously shown that the known subcomponents of ESCRT-I cofractionate with a molecular mass corresponding to ~350 kDa in yeast (Katzmann *et al.*, 2001) and mammalian cells (Bishop and Woodman, 2001). To further verify that HCRP1 is in complex with Tsg101 and hVps28, we compared the gel filtration elution profile of the three proteins. Fractions were precipitated with 5% trichloroacetic acid and analyzed by SDS-PAGE followed by immunoblotting with anti-Tsg101, anti-hVps28, and anti-HCRP1 antibodies. As shown in Figure 5, all three proteins were present in the same fractions. According to the standards included, the proteins migrated with an apparent molecular mass between 300 and 450 kDa. This is in agreement with previously published results and consistent with the conclusion that Tsg101, hVps28, and HCRP1 are present in the same complex.

Cellular Levels of hVps28 and HCRP1 Are Reduced When Tsg101 Is Depleted by siRNA

Because HCRP1 interacts with Tsg101, we wanted to investigate whether Tsg101 had any influence on the membrane localization of HCRP1. Should this be the case, a reduction of cellular levels of Tsg101 might lead to a decrease in membrane associated HCRP1. HeLa cells treated with siRNA against Tsg101 were fractionated into membrane and cytosolic fractions and analyzed by SDS-PAGE. Surprisingly, we found decreased amounts of hVps28 and HCRP1 both in the membrane and cytosolic fractions of Tsg101-depleted cells (Figure 6A). This suggests that these proteins

Figure 3. HCRP1 interacts with Tsg101, hVps28, and Hrs. (A) Interaction between HCRP1, Tsg101, and hVps28 in HeLa cells. GST alone or fused to HCRP1 was immobilized on glutathione-Sepharose beads and incubated with cell lysate from HeLa cells. The beads were washed and analyzed by SDS-PAGE and Western blotting with antibodies against Tsg101 and hVps28; 2.5 and 4% of the inputs of Tsg101 and hVps28, respectively, were loaded in lane 1. The amounts of Tsg101 and hVps28 pulled down with GST-HCRP1 correspond to 2–3 and 1–2% of the input amounts. (B) HCRP1 interacts with hVps28 in vivo. Protein A-Sepharose alone or bound to anti-HCRP1 was incubated with cell lysate from HeLa cells for 1 h at 4°C. The beads were washed and analyzed on SDS-PAGE and Western blotting with antibodies against hVps28. About 10% of hVps28 coimmunoprecipitated with HCRP1. (C) GST alone or fused to hVps28 was immobilized on glutathione-Sepharose beads and incubated with in vitro translated, ^{35}S-labeled HCRP1 for 1 h at 4°C. The beads were then washed and analyzed by SDS-PAGE and fluorography. The amount bound corresponds to 1–2% of the input amount. (D) GST alone or fused to HCRP1 was immobilized on glutathione-Sepharose beads and incubated with in vitro translated, ^{35}S-labeled Hrs or Tsg101 for 1 h at 4°C. The beads were washed and analyzed by SDS-PAGE and fluorography. (E) interactions of HCRP1 with hVps28 and Hrs in the yeast two-hybrid system. HCRP1 was used as prey and hVps28 and Hrs as baits. The values indicate β-galactosidase activities presented as fold reporter activation, and control bars (hVps28 and Hrs) represent background activation. The HCRP1 prey construct did not show any reporter activation in the absence of a bait construct (unpublished data).

