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COMMUNICATION

Hrs inhibits citron kinase-mediated HIV-1 budding via its FYVE domain

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ABSTRACT

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) is a key component of the endosomal sorting complexes required for transport and has been demonstrated to play a regulatory role in endocytosis/exocytosis and the accumulation of internal vesicles in multivesicular bodies. Citron kinase is a Ser/The kinase that we previously reported to enhance human immunodeficiency virus type 1 (HIV-1) virion production. However, the relationship between Hrs and citron kinase in HIV-1 production remains elusive. Here, we report that Hrs interacts with citron kinase via its FYVE domain. Overexpression of Hrs or the FYVE domain resulted in a significant decrease in HIV-1 virion production. Depletion of Hrs by RNA interference in HEK293T cells increased HIV-1 virion production and enhanced the activity of citron kinase. These data suggest that Hrs inhibits HIV-1 production by inhibiting citron kinase-mediated exocytosis.

KEYWORDS citron kinase, Hrs, HIV-1 budding

INTRODUCTION

Viral particle budding is a key step in the life cycle of human immunodeficiency virus type 1 (HIV-1) replication. HIV-1 structural protein $Pr55^{Gag}$ can direct virion assembly and budding in the absence of other HIV-1 viral proteins. HIV-1 Gag is composed of four distinct domains: the matrix (MA), capsid (CA), nucleocapsid (NC), and late (L) domains. The N-terminus of the MA domain contains a series of basic amino acids, which are required for the interaction with acidic phospholipids, and a myristyl group (Paillart and Gottlinger, 1999), which is believed to facilitate Gag targeting to membranes (Zhou and Resh, 1996; Spearman et al., 1997; Tang et al., 2004). The L domain catalyzes the pinching of viral particles from the cell surface (VerPlank et al., 2001; Bouamr et al., 2003; Strack et al., 2003). The PAT/SP and YPXL tetrapeptide motifs in the L domain of HIV-1 Gag, which interact with tsg101 and Alix, respectively, are required for viral budding (VerPlank et al., 2001; Pornillos et al., 2002; Strack et al., 2003; Zhai et al., 2008). HIV-1 facilitates its budding through the endosomal sorting complexes required for transport (ESCRTs), which include ESCRT 0, ESCRT I, ESCRT II, and ESCRT III. ESCRT complexes are sequentially recruited to budding sites to facilitate viral budding, with ESCRT III ultimately dissociated by the VSP4 ATPase (Stuchell-Brereton et al., 2007; McCullough et al., 2008).

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) was first identified as a protein that is phosphorylated in response to hepatocyte growth factor. The N-terminal region of Hrs contains VHS and FYVE domains, and the C-terminal region contains a glutamine/proline-rich domain. The FYVE domain (which shares a conserved sequence with Fab1, YOTB, Vac1, and EEA1) is a small, cysteine-rich domain that binds to two zinc ions. FYVE domain-containing proteins have diverse biological functions, including endocytic transport, regulation of endosomal membrane fusion, and signal transduction (Mills et al., 1998; Simonsen et al., 1998). Hrs contains four PXXP motifs that potentially interact with SH3-containing proteins and a PPXY motif that is the putative binding site for WW-domain (Kwong et al., 2000). In addition, Hrs contains a ubiquitin-interacting motif (UIM), which is presumed to bind to various ubiquitinated proteins (Pridgeon et al., 2009).

Hrs interacts with tsg101, which further recruits other ESCRT I subunits, as well as the ESCRT II and ESCRT III

complexes (Bache et al., 2003). The C-terminal portion of Hrs alone interacts with tsg101 and interferes with HIV-1 Gag particle production (Bouamr et al., 2007). However, the mechanism by which Hrs suppresses HIV-1 virion production remains unclear.

We previously reported that citron kinase, a Ser/The kinase, co-localizes with HIV-1 Gag and enhances HIV-1 virion production (Loomis et al., 2006). Overexpression of citron kinase increases HIV-1 virion production, whereas knockdown of citron kinase reduces virion production. We further mapped the third and fourth leucine zipper domains RBD domain and the zinc finger domain to be the shortest sequences required for HIV-1 production enhancement. In the present study, we report that Hrs interacts with citron kinase through its FYVE domain and inhibits citron kinase-mediated enhancement of HIV-1 budding.

