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CART: An Hrs/Actinin-4/BERP/Myosin V Protein Complex Required for Efficient Receptor Recycling

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Altering the number of surface receptors can rapidly modulate cellular responses to extracellular signals. Some receptors, like the transferrin receptor (TfR), are constitutively internalized and recycled to the plasma membrane. Other receptors, like the epidermal growth factor receptor (EGFR), are internalized after ligand binding and then ultimately degraded in the lysosome. Routing internalized receptors to different destinations suggests that distinct molecular mechanisms may direct their movement. Here, we report that the endosome-associated protein hrs is a subunit of a protein complex containing actinin-4, BERP, and myosin V that is necessary for efficient TfR recycling but not for EGFR degradation. The hrs/actinin-4/BERP/myosin V (CART [cytoskeleton-associated recycling or transport]) complex assembles in a linear manner and interrupting binding of any member to its neighbor produces an inhibition of transferrin recycling rate. Disrupting the CART complex results in shunting receptors to a slower recycling pathway that involves the recycling endosome. The novel CART complex may provide a molecular mechanism for the actin-dependence of rapid recycling of constitutively recycled plasma membrane receptors.

INTRODUCTION

Endocytosis is required for the uptake of essential nutrients from the extracellular environment as well as for retrieval of proteins and lipids that are added to the plasma membrane during fusion of regulated and constitutive secretory vesicles (De Camilli and Takei, 1996; Koenig and Ikeda, 1996; Robinson *et al*., 1996; Mukherjee *et al*., 1997; Schmid, 1997; Betz and Angleson, 1998; Koenig *et al*., 1998; Stoorvogel, 1998; D'Hondt *et al*., 2000; Gruenberg, 2001). The endocytic pathway can be separated into numerous stages based on the movement of cargo and the identification of morphologically defined compartments (De Camilli and Takei, 1996; Koenig and Ikeda, 1996; Robinson *et al*., 1996; Mukherjee *et al*., 1997; Schmid, 1997; Betz and Angleson, 1998; Koenig *et al*., 1998; Stoorvogel, 1998; D'Hondt *et al*., 2000; Gruenberg, 2001). Early events in the endocytic process include membrane invagination and vesicle budding from the plasma membrane, formation of transport vesicles, and fusion with early endosomes. Later events include cargo sorting, and additional transport/fusion steps, including those responsible for transport to the lysosome for degradation, and those responsible for recycling back to various compartments (De Camilli and Takei, 1996; Koenig and Ikeda, 1996; Robinson *et al*., 1996; Mukherjee *et al*., 1997; Schmid, 1997; Betz and Angleson, 1998; Koenig *et al*., 1998; Stoorvogel, 1998; D'Hondt *et al*., 2000; Gruenberg, 2001). Although much progress has been made in elucidating the molecular processes involved in early endocytic events, such as those involved in the genesis of clathrin-coated endocytic transport vesicles, an equally clear understanding of later events remains elusive.

The early endosome is a crucial point in the endocytic pathway to sort cargo for transport to late endosomes for eventual degradation in the lysosome, or for recycling to the plasma membrane (Hopkins *et al*., 1985; Gruenberg and Maxfield, 1995; Ward *et al*., 1995). The early endosome is composed of at least two forms, including the vacuolar or tubulovesicular endosome, containing early endosome antigen 1 (EEA1) and rab 5, as well as the recycling endosome, containing rab 11 (Hopkins *et al*., 1985; Gorvel *et al*., 1991; Gruenberg and Maxfield, 1995; Ward *et al*., 1995; Ullrich *et al*., 1996; Trischler *et al*., 1999). Recycling of cargo to the plasma membrane occurs from both early endosomal compartments as can transport to late endosomes (Hopkins *et al*., 1985; Gorvel *et al*., 1991; Ward *et al*., 1995; Ullrich *et al*., 1996; Trischler *et al*., 1999; Gruenberg, 2001). It is likely that a combination of maturation and vesicular transport mechanisms allows for tight control of the sorting, transport, and recycling functions in the early endosomal compartment (Gruenberg, 2001). Routing internalized receptors to different destinations suggests that distinct molecular mechanisms are required to direct their movement.

A role for the actin cytoskeleton in endocytosis has been controversial (Qualmann *et al*., 2000; Engqvist-Goldstein and Drubin, 2003). In yeast, mutations in actin or fimbrin inhibit endocytosis (Kubler and Riezman, 1993). Additionally, genetic screens for mutants affecting endocytosis have revealed mutations in several genes that disrupt both endocy-

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Abbreviations used: CART, cytoskeleton-associated recycling or transport; EGF, epidermal growth factor; Hrs, hepatocyte growth factor stimulated serum phosphoprotein; Tf, transferrin.

tosis and actin organization (Benedetti *et al*., 1994; Munn *et al*., 1995; Tang *et al*., 1997). However, some yeast mutants that disrupt actin organization have no effect on endocytosis (Riezman *et al*., 1997), and some suppressors of an endocytosis mutant that also affects actin organization (*end5*) can rescue one or the other phenotype, but not both, suggesting that they are separable (Riezman *et al*., 1997). In mammalian cells, many reports exist describing the necessity, or lack thereof, of the intact actin cytoskeleton for endocytosis (Durrbach *et al*., 1996; Lamaze *et al*., 1997; Fujimoto *et al*., 2000; Qualmann *et al*., 2000; Taunton *et al*., 2000; Lanzetti *et al*., 2001; Zaslaver *et al*., 2001). These reports have concentrated on the effect of disruption of the integrity of the actin cytoskeleton on early events in the endocytic process such as vesicle budding from the plasma membrane, but they have not focused on later events such as the motility of endosomes. GFP-actin tails occur on newly pinched off pinosomes in RBL cells and have been proposed to move these organelles from the membrane into the cell (Merrifield *et al*., 1999). Actin tails also associate with endosomes in a cell-free system (Moreau and Way, 1998; Taunton *et al*., 2000). These data suggest a role for the actin cytoskeleton in the motility of endosomes after initial endocytic events. Thus, although endosomes associate with the actin cytoskeleton and this association is likely involved in endosome motility (Durrbach *et al*., 1996; Lamaze *et al*., 1997; Nakagawa and Miyamoto, 1998; Qualmann *et al*., 2000; Gruenberg, 2001; Lanzetti *et al*., 2001), the precise role of the actin cytoskeleton in endocytosis is unclear.

Hrs is a mammalian protein, predominantly localized on early endosomes (Komada *et al*., 1997; Tsujimoto *et al*., 1999), that physically interacts with a number of proteins, including eps15 (Bean *et al*., 2000), SNX-1 (Chin *et al*., 2001), TSG101 (Pornillos *et al*., 2003), and SNAP-25 (Bean *et al*., 1997), previously implicated in membrane trafficking. Hrs has homologues in insects (Lloyd *et al*., 2002) and fungi (Raymond *et al*., 1992). Deletion or mutation of hrs results in an enlarged endosomal phenotype in mouse (Komada and Soriano, 1999), fly (Lloyd *et al*., 2002), and yeast (Raymond *et al*., 1992). This suggests that hrs deletion may decrease efflux from early endosomes to certain destinations (e.g., the plasma membrane) and that hrs may play a role in cargo sorting and/or endosomal trafficking at the early endosome.

Here, we report that the endosome-associated protein hrs is a subunit of the cytoskeleton-associated recycling or transport (CART) complex that also contains actinin-4, BERP, and myosin V. The CART complex is necessary for efficient recycling of the transferrin (Tf) receptor to the plasma membrane, but not movement to lysosomes and degradation of the EGFR. These results not only identify the CART complex but also support a model that describes how sorting endosomes are linked with the actin cytoskeleton to enable efficient retrieval of constitutively recycled receptors to the plasma membrane.

MATERIALS AND METHODS

Antisera and DNA Constructs

A rabbit was immunized with a peptide (MGDYMAQEDDWC) that had been coupled to keyhole limpet hemocyanin and corresponds to the first 11 amino acids of the actinin-4 protein (Honda *et al*., 1998). This region of actinin-4 has limited homology with sequences of other actinins (Honda *et al*., 1998) and has been used previously to produce specific actinin-4 antisera (Honda *et al*., 1998). The resulting serum was affinity purified and used for immunohistochemistry and Western blotting. The myosin Vb antibody was obtained from J. Leonard (University of Massachusetts Medical School, Worcester, MA); BERP, N-BERP, and rat myosin Vb (myr6) constructs were obtained from S. R. Vincent (University of British Colombia, Vancouver, BC, Canada); and pBKactinin-4 was obtained from T. Yamada and S. Hirohashi (National Cancer Center Research Institute, Tokyo, Japan). Other DNA constructs and antibodies are described here.

Immunohistochemistry

HeLa cells were maintained in DMEM (Mediatech, Herndon, VA) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Equitech-Bio, Ingham, TX). Cells were plated 1 d before experiments. Cells with or without drug treatment were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 10 min. Fixed cells were then extracted with 0.2% Triton X-100 (vol/vol) (in 0.1 M phosphate buffer, pH 7.4) for 1 min (4°C) and washed three times with PBS before immunolabeling. For immunolabeling, cells were incubated with primary antibodies in 0.1 mM phosphate buffer, pH 7.4 containing 2% normal goat serum and 0.25% saponin overnight at 4°C. After washing three times with PBS, the cells were incubated with secondary antibodies at 37°C for 30 min. After washing as described above, coverslips were mounted with paraphenylenediamine in 50% glycerol/0.1 M phosphate buffer, pH 7.4.