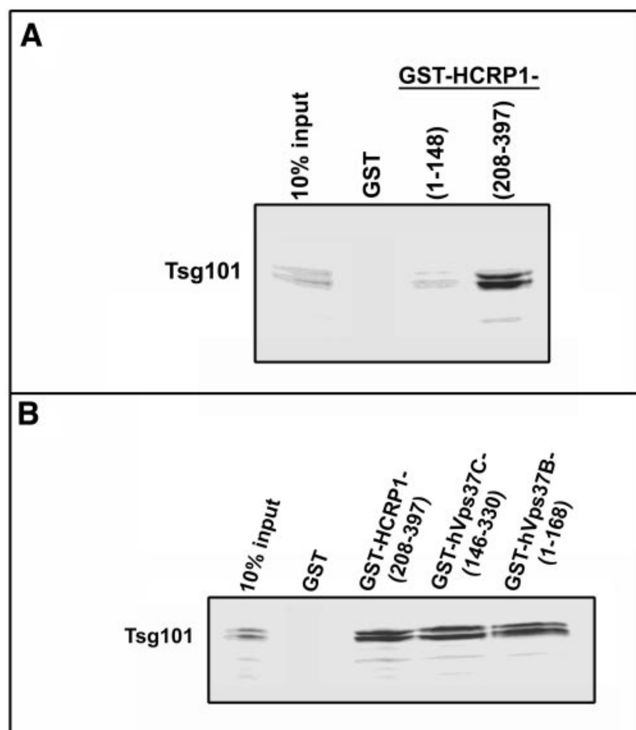

Figure 4. The mod(r) domains of HCRP1 and hVps37B/C interact with Tsg101. (A) GST alone or fused with HCRP1(1–148) or HCRP1(208–397) was immobilized on glutathione-Sepharose beads and incubated with in vitro-translated ^{35}S-labeled Tsg101 for 1 h at 4°C. The beads were then washed and analyzed by SDS-PAGE and fluorography. The input amount is indicated. (B) GST alone or fused with HCRP1 (208–397), hVps37C (146–330), or hVps37B (1–168) was immobilized on glutathione-Sepharose beads and incubated with in vitro-translated ^{35}S-labeled Tsg101 as described in A. The doublet band representing Tsg101 is probably due to translational initiation at alternative positions in vitro (Bache *et al.*, 2003).

need to be in a complex to be stable. To test the specificity of this result, we investigated whether Tsg101 depletion would affect another Tsg101-interacting protein, the mammalian orthologue of the yeast Vps class E protein Bro1, named Alix/AIP-1 (Odorizzi *et al.*, 2003; Vincent *et al.*, 2003). Alix interacts with both ESCRT-I (via Tsg101) and ESCRT-III, but it is not thought to be a subunit of ESCRT-I (von Schwedler *et al.*, 2003). Significantly, cellular levels of Alix were not reduced in the absence of Tsg101 (Figure 6A), indicating that only a subset of Tsg101-interacting proteins (those found in the same complex) are affected by its depletion.

To assess whether depletion of HCRP1 might cause similar effects on other ESCRT-I subunits as Tsg101 depletion, we designed an siRNA duplex for the knock-down of HCRP1. As shown in Figure 6B, this siRNA caused a strong depletion of HCRP1. However, in contrast to Tsg101 depletion, siRNA against HCRP1 did not have any effect on Tsg101 and hVps28, as assessed with Western blotting with antibodies specific for Tsg101 and hVps28 on membrane and cytosolic fractions from control cells and siRNA-treated cells (Figure 6B). Neither did depletion of HCRP1 influence the membrane association of Tsg101 and hVps28 (Figure 6B), but the elution peak of Tsg101 shifted toward a smaller M_r when lysate from HeLa cells treated with siRNA against HCRP1 was analyzed by gel filtration (Figure 6C). Together, these results indicate that Tsg101, hVps28, and HCRP1 are subunits of the same complex and that Tsg101 has a key role in keeping the complex stable. Moreover, although HCRP1 contributes to the overall size of ESCRT-I, it does not seem to be essential for the stability of the complex.