RESULTS

Citron kinase co-localizes with Hrs in the early endosome

We previously reported that citron kinase co-localizes with HIV-1 Gag on the membrane of certain cytoplasmic compartments (Loomis et al., 2006). There are several types of cytoplasmic membrane organelles, including the early endosome, multivesicular bodies (MVBs), recycling endosome, the late endosome, and lysosome. To determine whether citron kinase is specifically located in any of these endosomes, it was co-expressed with the endosomal markers FYVE-GFP (early endosome/MVB marker), EHD1-GFP (recycling endosome marker), and Lamp-1-GFP (late endosome/lysosome marker), and the locations of citron kinase and these markers were observed by confocal microscopy. As shown in Fig. 1A–C, citron kinase displayed no obvious co-localization with EHD1-GFP (Fig. 1A) or Lamp-1-GFP (Fig. 1C). In contrast, citron kinase did co-localize with FYVE-GFP (Fig. 1B), indicating that citron kinase was located in the early endosome/MVB.

The FYVE domain used above was derived from the *Mus musculus* Hrs. To explore whether citron kinase co-localizes with Hrs, myc-tagged citron kinase and FLAG-tagged Hrs were co-expressed in HeLa cells and analyzed for co-localization by immunostaining. The results demonstrate that citron kinase partially co-localizes with Hrs (Fig. 1D).

To determine whether the Hrs FYVE domain is essential for co-localization of citron kinase and Hrs, myc-tagged citron kinase and HA-tagged HrsΔFYVE, in which the FYVE domain was deleted, were co-expressed in HeLa cells and analyzed for co-localization by immunostaining. We found that citron kinase failed to co-localize with HrsΔFYVE (Fig. 1E), suggesting that the FYVE domain is the binding module for citron kinase and Hrs.

Hrs interacts with citron kinase via its FYVE domain

The above results suggest that citron kinase interacts with the FYVE domain of Hrs. To test this possibility, FLAG-tagged Hrs and myc-tagged citron kinase were co-expressed in HEK293T cells, and the interaction between the two proteins was assayed by co-immunoprecipitation. Indeed, immunoprecipitation of citron kinase co-precipitated Hrs (Fig. 2A). To further determine whether the FYVE domain alone interacts with citron kinase, FYVE-GFP was co-expressed with citron kinase in HEK293T cells, and its interaction with citron kinase was analyzed. As expected, immunoprecipitation of citron kinase co-precipitated FYVE-GFP (Fig. 2B). These results establish that citron kinase interacts with Hrs via its FYVE domain.

Hrs inhibits citron kinase-mediated HIV-1 production

It has been reported that Hrs inhibits exocytosis and that citron kinase enhances HIV-1 production by promoting exocytosis. Considering the interaction between Hrs and citron kinase, we analyzed the effect of Hrs on citron kinase-mediated HIV-1 production. Full-length Hrs, the FYVE domain of Hrs, and Hrs△FYVE were co-transfected into HEK293T cells with HIV-1 vector-producing constructs in the presence or absence of citron kinase. The HIV-1 vector, which expresses a luciferase reporter, was harvested to infect HeLa cells, and luciferase activity was measured in both producer HEK293T cells and recipient HeLa cells. It has been reported that the expression of citron kinase or Hrs affects the number of virions produced from cells without changing the infectivity of the virions (Loomis et al., 2006; Bouamr et al., 2007). Hence, the luciferase activity reflects virus production. Here, the virus production efficiency is presented as the luciferase activity in the recipient cells divided by the normalized luciferase activity in the producer cells. Overexpression of full-length Hrs, the FYVE domain of Hrs, and Hrs△FYVE reduced HIV-1 production by approximately 4.5-, 5.3-, and 3.0-fold, respectively (Fig. 3; columns 3, 5 and 7). Consistent with our previous results, overexpression of citron kinase increased HIV-1 production by ~8-fold (Fig. 3; column 2). When citron kinase was co-expressed with full-length Hrs or the Hrs FYVE domain, the enhancement of HIV-1 production was significantly compromised (Fig. 3; comparing column 2 with columns 4 and 6). In comparison, the effect of HrsΔFYVE on citron kinase was much less significant (Fig. 3; column 8). Together, these results indicate that Hrs inhibits citron kinase-mediated HIV-1 production via its FYVE domain. The residual inhibitory effect of HrsΔFYVE suggests that Hrs may also inhibit HIV-1 production in a citron kinase-independent manner.