The antibodies used in this study and their dilutions used for immunohistochemistry were rabbit anti-actinin-4 (described above), 1:100; rabbit anti-actin (recognizing both F- and G-actin (Sigma-Aldrich, St. Louis, MO), 1:100; rabbit anti-Eps15 (Santa Cruz Biotechnology, Santa Cruz, CA), 1:200; rabbit anti-EEA1 (BD Biosciences Transduction Laboratories, San Diego, CA), 1:200; mouse anti-α-actinin-1 (Sigma-Aldrich), 1:200; and mouse anti-transferrin receptor (Santa Cruz Biotechnology), 1:50. The secondary antibodies were Alexa 488-labeled goat anti-mouse IgG, dichlorotriazin amino fluorescein-labeled goat anti-mouse IgG, Alexa 594 goat anti-mouse IgG, Texas Red-labeled donkey anti-rabbit IgG, and Alexa 488-labeled donkey anti-rabbit IgG. All secondary antibodies were purchased from Molecular Probes (Eugene, OR) and were used at a 1:1000 dilution. Fluorescence images were acquired using a Zeiss Axiovert microscope with a Hamamatsu ORCA charge-coupled device (CCD) camera and viewed using MetaView software (Universal Imaging, Downingtown, PA).

Immunogold Electron Microscopy

HeLa cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M PHEM buffer (0.2 M, containing 240 mM PIPES, 100 mM HEPES, 8 mM $MgCl_2$, and 40 mM EGTA, pH 6.9) containing paraformaldehyde (2%) and glutaraldehyde (0.2%) for 2 h at room temperature. Fixed cells were stored as pellets in PHEM (0.1 M) and paraformaldehyde (0.5%) at 4°C until they were processed for ultrathin cryosectioning and immunolabeling according to the protein A-gold method (Slot *et al*., 1991). Briefly, fixed cells were washed with 0.02 M glycine in PBS, scraped gently from the dish in PBS with 1% gelatin, and pelleted in 12% gelatin in PBS. The gelatin cell pellet was solidified on ice and cut into small blocks. For cryoprotection, blocks were infiltrated overnight with 2.3 M sucrose at 4°C and mounted on aluminum pins then frozen in liquid nitrogen. Ultrathin cryosections with an average thickness of 60 nm were cut with a diamond knife (Diatome, Biel, Switzerland) at −120°C and picked up in a 1:1 mixture of 2.3 M sucrose and 1.8% methyl cellulose in distilled water (Liou *et al*., 1996). The sections were thawed to room temperature, incubated with primary antibodies (rabbit anti-actinin-4, 1:30 or mouse monoclonal anti-Hrs, 1:100 [Axxora, San Diego, CA]; 1 h, room temperature) and protein A coupled to 15-nm-gold particles (University Medical Centre of Utrecht, Utrecht, The Netherlands). For the Hrs labeling rabbit anti-mouse IgG antibodies (Pierce Chemical, Rockford, IL) were used as bridging antibodies before applying the protein A-gold. Negative controls included labeling without primary antibodies. Finally, sections were contrasted and embedded within a mixture of 0.4% uranylacetate in 2% methyl cellulose, after which they were examined in a Tecnai 12 transmission electron microscope (FEI, Eindhoven, The Netherlands). Photographic negatives of light microscopic and electron microscopic images were digitized with an Epson Perfection 2450 scanner.

For morphometric quantification, the distribution of total gold particles on 50 cell profiles for actinin-4 and 30 for hrs, respectively, were counted at 20,000× magnification and divided into the following eight subcellular compartments proved relevant for their distribution: 1) plasma membrane (i.e., gold particles within the distance of 20 nm of the membrane); 2) filopodia (i.e., actin-rich cellular extensions from plasma membrane); 3) terminal web under plasma membrane (actin-rich region <200 nm beneath the plasma membrane; 4) early/recycling endosomes; 5) late endosomes/lysosomes; 6) Golgi/*trans*-Golgi network (TGN); 7) endoplasmic reticulum (ER); and 8) other.

Two-Hybrid Screen

Full-length hrs was subcloned into the pGBT vector and used to screen a human brain cDNA library inserted downstream of the GAL4 activation domain in the pGAD10 vector (BD Biosciences Clontech, Palo Alto, CA) as described previously (Bean *et al*., 2000). Twelve days after plating, large colonies (>3 mm in diameter, n = 190) were replica plated onto new plates and β-galactosidase activity was assessed on filter lifts. Single colonies from clones that turned blue within 1 h (n = 40) were grown overnight in SD medium lacking leucine, tryptophan, and histidine and DNA was extracted. DNA was electroporated into HB101 *Escherichia coli* cells, and DNA was

isolated from single colonies. After restriction digests confirmed the presence of the activation domain plasmid, multiple cotransformations were performed with the candidate DNA and the pGBT9/h, as well as pGBT9/p53. Clones that reacted positively for β-galactosidase activity with the pGBT9/h but not either by themselves or with the control plasmids containing the binding domain alone or the binding domain fused to the tumor suppressor gene p53, were considered for further study (n = 36).

In Vitro Binding Assay

The His-tagged hrs fusion protein and all glutathione *S*-transferase (GST) fusion proteins were prepared as described previously (Tsujimoto and Bean, 2000). GST-fused proteins were cleaved from the GST moiety by using thrombin (7.5 U/ml; Amersham Biosciences, Sunnyvale, CA) at room temperature for 2–4 h. The cleavage reaction was stopped by adding 0.1 mM phenylmethylsulfonyl fluoride. All soluble proteins were precleared with glutathione-agarose before quantitation and binding. Protein concentrations were estimated by Coomassie Blue stain following SDS-PAGE by using bovine serum albumin (BSA) as a standard.

To identify the region of hrs responsible for actinin-4 binding, a cleaved GST-fusion of actinin-$4_{(359–913)}$ and increasing concentrations of various GST-fused hrs fragments immobilized on glutathione-agarose beads were incubated in binding buffer (20 mM HEPES, pH 7.4, 150 mM KCl, and 0.05% Tween 20) for 1 h at 4°C. Beads were washed three times with buffer containing 0.1 M PBS and 0.05% Tween 20, solubilized in 12 μl of SDS sample buffer, and separated by SDS-PAGE. The bound actinin-4 was detected by immunoblot analysis by using enhanced chemiluminescence (Pierce Chemical).

To quantify the binding of hrs and actinin-4, 0.06 μM GST-actinin-4 was immobilized and incubated with various concentrations of purified soluble hrs. After resolution by SDS-PAGE, the bound hrs was detected by immunoblot with ^{125}I-conjugated secondary antibody. The hrs bands on the phosphorimage were subjected to a quantitation with IQMac software (version 1.2; Amersham Biosciences). Hrs did not bind to GST alone (our unpublished data).

To examine the inhibition of actinin-4/h binding by hrs or actinin-4 fragments, we incubated hrs (0.5 μM, 4°C for 1 h) and immobilized GST-actinin-4 (0.1 μM) with purified $hrs_{(1–449)}$ or actinin-$4_{357–469}$ (8:1 M ratio) (4°C for 1 h). The hrs that remained bound to actinin-4 in the presence and absence of hrs or actinin-4 fragments was detected by immunoblot with ^{125}I-conjugated secondary antibody after SDS-PAGE and quantified by phosphorimaging as described above.

Cell Culture and Transfection

HeLa cells were cultured as a monolayer in 10-cm plastic plates in DMEM containing 10% FBS under 5% CO_2 at 37°C. Before each experiment, cells were split with trypsin/EDTA and seeded in six-well tissue culture plates.

Plasmid DNA was prepared (QIAGEN, Hilden, Germany) for the transfection of HeLa cells. Transient transfections of plasmid DNA were performed (Effectene transfection kit, QIAGEN) on HeLa cells according to the manufacturer's protocol. The constructs used in each transfection are as indicated. Cells remained in growth medium with the transfection mixture for 4 h and were subsequently washed three times with fresh media and incubated overnight in conditioned media before use.

For trafficking studies in which $hrs_{(1–449)}$ was expressed, HeLa cells were first infected with recombinant vaccinia virus (vT7) bearing the T7 RNA polymerase gene. Vaccinia virus stock was trypsinized at 37°C for 30 min with vigorous vortexing every 5–10 min, and then 10 μl was added to HeLa cells (de Bernard *et al.*, 1997). After infection for 30 min at 37°C, the virus stock was removed, and HeLa cells were transfected with pCDNA3-myc-$hrs_{(1–449)}$ or the pCDNA3 vector. Cells were incubated for 4 h at 37°C, after which time the transfection reagent was removed. The cells were washed twice with PBS and incubated for 20 h before beginning trafficking assays.

Actinin-4 Depletion by Using RNA Interference

Duplexes of RNA oligonucleotides targeting human actinin-4 were designed as 21 nucleotide RNAs and were chemically synthesized (Dharmacon Research, Boulder, CO) The sequences of each RNA pair were as follows: actinin-4, 5′-AAG CAG CAG CGC AAG ACC UUC dTdT-3′ and scrambled (control), 5′-CAG UCG CGU UUG CGA CUG GdTdT. RNA oligonucleotides were transfected (Oligofectamine; Invitrogen, Carlsbad, CA) into 40–50% confluent HeLa cells. A second transfection was performed 24 h after the first. Trafficking was assessed 24 h after the second transfection, and the efficiency of the depletion was analyzed by quantitative Western blotting using ^{125}I-secondary antibodies and phosphorimage analysis.