HCRP1 Is Important for EGF Receptor Down-Regulation

Our findings that HCRP1 is a subunit of mammalian ESCRT-I prompted us to ask whether this protein has a function in the sorting and down-regulation of endocytosed receptors. Cells expressing decreased levels of Tsg101 have previously been shown to be impaired in mitogenic receptor down-regulation (Babst *et al.*, 2000), and depletion of both Tsg101 and hVps28 results in accumulation of endocytosed EGF in HeLa cells (Bishop *et al.*, 2002). To investigate the possible role of HCRP1 in receptor down-regulation, we incubated HeLa cells with siRNA duplexes against HCRP1 and observed whether this had any effect on the ligand-induced degradation of EGF receptors. EGF was added to the cells, and 0, 3, and 6 h later they were lysed and the amount of EGF receptor was measured by Western blotting with anti-EGF receptor antibodies. As shown in Figure 7A, EGF receptor levels decreased significantly in control cells after 3 and 6 h of EGF stimulation, whereas this effect was largely abolished in siRNA-treated cells. By quantitating Western blots, we found that the amount of EGF receptors decreased to ~40% in control cells, whereas in RNAi-treated cells the EGF receptor levels remained at ~80%, even after 6 h of EGF stimulation (Figure 7B). This shows that HCRP1 is necessary for proper degradation of EGF receptor, which is consistent with a role for HCRP1 in the ESCRT-I complex.

DISCUSSION

The machinery involved in the endosomal sorting and down-regulation of ubiquitinated growth factor receptors is starting to be elucidated. It mediates the recognition and guidance of ubiquitinated proteins to the internal vesicles of MVBs and delivery to the lumen of lysosomes upon fusion of the MVB with organelles containing hydrolases. Subsequently, both the vesicles and the proteins contained therein are degraded. In yeast, it has been demonstrated that ESCRT-I, which consists of Vps23, Vps28, and Vps37, interacts with ubiquitinated cargo directly via the UEV-domain of Vps23 and thereby actively drives the sorting away from nonubiquitinated cargo, which remains in the limiting membrane of the MVB (Katzmann *et al.*, 2001; Conibear, 2002). In

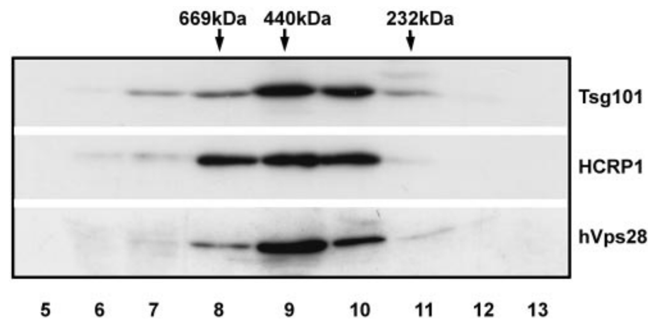

Figure 5. Size exclusion chromatography of ESCRT-I. HeLa cells were lysed, followed by size exclusion chromatography. In total, 18 fractions were analyzed by SDS-PAGE and anti-Tsg101, HCRP1, and hVps28 blotting. Only fractions 5–13 are shown; no immunoreactivity was detected in the other fractions. The molecular mass standards and fraction numbers are indicated.