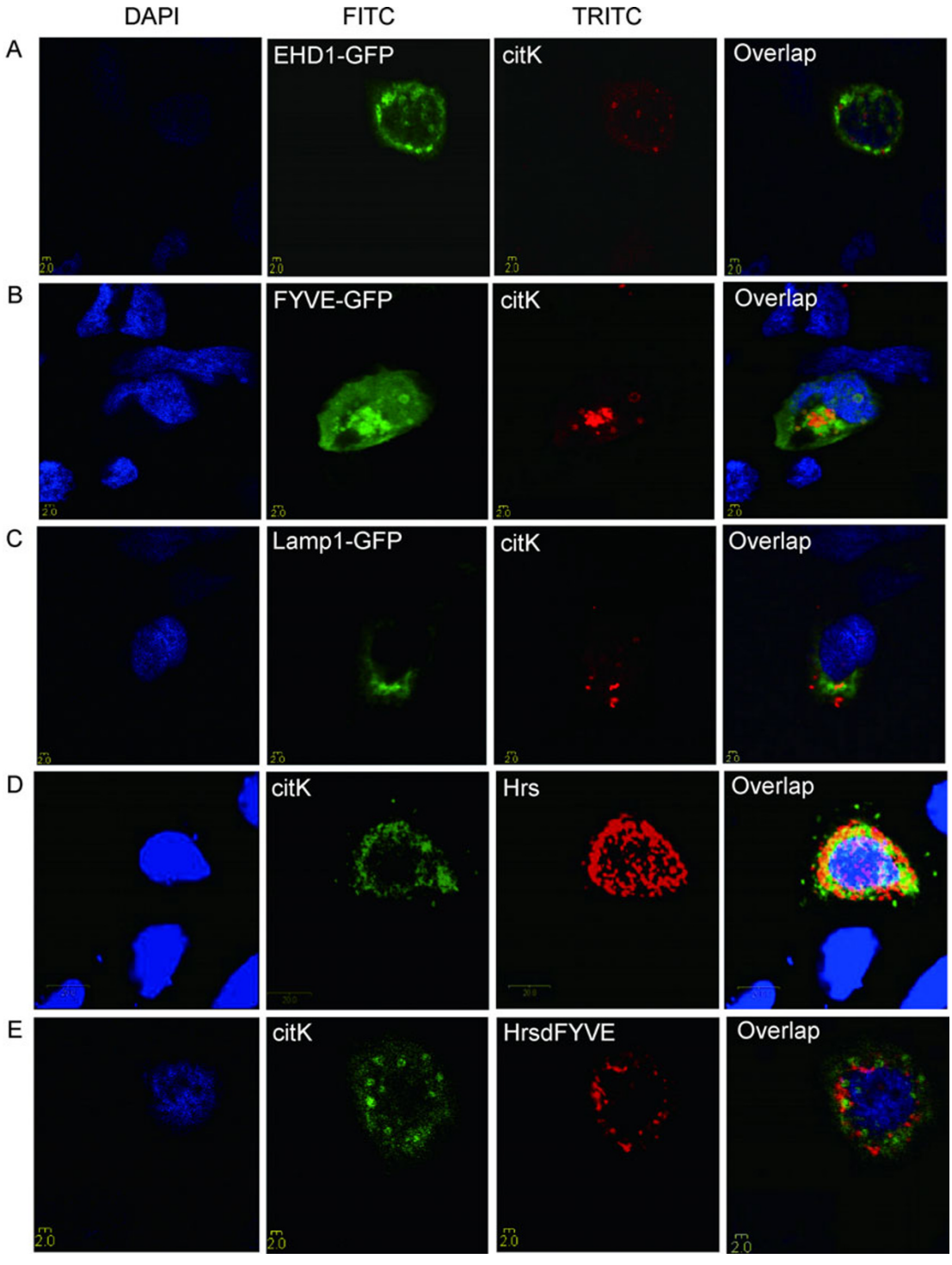

Figure 1. Citron kinase co-localizes with Hrs. HeLa cells were transfected with plasmids expressing myc-tagged citron kinase (citK) and endosomal markers EHD1-GFP (A), FYVE-GFP (B) or Lamp-1-GFP (C). At 24 h post-transfection, the cells were incubated with anti-myc antibody and then TRITC-conjugated secondary antibody. (D) HeLa cells expressing myc-tagged citron kinase (citK) and Flag-tagged Hrs were stained with rabbit polyclonal anti-citron antibody and mouse monoclonal anti-Flag antibody, and then FITC-conjugated goat anti-rabbit antibody and TRITC-conjugated anti-mouse antibody. (E) HeLa cells expressing myc-tagged citron kinase (citK) and HA-tagged HrsdFYVE were stained with rabbit polyclonal anti-citron antibody and mouse monoclonal anti-HA antibody, and then FITC-conjugated goat anti-rabbit antibody and TRITC-conjugated anti-mouse antibody. The cells were observed under an Olympus FV500 confocal microscope.