^{125}I-Epidermal Growth Factor Trafficking

After transfection of HeLa cells with either duplexed RNA oligonucleotides, pCDNA3-myc-$hrs_{(1–449)}$, pCDNA3-myc-actinin-$4_{357–469}$, or controls (scrambled RNA duplexes or pCDNA3 vector), cells were starved (medium A, 1% BSA in DMEM, 60 min at 37°C), labeled with ^{125}I-epidermal growth factor (1 ng/ml) for 30 min at 37°C, and then rinsed with cold medium A twice, acidic solution (0.15 M NaCl and 0.1 M glycine, pH 3.0) twice, and once again with medium A. Cells were chased with medium A for the indicated times. The media and cells were collected at each time point. Cells were harvested by scraping with 1 M NaOH. Proteins in the media were precipitated (20% trichloroacetic acid [TCA]), and cells (internalized EGF), media pellet (recycled EGF), and media supernatant (degraded EGF) were counted in a gamma counter. Each data point was collected in duplicate. Recycled ^{125}I-epidermal growth factor was expressed as the ratio of recycled ^{125}I-epidermal growth factor versus internalized ^{125}I-epidermal growth factor. Degraded ^{125}I-epidermal growth factor was expressed as the ratio of degraded ^{125}I-epidermal growth factor versus internalized ^{125}I-epidermal growth factor. The data were corrected for nonspecific cell-associated ^{125}I-epidermal growth factor (<10%) as determined in parallel experiments in which an excess (200 ng/ml) of unlabeled EGF was present during labeling. Kinetic parameters were obtained by fitting data with a linear regression over time. Bar graphs present mean slopes obtained by regression analysis. Differences between rates were analyzed using a Student's *t* test.

^{125}I-Transferrin Trafficking

Tf (Sigma-Aldrich) was saturated with Fe^{3+} and labeled with ^{125}I by using iodo-beads (Pierce Chemical) according to the manufacturer's procedures. After transfection of HeLa cells with either duplexed RNA oligonucleotides, pCDNA3-myc-$hrs_{(1–449)}$, pCDNA3-myc-actinin-$4_{357–469}$, or controls (scrambled RNA duplexes or pCDNA3 vector), cells were starved (medium A, 1% BSA in DMEM, 60 min at 37°C). To examine Tf recycling and degradation, cells are labeled with ^{125}I-transferrin (1 μg/ml) for 30 min at 37°C and then rinsed once with cold medium A, once with acidic solution (0.15 M NaCl and 0.1 M glycine, pH 3.0), and once again with medium A. Cells were chased with medium A for the indicated times. The media and cells were collected at each time point. Cells were harvested by scraping with 1 M NaOH. Proteins in the media were precipitated (20% TCA), and cells (internalized transferrin), media pellet (recycled transferrin), and media supernatant (degraded transferrin) were counted in a gamma counter. Each data point was collected in duplicate. Recycled ^{125}I-Tf was expressed as the ratio of recycled ^{125}I-Tf versus internalized ^{125}I-Tf over time. The data were corrected for nonspecific cell-associated ^{125}I-Tf (<10%) as determined in parallel experiments in which an excess (200 μg/ml) of unlabeled Tf was present during labeling. Kinetic parameters were obtained by fitting data with a linear regression over time. Bar graphs present mean slopes obtained by regression analysis. Differences between rates were analyzed using a Student's *t* test.

Transferrin-Alexa 594 Trafficking

After transfection of HeLa cells with either duplexed RNA oligonucleotides specific for actinin-4 or scrambled oligonucleotides, cells were starved (medium A, 1% BSA in DMEM, 60 min at 37°C) and then incubated with Alexa 594-transferrin (5 μg/ml, 60 min at 0°C). The cells were washed three times with medium A and transferred to a 37°C incubator for 10 min before washing once with medium A, then with acidic solution (0.15 M NaCl and 0.1 M glycine pH 3.0), followed by another wash with medium A. Some cells (0-min time point) were immediately fixed (4% paraformaldehyde at 0°C); other cells were incubated in media A for 30 min before fixation (30 min time point). Cells were examined using epifluorescence and images were obtained using a Zeiss Aviovert microscope with a Hamamatsu ORCA CCD camera and optimized with MetaMorph software (Universal Imaging).

RESULTS

Hrs Interacts with Actinin-4

We identified an interaction between hrs and actinin-4, an actin binding protein, in a yeast two-hybrid screen. Actinin-4 transiently associates with both macropinosomes and phagosomes, structures that perform endocytic functions (Araki *et al.*, 2000). Actin filaments associate with endosomes (Pol *et al.*, 1997; Nakagawa and Miyamoto, 1998), suggesting a role for this molecule in endocytosis. To confirm the interaction between hrs and actinin-4 observed in the two-hybrid screen, we performed binding experiments that showed recombinant hrs and actinin-4 interact in a saturable manner in the absence of other proteins (Figure 1A). The affinity of hrs for immobilized actinin-4 (the concentration of protein at which half-maximal binding occurs (EC_{50}) was ~0.4 μM. The apparent stoichiometry based on recombinant protein binding experiments was 0.1–0.5:1 (actinin-4:hrs).

The interaction of hrs and actinin-4 also was observed after antibodies specific for hrs coimmunoprecipitated both hrs and actinin-4 from rat brain extracts (Figure 1B, lane 2) or HeLa cells (Figure 1B, lane 3). The domain of hrs required

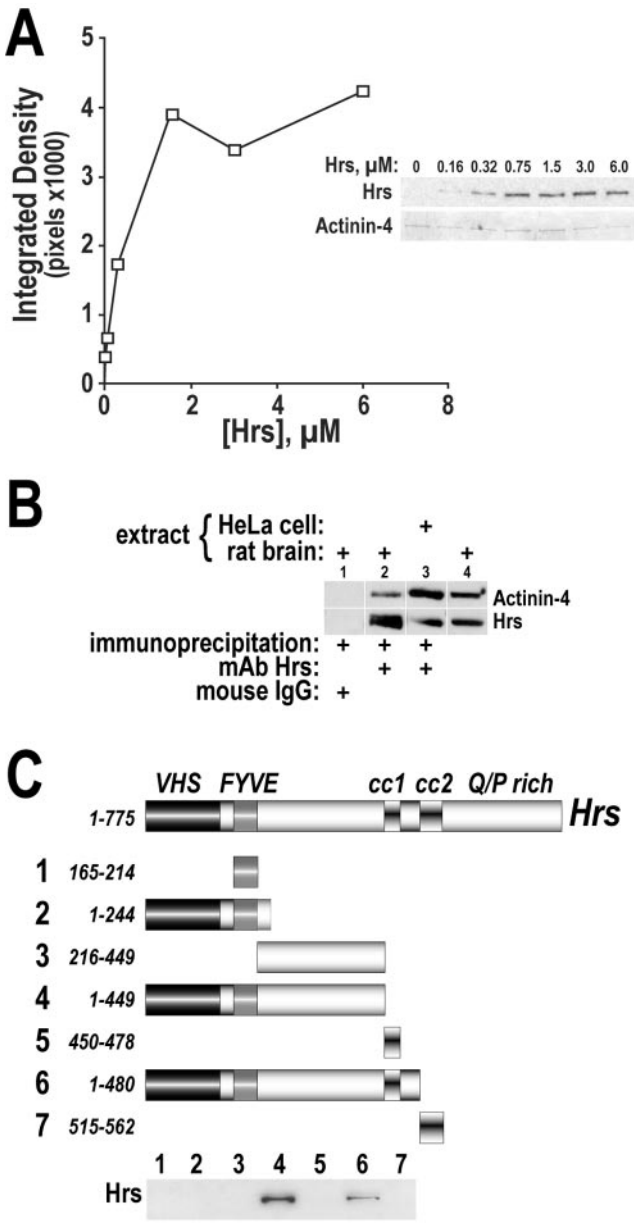

Figure 1. Hrs binds directly to actinin-4 through distinct domains. (A) Recombinant hrs was incubated with immobilized actinin-4, washed, eluted, separated by SDS-PAGE, and bound hrs was quantitated using ^{125}I-secondary antibody and phosphorimaging. The EC_{50} is ~0.4 μM, and the stoichiometry is 0.1–0.5–1.0, depending on which protein is immobilized. A Ponceau S stain of actinin-4 is shown for a loading control. (B) Actinin-4 coimmunoprecipitates with hrs. Rat brain postnuclear supernatant (lane 4) was incubated with purified mouse IgG (lane 1) or a monoclonal anti-hrs (lanes 2 and 3). Hrs and actinin-4 also were coimmunoprecipitated from HeLa cell lysate (lane 3). (C) Seven GST-hrs fusion proteins (designated 1–7) were immobilized on glutathione-agarose and assayed for binding to actinin-$4_{(359–913)}$ as in A. These results identify a specific domain in hrs essential for its interaction with actinin-4.

for the interaction with actinin-4 was determined by examining the binding of hrs fragments to actinin-4 (Figure 1C). The minimal fragment of hrs necessary and sufficient for actinin-4 binding consists of amino acids 1–449 (Figure 1C, lane 4). The FYVE or the VHS domains within the N-terminal region of hrs were not sufficient for actinin-4 binding (Figure 1C, lanes 1 and 2). These data suggest that actinin-4 binding to hrs does not require the helical coiled coil regions and imply that the tertiary structure of the N-terminal region of hrs is an actinin-4 binding determinant. The domain of actinin-4 required for hrs interaction was identified based on overlapping fragments of actinin-4 obtained in our two-hybrid screen. Four independent clones [22-1 (identical to 359–911 of human actinin-4), 47-2 (359–911 of actinin-4), 52-1 (233–416 of actinin-4), and 123-8 (232–412 of actinin-4)] were isolated in our screen. These data suggested that the minimal domain of actinin-4 necessary for hrs binding is contained within the central coiled region (amino acids 359–412).