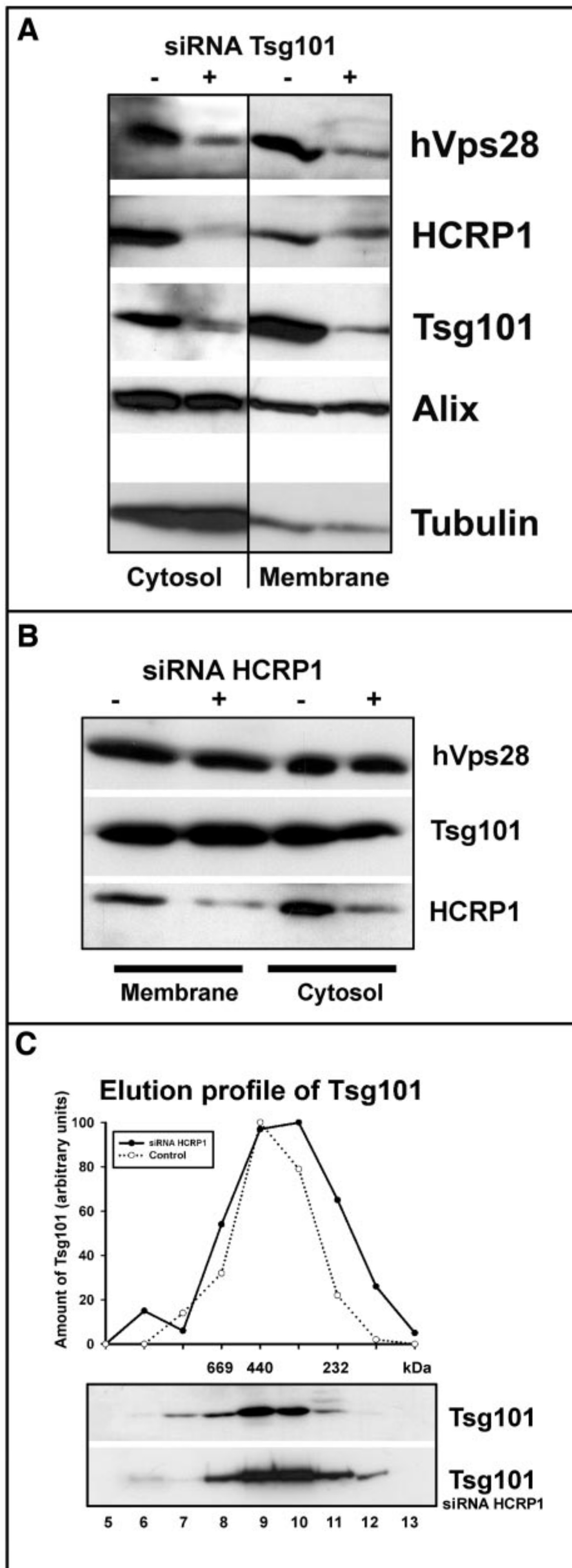

Figure 6. Depletion of Tsg101 by siRNA reduces cellular levels of hVps28 and HCRP1. (A) HeLa cells treated with control RNA (−) or with siRNA against Tsg101 (+) were fractionated into membrane and cytosolic fractions as described in *Materials and Methods*. Tsg101 left in the cytosol and on membranes after siRNA treatment as described in *Materials and Methods* was analyzed by SDS-PAGE, and