Hrs and Actinin-4 Are Localized on Early Endosomes

To understand a cellular basis for the hrs/actinin-4 interaction, we have examined the ultrastructural localization of both hrs and actinin-4 in HeLa cells by using hrs (Tsujimoto *et al*., 1999) and actinin-4 specific antibodies. The actinin-4 antisera labeled a single protein of ~100 kDa on an immunoblot of HeLa cell lysate, which was completely blocked after preincubation of the antisera with the immunogen peptide (our unpublished data). Furthermore, Western analysis of this antisera revealed no cross-reactivity with other actinins (our unpublished data). Hrs localizes both in the cytoplasm and on the cytoplasmic face of endosomal membranes (Figure 2A). In addition to hrs, we also detected actinin-4 on endosomal membranes (Figure 2B). Quantification of gold particles on various organelles confirmed that hrs labeling was found predominantly associated with early endosomes but also on the actin-rich terminal web region (<200 nm beneath the plasma membrane) and the late endosomes/lysosomes, with some labeling on the plasma membrane (Figure 2E). Actinin-4 labeling was found predominantly associated with the terminal web and early endosomes, with some labeling of filopodia and on the plasma membrane (Figure 2C,D,E). These data suggested that early endosomes contained a significant overlap in the localization of hrs and actinin-4.

Actinin-4 Depletion Affects Tf Trafficking

Endosomes or endocytic transport vesicles likely use cytoskeletal elements for motility (Durrbach *et al*., 1996; Nakagawa and Miyamoto, 1998; Durrbach *et al*., 2000; Neuhaus and Soldati, 2000; Lanzetti *et al*., 2001; Lapierre *et al*., 2001; Zaslaver *et al*., 2001; Shupliakov *et al*., 2002; Engqvist-Goldstein and Drubin, 2003). Actinin-4 is predicted to have an actin-binding domain, and we have observed that it binds actin (our unpublished data). We hypothesized that the hrs/actinin-4 interaction may play a role in actin-associated trafficking of endosomes. We tested this notion and examined how depletion of actinin-4 affects the trafficking of endocytic cargo. We determined the kinetics of ^{125}I-epidermal growth factor receptor and ^{125}I-Tf receptor internalization, recycling, and degradation in cells that had been depleted of actinin-4 by RNA interference (Figure 3). Transfection of HeLa cells with RNA duplexes resulted in an 87 ± 13% decrease in actinin-4 levels (Figure 3A, lane 2), whereas other actinin isoforms remained present in these cells (Figure 3A, lane 4). There was no significant alteration in actin, hrs, or eps15 levels (Figure 3A, lanes 5–7). Furthermore, no alterations in the localization or morphology of early endosomes or actin filaments were evident after depletion of actinin-4 (Figure 3A, right). Depletion of actinin-4 had no significant effect on the internalization of Tf or EGF or the degradation of Tf (our unpublished data) nor were there significant differences in plasma membrane Tf versus

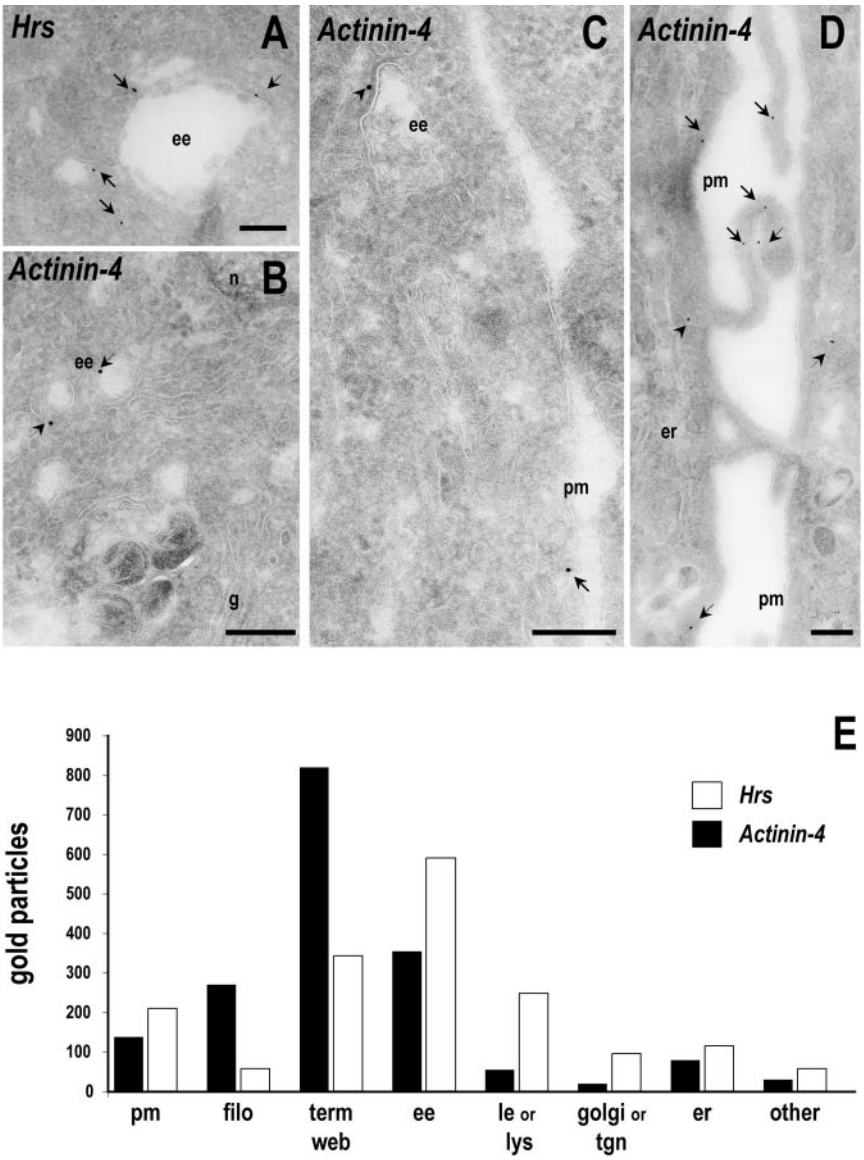

Figure 2. Hrs and actinin-4 are localized on early endosomes. Ultrathin cryosections of HeLa cells were labeled with specific hrs and actinin-4 antisera and visualized with 15-nm gold particles conjugated to protein A. (A) Hrs is mostly found associated with early endosomal vacuoles and recycling tubules. Actinin-4 (B and C) is occasionally found associated with early endosomal vacuoles, but it is predominantly enriched to the proximity to the plasma membrane (D), at the actin-rich terminal web within 200 nm of the plasma membrane and the filopodial extensions. Arrowheads indicate the location of gold particles. ee, early endosome; er, endoplasmic reticulum; g, Golgi; pm, plasma membrane; n, nucleus. Bars, 200 nm. (E) Quantitation of hrs and actinin-4 labeling in eight subcellular compartments relevant for their distribution; including the plasma membrane (i.e., label on pm ± 20 nm), filopodia (i.e., actin rich cellular extensions from plasma membrane), terminal web under plasma membrane (actin-rich region <200 nm beneath the plasma membrane), early/recycling endosomes, late endosomes/lysosomes, Golgi/TGN, ER, and all other particles.

total cellular Tf at time points before recycling (0–3 min) in actinin-4 depleted cells versus control cells. However, actinin-4 depletion inhibited the rate of Tf recycling by 63.6 ± 2.8% with no significant effect on the EGF recycling or degradation rate (Figure 3B). These data suggested that actinin-4 plays a role in efficient recycling of the Tf receptor.

The reduction in Tf recycling by depletion of actinin-4 was not accompanied by an alteration in its degradation. This suggested that reduction in the rate of recycling reflected an alteration in the trafficking of recycling cargo. We examined the localization of internalized Tf-Alexa 594 after cells were transfected with either control (scrambled) or actinin-4-specific RNA duplexes (Figure 3C). In the presence of actinin-4, Tf moved through the recycling endosome and seemed to be largely recycled after a 30 min chase period, consistent with previous data (Hopkins and Trowbridge, 1983; Hopkins *et al.*, 1985; Mayor *et al.*, 1993; Sheff *et al.*, 2002). In contrast, Tf-Alexa 594 was concentrated in the recycling endosome after 30 min in cells lacking actinin-4 (Figure 3C). This suggested that the decreased recycling rate observed after depletion of actinin-4 likely reflected either decreased egress or rate of movement from the early endosome to the recycling endosome. Equally probable is that a greater percentage of Tf took the longer route through the recycling endosome because the pathway for direct recycling from the early endosome was inhibited. Thus, the reduction in rate of recycling after actinin-4 depletion reflected an alteration in the trafficking of recycling cargo, perhaps by diverting it through a longer recycling pathway.

Disruption of Hrs/Actinin-4 Binding Affects Tf Trafficking

Because $hrs_{(1-449)}$ was the minimal fragment required for actinin-4 binding (Figure 1C) and we deduced the actinin-4 binding site to be $actinin\text{-}4_{(359-412)}$ (based on the minimal interacting fragment of actinin-4 found in our two-hybrid screen), we tested whether these regions might inhibit the binding of full-length hrs to actinin-4. In the presence of increasing amounts of $hrs_{(1-449)}$ or $actinin\text{-}4_{(357-469)}$, the binding of hrs to actinin-4 was reduced as much as 99%, which was likely due to competition with full-length hrs for actinin-4 binding (our unpublished data). The role of the hrs/actinin-4 interaction was probed by expressing the fragment of

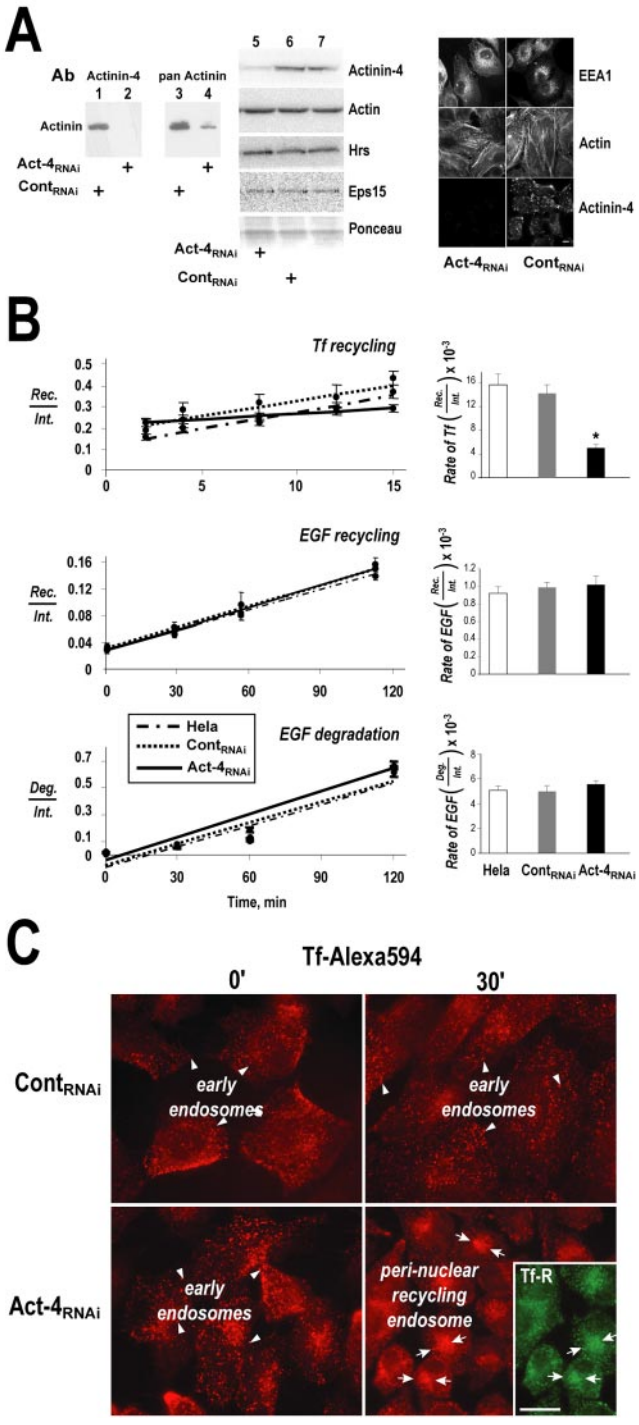

Figure 3. RNA interference specifically depletes actinin-4 and inhibits transferrin receptor recycling. (A) Extracts from HeLa cells transfected with either duplexed RNA oligonucleotides directed against the actinin-4 sequence (Act-4_{RNAi}) or a random RNA sequence ($Cont_{RNAi}$) were subjected to quantitative western-blots for actinin-4 (lanes 1 and 2). The same samples were probed with a pan actinin antibody that likely recognizes all actinin isoforms (lanes 3 and 4). Extracts also were probed with antibodies to actin, hrs, or eps15 (lanes 5–7). Ponceau S staining shows equal protein loading. HeLa cells were plated on coverslips (right), transfected with either Act-4_{RNAi} or $Cont_{RNAi}$, and probed with antibodies against EEA1, actin, and actinin-4. Bar, 4 μm. (B) Actinin-4 was depleted from HeLa cells as described above. Cells were incubated with ^{125}I-epidermal growth factor or ^{125}I-transferrin as described in *Materials and Methods*. At each time point, the amount of Tf or EGF internalized (Int.), recycled (Rec.), and degraded (Deg., EGF only) was determined as described (see Materials and Methods). The ratio of the amount of recycled or degraded to the amount internalized is plotted over time for Tf and EGF. Histograms of the mean slope (rate) of these ratios are shown for each condition (n = 3). The rate of recycling of transferrin was significantly inhibited by depletion of actinin-4 (n = 3, p = 0.0021; recycling rate in actinin-4–depleted cells, 0.0051 ± 0.0004; scrambled, 0.014 ± 0.001). The rate of recycling and degradation of EGF were not altered by depletion of actinin-4 (rate of recycling: scrambled, 0.001, and actinin-4, 0.001, n = 3; rate of degradation: scrambled, 0.0052, and actinin-4, 0.0057, n = 3). C. HeLa cells transfected with either Act-4_{RNAi} or $Cont_{RNAi}$ were loaded with Tf-Alexa594 at 0°C for 60 min then shifted to 37°C for 10 min (0′). After 0- or 30-min chase period the location of Tf-Alexa594 was determined. Early endosomes (arrowheads alone) and perinuclear recycling endosomes (arrows) are indicated. The inset of the Act-4_{RNAi} panel at 30 min shows colocalization of the Tf receptor with Tf-Alexa 594. Bar, 16 μm.

hrs or actinin-4 required for hrs/actinin-4 binding (Figure 4B) in HeLa cells and then examining the trafficking of ^{125}I-epidermal growth factor and ^{125}I-Tf. Expression of hrs_{1-449} blocked the ability of actinin-4 to be precipitated with hrs (Figure 4A) and inhibited the rate of Tf recycling by 67.7 ± 3.1% (Figure 4C). Actinin-$4_{357-469}$ also blocked the ability of actinin-4 to be precipitated with hrs (Figure 4A) and inhibited the rate of Tf recycling by 46.1 ± 2.2% (Figure 4D) with no significant effect on the EGF recycling or degradation rate (our unpublished data). We observed no significant effect on the internalization of Tf or EGF after expressing the hrs or actinin-4 fragments hrs_{1-449} or actinin-$4_{357-469}$ (our unpublished data). Thus, blocking the hrs/actinin-4 interaction by using fragments of either hrs or actinin-4 produced the same effect as reducing the levels of actinin-4, a marked inhibition of Tf recycling with no significant effect on EGF trafficking or Tf internalization. This suggested that the hrs/actinin-4 interaction was required for efficient recycling of Tf.

Actinin-4 Is Present in a Complex Containing Hrs, BERP, and Myosin V

Sorting endosomes or vesicles that bud from this compartment must move to the plasma membrane to recycle cargo. It seems likely that this vectorial movement would involve the cytoskeleton and a molecular motor that propels the membrane compartment to the plasma membrane. Interestingly, myosin Vb, a processive motor (Yildiz *et al*., 2003), is present on early endosomes and is involved in receptor recycling (Evans *et al*., 1997; Lapierre *et al*., 2001). Moreover, myosin V binds to BERP (El-Husseini and Vincent, 1999), a ring-finger protein of unknown function that also interacts with actinin-4 (El-Husseini *et al*., 2000). We reasoned that an hrs/actinin-4/BERP/myosin V protein complex could link sorting endosomes with the actin cytoskeleton and enable their motility for receptor recycling.

We first examined whether BERP was enriched on early endosomes because its cellular location was unknown. HeLa cell lysate contained the early endosomal marker protein EEA1, as well as hrs, actinin-4, and BERP proteins (Figure 5A). Isolation of early endosomes that did not contain detectable levels of the plasma membrane marker Na^+/K^+ ATPase, the ER marker calnexin, or the lysosomal marker LAMP1 (Figure 5A) that are enriched for the endosomal marker EEA1 and hrs (Yan *et al*., 2004) were also enriched in actinin-4 and BERP, suggesting that BERP was present on early endosomes (Figure 5A).