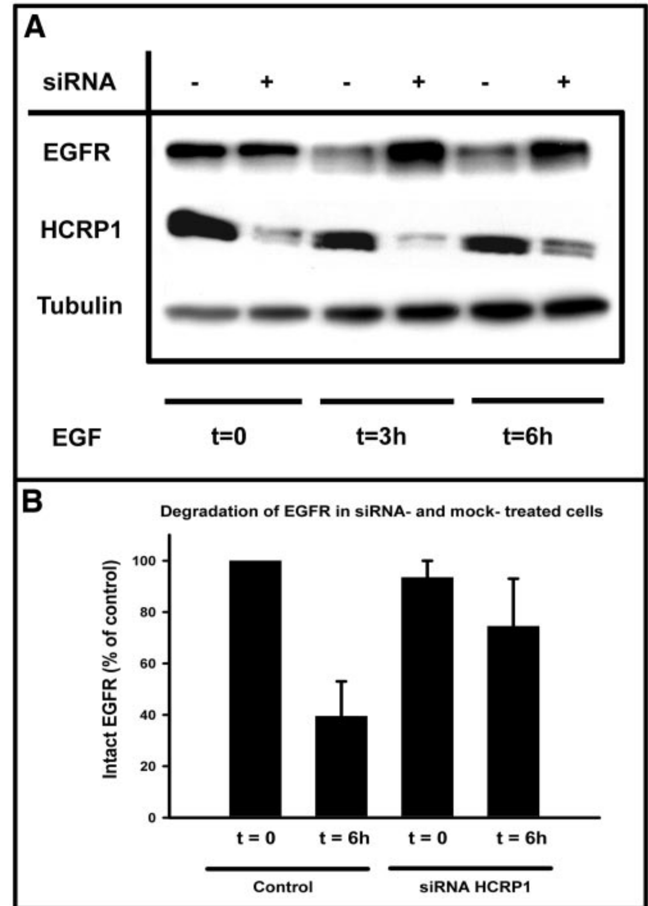

Figure 7. Depletion of HCRP1 retards EGF receptor down-regulation. (A) HeLa cells treated with control RNA (−) or with siRNA against HCRP1 (+) were stimulated for 0, 3, and 6 h with EGF. The cells were lysed and analyzed by SDS-PAGE and sequential blotting with antibodies against EGF receptor and HCRP1. The same membrane was then reblotted with anti-tubulin to verify equal loadings. (B) Relative intensities of the bands from control and siRNA-treated cells incubated with EGF for 0 or 6 h were quantified using ImageQuant 5.0 and are presented as the average of two experiments. Error bars denote SEM.

mammalian cells, there is evidence for a homologous pathway, in which Tsg101 and hVps28 recognize ubiquitin and act in the lysosomal sorting of ubiquitin-protein conjugates

Figure 6 (continued). the corresponding levels of hVps28, HCRP1, and Alix were shown by sequential blotting of the same membrane with anti-hVps28, anti-HCRP1, or anti-Alix. Equal amount of loaded protein is verified by blotting with anti-tubulin. (B) HeLa cells treated with control RNA (−) or with siRNA against HCRP1 (+) were fractionated into membrane and cytosolic fractions as described in *Materials and Methods*. The fractions were analyzed by Western blotting by using antibodies against HCRP1, Tsg101, and hVps28. (C) Lysates from HeLa cells treated with siRNA against HCRP1 (bottom gel) or not (top gel) were run on size exclusion chromatography. Eighteen fractions were analyzed by SDS-PAGE and Western blotting by using antibodies against Tsg101. Only fraction 5–13 are shown, because no Tsg101 was detected in any other fractions. The intensity of the bands was measured using ImageQuant 5.0 and plotted as percentage of the strongest band (top). Control cells, open symbols; HCRP1 siRNA-treated cells, closed symbols.

(Bishop *et al.*, 2002). A mammalian counterpart of Vps37 has, however, so far proven difficult to reveal.

In this study, we present evidence that a recently identified growth-regulatory protein named HCRP1 (Xu *et al.*, 2003) has regional sequence similarities with Vps37 and is part of the ESCRT-I complex. We find that it colocalizes with hVps28 on LAMP1-positive late endosomes and that it interacts with Tsg101 and hVps28. In addition, it shows the same elution profile as Tsg101 and hVps28 on gel filtration analysis. Furthermore, we provide evidence that it is important for proper EGF receptor down-regulation. We therefore conclude that HCRP1 is a human counterpart of Vps37 (therefore named hVps37A), with a function in endosomal sorting. This is consistent with a role in the ESCRT-I complex.

Why has a putative mammalian Vps37 counterpart been so difficult to reveal? In contrast to all other Vps proteins involved in the ESCRT complexes, BLAST searches with the sequence of Vps37 did not pick up any strong candidate for a human homologue. The closest hit was the rat counterpart of HCRP1, which is very similar to human HCRP1. The low sequence similarity between yeast Vps37 and mammalian HCRP1 suggests that although the complexes as such are evolutionarily conserved, this specific subcomponent of ESCRT-I has diverged from its precursors during evolution. Many functionally and evolutionarily important protein similarities are recognizable only through comparison of three-dimensional structures (Brenner *et al.*, 1998). Positional specific iteration-BLAST algorithm is a program that has the advantage of uncovering protein relationships that are usually missed by single-pass database-search methods (Mushegian *et al.*, 1997; Huynen *et al.*, 1998). This method uncovered the relationship between Tsg101 and HCRP1 (Xu *et al.*, 2003). However, we find it unlikely that HCRP1 represents a counterpart of Tsg101/Vps23, as the sequence similarity is very weak, and only the latter has been found to bind ubiquitin. Because the coimmunoprecipitation of hVps28 with HCRP1 was not quantitative, we cannot exclude the possible existence of additional human Vps37 counterparts, or other proteins that form alternative complexes with Tsg101 and hVps28. The other mod(r) domain proteins, which we assign the names hVps37B, hVps37C, and hVps37D, represent possible candidates. In any case, the strong inhibitory effect of HCRP1 depletion on EGF receptor degradation argues that HCRP1 is an important protein for receptor down-regulation, as expected for a true Vps37 homologue.