We next immunoprecipitated BERP from HeLa cells and found that actinin-4, hrs, and myosin Vb were coprecipi-

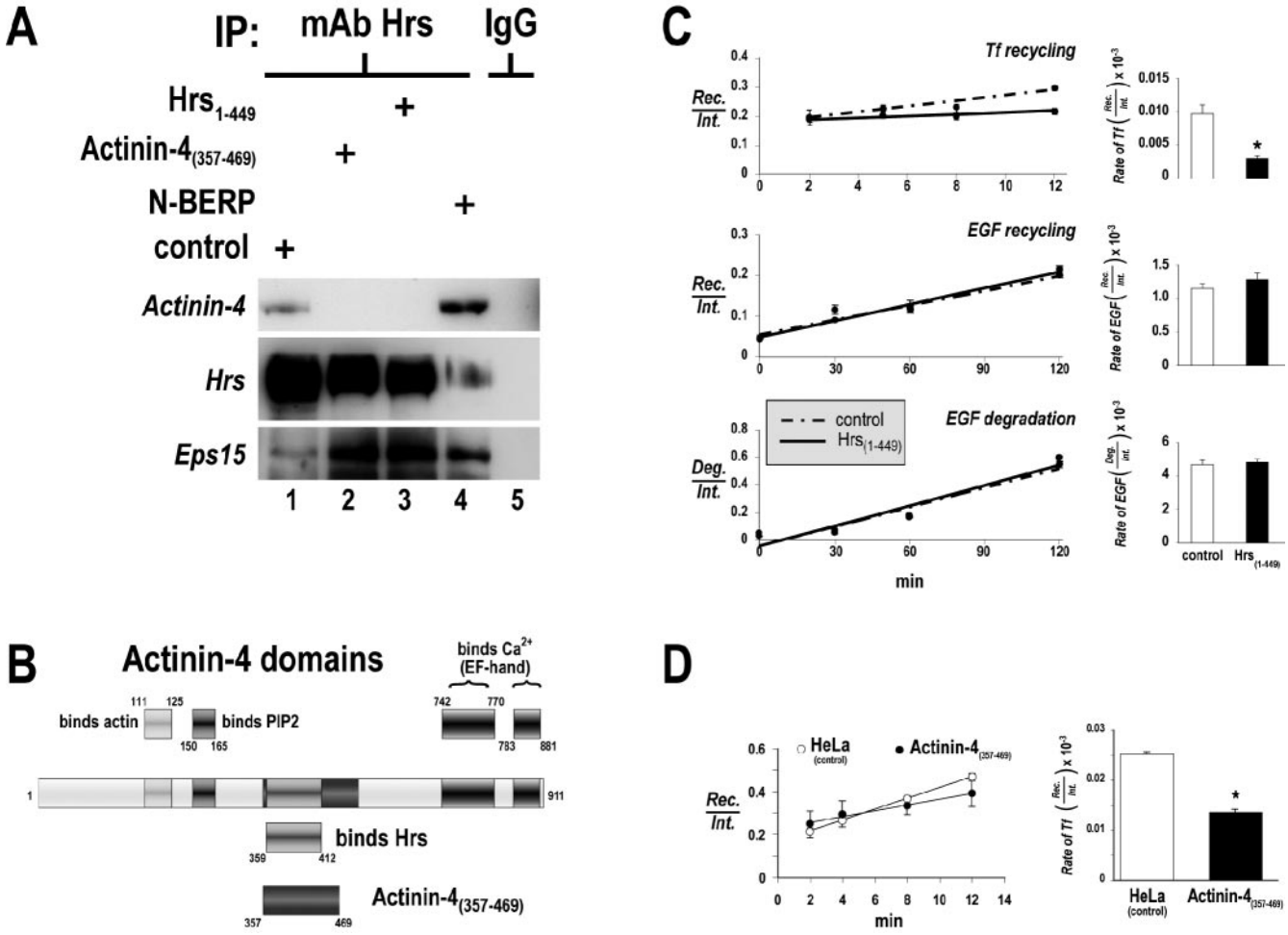

Figure 4. Inhibition of hrs/actinin-4 interaction reduces the rate of transferrin recycling. (A) Expression of $hrs_{(1-449)}$ or actinin-$4_{(357-469)}$ inhibits the interaction of hrs and actinin-4 in situ. Immunoprecipitation of endogenous hrs coprecipitates actinin-4 in wt control HeLa cells (lane 1), whereas expression of either actinin-$4_{(357-469)}$ (lane 2) or $hrs_{(1-449)}$ (lane 3), but not N-BERP (lane 4) inhibited the coprecipitation. Moreover, eps15 was coprecipitated with hrs under all conditions. Mouse IgG did not precipitate any proteins. (B) The $hrs_{(1-449)}$ fragment was expressed in HeLa cells as described in *Materials and Methods*. The fate of both transferrin and EGF was then determined and represented as in Figure 3. (C) Schematic representation of the domain structure of actinin-4 with the region required for hrs binding (binds hrs), and a depiction of the region used to inhibit hrs/actinin-4 binding (actinin-$4_{357-469}$). Expression of $hrs_{(1-449)}$ also significantly inhibited transferrin recycling (rate of control is 0.0096 ± 0.0012, $hrs_{(1-449)}$ is 0.0031 ± 0.0003, n = 3, p = 0.001). Expression of $hrs_{(1-449)}$ did not significantly affect EGF recycling or degradation (recycling rate in control is 0.0012 ± 5.77E-05, $hrs_{(1-449)}$ is 0.0013 ± 8.82E-05, n = 3; degradation rate in control is 0.0047 ± 0.0003, $hrs_{(1-449)}$ is 0.0049 ± 0.0002, n = 3). (D) The actinin-$4_{(357-469)}$ fragment was expressed in HeLa cells as described in *Materials and Methods*. Expression of actinin-$4_{(357-469)}$ did not significantly affect EGF recycling or degradation (recycling rate in control, 0.0025 ± 7.83E-05; actinin-$4_{(357-469)}$, 0.0018 ± 1.09E-04, n = 12; degradation rate in control, 0.0028 ± 0.0001; actinin-$4_{(357-469)}$, 0.0021 ± 0.0001, n = 12). The kinetics of transferrin recycling is shown (left) as is a bar graph representing the rate of transferrin recycling (right, recycling rate in control, 0.0252 ± 0.00036; actinin-$4_{(357-469)}$, 0.0136 ± 0.000548). * denotes $p \leq 0.05$.

tated (Figure 5B, lane 1). To further understand the nature of this protein complex, we tested whether it would form by using recombinant proteins (Figure 5C). Incubation of hrs, actinin-4, and BERP with the tail domain of myosin V that had been immobilized on agarose beads, but not on beads with immobilized GST, revealed that a complex forms in the absence of other components (Figure 5C, lane 1). Importantly, if BERP was immobilized, both actinin-4 and hrs would bind (Figure 5C, lane 2), but if actinin-4 was immobilized only hrs would bind (Figure 5C, lane 3). No complex could form when the tail domain of myosin V was immobilized and hrs and actinin-4 were added in the absence of BERP, suggesting that BERP links myosin V with hrs and/or actinin-4 (Figure 5C, lane 4). However, if the tail domain of myosin V was immobilized and hrs and BERP were added in the absence of actinin-4, then only BERP was bound to myosin V (Figure 5C, lane 5), suggesting that hrs binds to the complex via interacting with actinin-4.

BERP Links Actinin-4 to Myosin V and Is Required for Tf Recycling

The CART complex, composed of hrs, actinin-4, BERP, and myosin V, seemed to require BERP to link myosin V to hrs/actinin-4. We examined the effect of the N-terminal region of BERP (Figure 5E) that binds to actinin-4 but not to myosin V, on the trafficking of ^{125}I-epidermal growth factor and ^{125}I-Tf. Expression of N-BERP blocked the ability of myosin Vb to coprecipitate actinin-4 (Figure 5D) and inhibited the rate of Tf recycling by 49.5 ± 7.7% (Figure 5F) with