We have previously shown that Tsg101 is found on MVBs and late endosomes (Bache *et al.*, 2003). Given that HCRP1 is in the same complex as Tsg101 and hVps28, we expected to find colocalization between HCRP1 and the late endosomal marker LAMP1. When we coexpressed HCRP1 and hVps28 in the same cell, we were able to observe colocalization of the two proteins on late endosomes. Expressed alone, both HCRP1 and hVps28 tended to form aggregates, but this effect was reduced when they were expressed together. We also tried to coexpress Tsg101, but this protein still displayed strong aggregate formation (unpublished data). However, knowing that endogenous Tsg101 is found on late endosomes, the observed colocalization between hVps28 and HCRP1 on LAMP1-positive structures is in agreement with the conclusion that hVps28, Tsg101, and HCRP1 are in the same complex and that this complex is found mainly on late endosomes although its initial recruitment probably takes place on early endosomes (Bache *et al.*, 2003).

HCRP1 is found in complex with hVps28 in vivo, as observed by coimmunoprecipitation of the two proteins. A weak direct interaction was suggested by in vitro by pull-down assays and yeast two-hybrid analysis. In addition, a strong in vitro interaction was found between HCRP1 and Tsg101. The ability of GST-HCRP1 to pull down Tsg101 and hVps28 from a cell lysate was perhaps surprising, considering that, in yeast, the pool of uncomplexed ESCRT-I subunits seems to be low (Katzmann *et al.*, 2001). We assume that GST-HCRP1 may either displace endogenous HCRP1 from ESCRT-I, or capture the low amounts of Tsg101 and hVps28 that may be present in uncomplexed form.

Knockdown of one subunit within a complex often leads to destabilization of other subunits. For instance, siRNA-mediated knockdown of the $\mu 2$ subunit of the tetrameric clathrin adaptor complex AP-2 causes a codepletion of the α subunit, and vice versa (Motley *et al.*, 2003). Likewise, Hrs is important for the stability of STAM1 and STAM2 (Kanazawa *et al.*, 2003), and caveolin-1 knockout mice also lack caveolin-2, which is normally found in complex with caveolin-1 (Drab *et al.*, 2001). The observation that hVps28 and HCRP1 were depleted when Tsg101 was knocked down by siRNA therefore further supports the idea that these proteins are in the same complex. This suggests that Tsg101 plays a key role in the stability of the ESCRT-I complex. Our findings also imply that the effects of HCRP1 knockdown on EGF receptor degradation cannot be explained by a destabilization of Tsg101 or hVps28, indicating a crucial role for HCRP1 as such in EGF receptor sorting.

What is the role of HCRP1 in ESCRT-I? Knocking down HCRP1 with siRNA did not destabilize Tsg101 and hVps28, suggesting that HCRP1 is not important for their stability. Likewise, HCRP1 depletion did not result in disassembly of ESCRT-I, although we did observe a size shift of ESCRT-I (measured as Tsg101) when cell lysate from HCRP1 depleted cells was analyzed by gel filtration. The ESCRT-I size reduction upon HCRP1 depletion was somewhat smaller than that observed in in *vps37*Δ yeast cells (Katzmann *et al.*, 2001). We do not know the reason for this, but one possibility is that additional components, such as hVps37B-D, also might contribute to ESCRT-I in mammals. The poor sequence similarities between yeast Vps37 and HCRP1 could suggest that their respective functions have diverged throughout evolution. In contrast to the yeast protein, HCRP1 contains an N-terminal domain weakly similar to the UEV domain found in Tsg101 (unpublished data). However, we were not able to detect any interaction between HCRP1 and ubiquitin (unpublished data). The importance of this domain thus requires further investigation. One possible role of HCRP1 within ESCRT-I might be to regulate the function of the other subunits.

siRNA-mediated reduction of cellular levels of HCRP1 strongly inhibited EGF receptor down-regulation. MVB sorting and lysosomal degradation of growth factor receptor tyrosine kinases such as the EGF receptor constitutes an important mechanism to desensitize the cell and thereby regulate the potency of growth factor-induced signaling. It is interesting to note that HCRP1 functions as a growth inhibitory protein that is down-regulated in hepatocellular carcinoma (Xu *et al.*, 2003). Tsg101 represents another example of a protein in the endocytic pathway involved in growth regulation. As the product of a putative tumor susceptibility gene, it has been suggested to play a role in various cancer types (Li and Cohen, 1996; Ponting *et al.*, 1997; O'Boyle *et al.*, 1999; Hosokawa *et al.*, 2000). Tsg101 mutant cells also have been shown to be impaired in EGF receptor degradation, which results in the prolonged induction of the downstream signaling cascade (Babst *et al.*, 2000). Thus, two ESCRT-I subunits have now been implicated in growth inhibition,

and their functional inactivation seems to be associated with cancer. This supports the idea that the endosomal sorting machinery is important for proper cell signaling whose impairment may cause cancerous cell proliferation (Waterman and Yarden, 2001).

The interaction between Hrs and Tsg101 is of moderate strength and is probably transient (Pornillos *et al*., 2003). Still, it seems to be crucial for the recruitment of ESCRT-I to endosomes, because down-regulation of Hrs results in less membrane-associated ESCRT-I (Bache *et al*., 2003; Pornillos *et al*., 2003). Interestingly, we here uncovered an interaction between Hrs and a second ESCRT-I subunit, HCRP1, which, according to GST pull-down experiments, was of relatively low affinity. Because depletion of HCRP1 did not seem to inhibit membrane association of ESCRT-I, an interaction between Hrs and HCRP1 (as opposed to Tsg101) may not be important for the recruitment of ESCRT-I to endosome membranes. The biological significance of the Hrs–HCRP1 interaction therefore remains to be determined.

ESCRT-I plays a crucial role in the initial steps of MVB formation and cargo sorting. Here, we have identified a possible human counterpart of the yeast Vps37 protein HCRP1 and provided evidence for a functional role of this protein in the ESCRT-I complex. Tsg101 was originally implicated in the coordination and relay of diverse signals controlling cell growth and differentiation (Li and Cohen, 1996), and more recently, it also has been demonstrated to be crucial for budding of RNA viruses such as HIV and Ebola (Garrus *et al*., 2001; Martin-Serrano *et al*., 2001). To what extent are these tasks carried out by Tsg101 as such and to what extent do they depend on the function of the entire ESCRT-I complex? From this work, we have learnt that knock-down of Tsg101 also results in reduced levels of hVps28 and HCRP1, so a direct role for the latter proteins in viral budding cannot be excluded. It would be interesting to investigate viral budding in HCRP1-depleted cells, knowing that this protein can be knocked down without influencing the levels of Tsg101 and hVps28.

ACKNOWLEDGMENTS

We thank Eva Rønning for technical assistance and Wes Sundquist for sharing unpublished results and for agreeing upon a similar nomenclature for mod(r) domain proteins. This work was supported by the Top Research Programme, the Research Council of Norway, the Norwegian Cancer Society, the Novo-Nordisk Foundation, and the Anders Jahre's Foundation for the Promotion of Science.

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