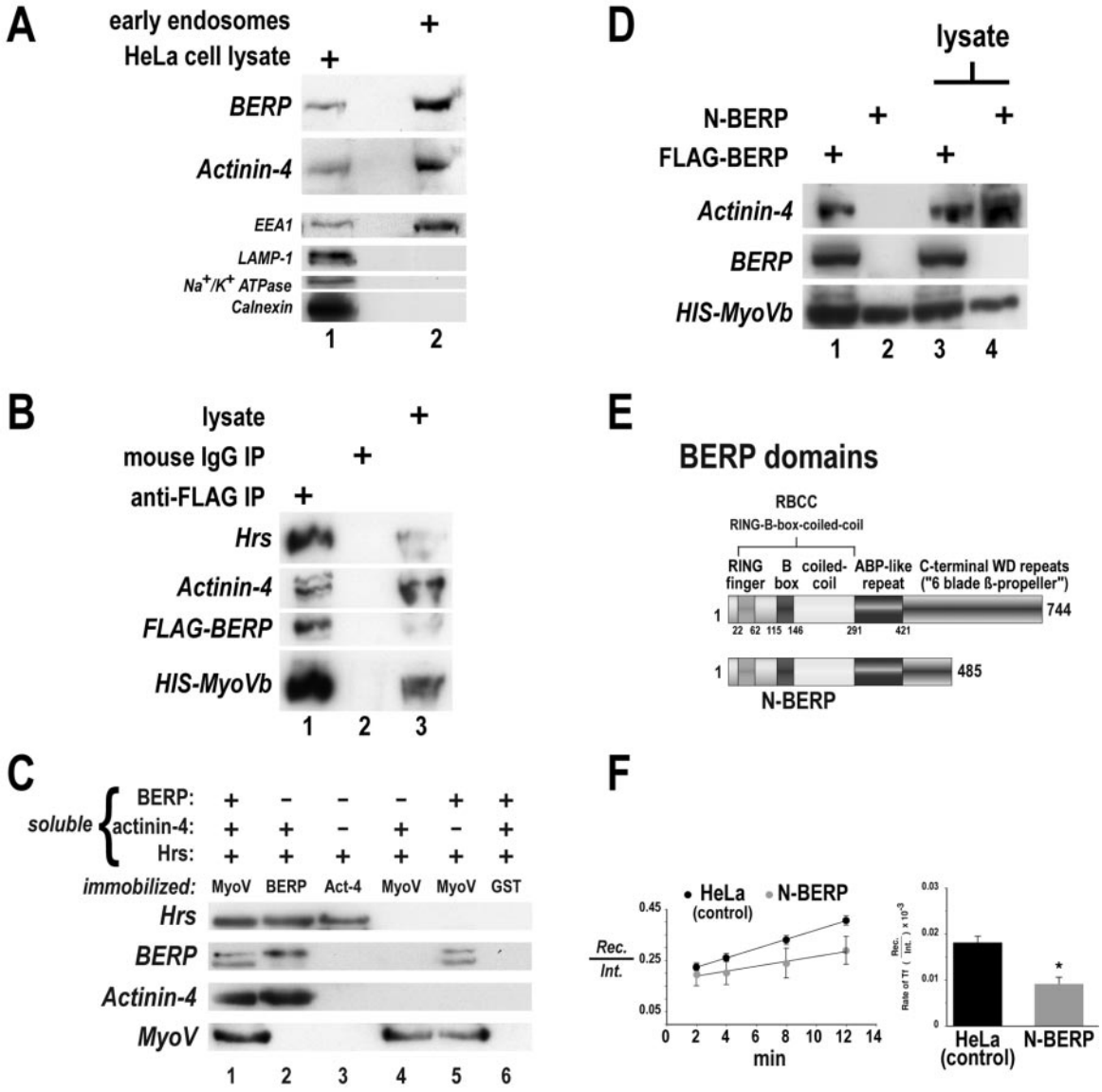

Figure 5. The CART complex is formed in situ and in vitro, and BERP interactions are required for transferrin recycling. (A) BERP and actinin-4 are enriched with early endosomal membranes. HeLa cell lysate and endosomal membranes were isolated as described previously (Sun *et al*., 2003; Yan *et al*., 2004), separated on SDS-PAGE, and examined for the presence of BERP and actinin-4. The endosomal fractions do not contain detectable Na^+/K^+ ATPase, calnexin, LAMP1, or rab 7, but they are enriched for the early endosomal marker protein, EEA1. (B) Immunoprecipitation of hrs from HeLa cell lysate. HeLa cells were transfected with myc-hrs, FLAG-BERP, and HIS-myosin V and precipitated with either FLAG antisera (lane 1) or mouse IgG (lane 2). The precipitated material was separated by SDS-PAGE, and resulting blots were probed with antisera against hrs, FLAG, actinin-4, and HIS. (C) Hrs, actinin-4, BERP, and myosin V interact in the absence of other proteins and assemble in an ordered manner. Recombinant proteins were either immobilized on Sepharose or purified in a soluble form and incubated in various combinations (as indicated). All proteins bound when incubated with immobilized myosin V (lane 1), actinin-4 and hrs bound to immobilized BERP (lane 2), and hrs bound to immobilized actinin-4 (lane 3). Neither hrs nor actinin-4 bound to immobilized myosin V in the absence of BERP (lane 4). Hrs did not bind to immobilized myosin V in the presence of BERP, but not actinin-4 (lane 5). None of the recombinant proteins bound to immobilized GST (lane 6). (D) Expression of BERP (lane 3), but not N-BERP (lane 2), allowed coprecipitation of actinin-4 with myosin Vb. Although BERP is seen in the lysate (lane 3), N-BERP is not seen in this lane because of significantly smaller size. However, it was detected when separated on another gel, transferred, and probed with FLAG antibody (our unpublished data). (E) The domain structure of BERP and the N-BERP fragment. (F) The N-terminal domain of BERP inhibits the rate of transferrin recycling. Linear domain structure of BERP and N-BERP (top, see text for details). The N-terminal domain of BERP binds to actinin-4, but not to myosin V, which allows the formation of the hrs/actinin-4/N-terminal BERP complex but inhibits association with myosin V. In the presence of this BERP fragment, the transferrin recycling rate was significantly slowed. Tf recycling rates for the HeLa control were 0.0180 ± 0.0013 (n = 6) and for N-BERP, 0.0091 ± 0.0014 (n = 6). * denotes $p \leq 0.05$.

no significant effect on the EGF recycling or degradation rate (our unpublished data). We observed no significant effect on the internalization of Tf or EGF after expressing the N-BERP fragment (our unpublished data).

DISCUSSION

The number of receptors and their residence time on the plasma membrane are critical determinants for the response

of a cell to extracellular cues. Recycling is an efficient mechanism to ensure rapid delivery of receptors to the plasma membrane and to regulate membrane dwell time. Although much examination has resulted in a detailed view of the internalization of plasma membrane receptors, the molecular mechanisms of their recycling are yet to emerge with the same clarity. Recent results have suggested a role for myosins in recycling (Neuhaus and Soldati, 2000; Lapierre *et al*., 2001), implying a role for the actin cytoskeleton in this process. Studies using drugs that depolymerize actin filaments also implicate actin at a recycling step (Durrbach *et al*., 1996; Cao *et al*., 1999; Zaslaver *et al*., 2001; Sheff *et al*., 2002; Engqvist-Goldstein and Drubin, 2003). We have found that an endosome-associated protein, hrs, is part of a protein complex that includes actinin-4, BERP, and myosin Vb, designated CART. The CART complex is required for efficient recycling of Tf receptors to the plasma membrane.

The interaction of an endosome-associated protein, hrs, with actinin-4, a nonmuscle actin binding protein, was unexpected. However, the hrs/actinin-4 interaction was observed using the two-hybrid approach and confirmed by both recombinant protein binding and immunoprecipitation experiments. The interaction seems specific for actinin-4, because other actinin isoforms were not found in the yeast two-hybrid screen. Actinin-4 is a member of a protein family that contains conserved structural and functional motifs. These motifs include: an F-actin binding domain, a pleckstrin homology domain containing a phosphatidylinositol bisphosphate binding site, and two EF-hand calcium-binding domains (Honda *et al*., 1998). Little is known about the function of actinin-4, although an actin-bundling activity is suggested by the known activity of other actinin family members (Honda *et al*., 1998). We have observed that actinin-4 does indeed bind to filamentous actin and has bundling, but not nucleation, activity (Lotfi and Bean, unpublished observations). In macrophages, actinin-4 is transiently associated with macropinosomes before their fusion with lysosomes, as well as with phagosomes (Araki *et al*., 2000), suggesting a role for this molecule in two mechanistically similar types of endocytic trafficking (Racoosin and Swanson, 1993). Interestingly, the depletion of actinin-4 or disruption of hrs/actinin-4 interaction results in a decrease in Tf recycling with no effect on EGF recycling or degradation and no effect on internalization of either Tf or EGF. These data suggest a specific role for actinin-4 and the hrs/actinin-4 interaction in receptor recycling from the early endosome to the plasma membrane.

We considered the existence of an hrs/actinin-4/BERP/myosin V complex based on the binary interactions between hrs and actinin-4, actinin-4 and BERP (El-Husseini *et al*., 2000), and BERP with myosin V (El-Husseini and Vincent, 1999). BERP is a ring-finger containing protein that interacts with the tail domain of myosin V (El-Husseini and Vincent, 1999) through its C-terminal region and interacts with actinin-4 through its N-terminal region (El-Husseini *et al*., 2000). BERP may function as an adapter protein between actinin-4 that has bound to endosomes and myosin V because it is enriched with endosomal membranes. Many ring finger-containing proteins have ubiquitin ligase activity that would be particularly interesting because receptor sorting is thought to be regulated by this protein modification. We have examined whether BERP is self-ubiquitinated or will ubiquitinate proteins from *E. coli* lysate, although we have not detected ligase activity with either potential substrate (Lotfi and Bean, unpublished observations). The CART complex most likely has an ordered assembly for its formation. Specifically, the binding of actinin-4 to hrs is required for BERP to bind and the binding of BERP to actinin-4 is required for myosin V to bind. Disruption of any of the binary interactions comprising the quaternary complex inhibits Tf recycling, as does disengaging myosin V from actin (Lapierre *et al*., 2001). These data suggest that the CART complex is required for a specific transport step necessary for recycling to the plasma membrane.

A role for the actin cytoskeleton in endocytosis has been controversial (Durrbach *et al*., 1996; Riezman *et al*., 1997; Fujimoto *et al*., 2000; Qualmann *et al*., 2000; Gruenberg, 2001; Engqvist-Goldstein and Drubin, 2003). In mammalian cells, many reports describe the dependence or independence of an intact actin cytoskeleton for early endocytic events such as internalization (Durrbach *et al*., 1996; Cao *et al*., 1999; Fujimoto *et al*., 2000; Qualmann et al., 2000; Gruenberg, 2001; Zaslaver *et al*., 2001). However, these studies have not focused on later events such as endosome motility or recycling. Studies using drugs that depolymerize actin filaments implicate actin at a recycling step (Durrbach *et al*., 1996; Cao *et al*., 1999; Zaslaver *et al*., 2001; Sheff *et al*., 2002). Furthermore, mutations or deletions of myosin subtypes associated with early endosomes specifically alter recycling from early endosomal compartments, further suggesting a role for actin in the motility of endocytic vesicles at a recycling step (Neuhaus and Soldati, 2000; Lapierre *et al*., 2001). Our data suggest a specific role for the CART complex in endosomal motility as well as a molecular mechanism linking endosomes to actin filaments essential for a particular step in the endocytic pathway. We propose that the CART complex facilitates recycling of molecules that are internalized and recycled by a constitutive, but not a ligand-induced, mechanism.

There are three myosin V family members in mammals (Mercer *et al*., 1991; Zhao *et al*., 1996; Rodriguez and Cheney, 2002) that have been implicated in genetic diseases and membrane trafficking (Mooseker and Cheney, 1995; Hasson and Mooseker, 1996; Titus, 1997). A role for myosin Vb in endocytic trafficking, specifically at a recycling step (Lapierre *et al*., 2001; Volpicelli *et al*., 2002), has been suggested based on the ability of the myosin Vb tail domain to retard recycling of the M4 muscarinic acetylcholine receptor (Volpicelli *et al*., 2002) and the Tf receptor (Lapierre *et al*., 2001) and to result in the accumulation of Tf in pericentriolar vesicles (but see Provance *et al*., 2004). Overexpression of tail fragments prevents binding of the myosin to its cargo, disengaging it from actin. The model proposed for the role of myosin Va in melanosome transport (Gross *et al*., 2002) suggests that retrograde microtubule-based transport by dynein antagonizes anterograde transport by kinesin. Myosin V contributes to anterograde transport by capturing melanosomes in the cell periphery. Similar models have been proposed for chromaffin cell exocytosis (Rose *et al*., 2003) and for vesicle transport in neurons (Bridgman, 1999). The slow recycling kinetics and the pericentriolar localization of Tf when actinin-4 is depleted or when the hrs/actinin-4, actinin-4/BERP, or BERP/myosin V interactions are disrupted are consistent with this model.

The association of endocytic organelles with cytoskeletal networks would allow guided vesicular trafficking to subsequent cellular compartments. The complementary roles played by the actin cytoskeleton and microtubule network in the endocytic pathway (van Deurs *et al*., 1995; Durrbach *et al*., 1996; Maples *et al*., 1997; Murray *et al*., 2000) suggest that endosomes contain proteins allowing for movement on both types of cytoskeletal network. Small GTPases of the rab family have been suggested to be required for endosome motility and recycling. For example, rab5a is present on

endosomes, is involved in endosome-endosome fusion (Gournier *et al*., 1998), in linking early endosomes to microtubules, and in minus-end–directed movement (Nielsen *et al*., 1999), although rab5a has not yet been demonstrated to interact with any motor protein. Rab 4 and rab 11 are endosome-associated proteins thought to be involved in recycling (Van Der Sluijs *et al*., 1991; McCaffrey *et al*., 2001; Lindsay and McCaffrey, 2002; Peden *et al*., 2004) but their precise roles and which of the many possible effectors are relevant for this function remains unclear (Nagelkerken *et al*., 2000; Cormont *et al*., 2001; van der Sluijs *et al*., 2001; Lindsay *et al*., 2002; Fouraux *et al*., 2004; Peden *et al*., 2004). The present data suggest that hrs may link endosomes to actin through actinin-4, BERP, and myosin Vb. Thus, rab 4, 5, and 11 proteins and hrs can associate with endosomal vesicles and may be involved in sequential stages of endosomal motility/maturation. For example, rab5 may allow for microtubule-dependent trafficking of early endosomes, whereas hrs is involved in actin-based trafficking and has a specific role in recycling from the early endosome.

Our proposal for a role of hrs in endosomal recycling is consistent with data showing that inactivation of its yeast ortholog, Vps27p, results in accumulation of both recycling Golgi proteins and endocytosed proteins in a class E compartment and suggests a generalized role of this protein and other class E proteins in endosomal recycling (Piper *et al*., 1995). The role of hrs in endosomal recycling is not inconsistent with a hypothesis suggesting that hrs or Vps27p functions in endocytic protein sorting (Katzmann *et al*., 2001; Bilodeau *et al*., 2002; Lloyd *et al*., 2002) and in early endosome fusion (Sun *et al*., 2003). These studies have suggested that hrs/Vps27p is linked with proteins required for the ubiquitination and sorting of cargo (Katzmann *et al*., 2001; Bilodeau *et al*., 2002) and that hrs inhibits homotypic early endosome fusion (Sun *et al*., 2003). Hrs/Vps27p may bind ubiquitinated cargo with its UIM domain (Bilodeau *et al*., 2002; Polo *et al*., 2002; Shih *et al*., 2002), which is required for its cargo sorting function because mutation of that domain blocks sorting of ubiquitinated cargo proteins (Bilodeau *et al*., 2002; Shih *et al*., 2002). The endosomal sorting function also has been hypothesized to require a protein complex called ESCRT I (Katzmann *et al*., 2001). Hrs has been suggested to recruit the ESCRT 1 complex to early endosomes. The role of hrs in recruiting sorting or signaling components to the endosomal membrane likely is a function of a number of factors, including its oligomerization (Bean *et al*., 1997) and/or competition among binding proteins (Bean *et al*., 2000). Therefore, hrs may bind to an endosomal receptor, SNAP-25, by using its Q-SNARE domain and inhibit endosomal fusion (Sun *et al*., 2003), whereas it is involved in cargo sorting and/or endosome motility using N-terminal VHS, FYVE, or UIM domains or via actinin-4 interactions. Thus, a sorting step might occur before, or coincident with, the inhibition of fusion. Subsequently, if endosomes destined for different cellular destinations used a diverse assortment of molecules to associate with various cytoskeletal elements, this would allow them to achieve the sorting and distinct routing required for separating receptors to be recycled from those to be degraded.

We have presented a model for the role of the CART complex and the actin cytoskeleton in endosome recycling (Figure 6). Because much of hrs is cytosolic, it likely cycles on and off the early endosomal membrane (Komada *et al*., 1997; Tsujimoto *et al*., 1999), perhaps via binding to a protein receptor (Figure 6, step 1), before binding actinin-4. Our data suggest a sequential association of complex components such that once the actinin-4 has bound hrs, BERP may bind, followed by myosin V although subcomplexes may form and unite. Association of the endosomal CART complex with actin filaments would facilitate rapid movement of the

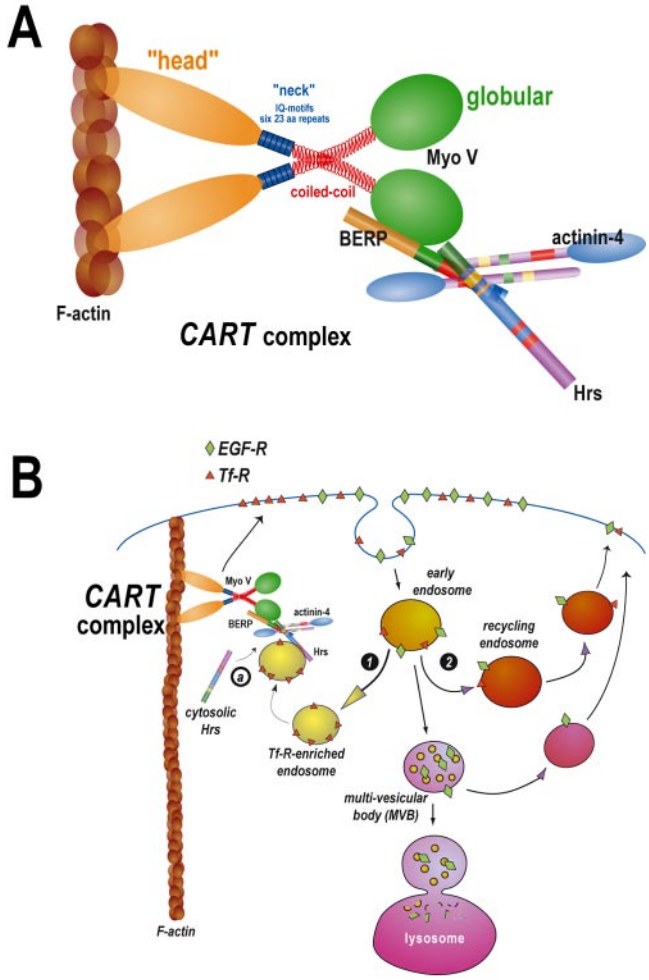

Figure 6. Model of how Tf-containing recycling endosomes may link to actin filaments via the CART complex. (A) Recombinant protein binding experiments suggest that there is an ordered assembly for the complex: 1) hrs binds to actinin-4; 2) BERP binds to hrs/actinin-4; and 3) myosin V binds to hrs/actinin-4/BERP. BERP has been shown to bind to myosin V via its tail domain, whereas myosin V binds to actin filaments and moves processively via its head domain. Actinin-4 binds to BERP in its C-terminal region and to hrs in the region after the EF hands. The quaternary complex could bind to actin filaments or the tertiary hrs/actinin-4/BERP complex might bind to myosin V already attached to actin filaments. (B) Model for the role of the CART complex and the actin cytoskeleton in endosome recycling. Because much of hrs is cytosolic, it likely cycles on and off the early endosomal membrane (Komada *et al*., 1997; Tsujimoto *et al*., 1999), perhaps via binding to a protein receptor (SNAP-25, step a), before actinin-4 binding. This would occur after, or coincident with, endosomal sorting of Tf-R (step 1). Once the hrs/actinin-4 complex was formed, association with BERP and myosin V would facilitate rapid movement of Tf-R-enriched endosomes back to the plasma membrane on actin filaments. The majority of EGF receptor would bypass this recycling step (step 2) after ligand-induced internalization and would instead travel through the MVB en route to the lysosome for degradation. A minority of EGF receptor would recycle to the plasma membrane (from the MVB or recycling endosome) along with a fraction of the Tf receptor (through the recycling endosome), in CART complex-independent pathways.

endosome back to the plasma membrane. The majority of EGF receptor would bypass this recycling step (Figure 6, step 2) after ligand-induced internalization and would instead travel through the multivesicular body (MVB) en route to the lysosome for degradation. A minority of EGF receptor would recycle to the plasma membrane (from the MVB or recycling endosome) along with some Tf receptor (through the recycling endosome), in slower CART complex-independent pathways. The large number and varied function of molecules that undergo constitutive endocytosis suggests that the CART molecular interaction may be critical for many cellular functions.

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