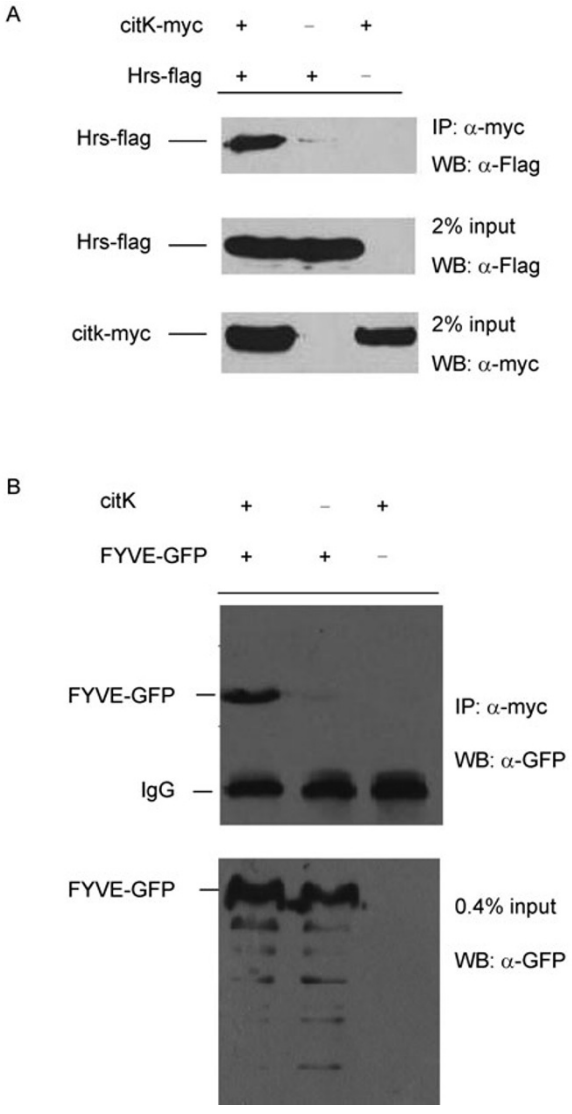

Figure 2. Hrs interacts with citron kinase. (A) HEK293T cells transfected with plasmids expressing myc-tagged citron kinase (citK) and Flag-tagged Hrs were lysed in CoIP buffer. The lysates were immunoprecipitated with anti-myc antibody, and detected by Western blotting using anti-Flag antibody. (B) HEK293T cells transfected with plasmids expressing myc-tagged citK and FYVE-GFP were lysed in CoIP buffer. The lysates were immunoprecipitated with anti-myc antibody, and detected by Western blotting using anti-GFP antibody.

Downregulation of Hrs improves HIV-1 production

To further determine whether Hrs inhibits citron kinase-mediated HIV-1 production under physiological conditions, Hrs levels were knocked down by RNA interference (RNAi). Plasmids expressing short hairpin RNAs (shRNAs) directed against different sequences of Hrs (Hrsi4 and Hrsi5) were transfected into HEK293T cells with the HIV-1 vector-producing constructs with or without citron kinase, and HIV-1 production efficiency was measured. The ability of the shRNAs to downregulate the expression of Hrs was first confirmed (Fig. 4A). Downregulation of Hrs reproducibly increased HIV production by ~2-fold (Fig. 4B; columns 3 and 5). Due to different experimental settings, the enhancement of HIV-1 production by the overexpression of citron kinase was not as significant as described above, i.e., only approximately 3-fold (Fig. 4B; column 2). In any event, the downregulation of Hrs augmented citron kinase-mediated enhancement of HIV-1 production (Fig. 4B; columns 4 and 6).

DISCUSSION

We previously demonstrated that citron kinase is essential for promoting HIV-1 budding by enhancing exocytosis (Loomis et al., 2006). Here, we report that citron kinase co-localizes with Hrs in early endosomes and MVBs by confocal microscopy. The interaction between citron kinase and Hrs was further confirmed by co-immunoprecipitation assays. Overexpression of Hrs and its FYVE domain attenuated citron kinase-mediated HIV-1 virion production, while depletion of Hrs enhanced HIV-1 production and augmented citron kinase-mediated enhancement of HIV-1 production. Hrs interacts with citron kinase via its FYVE domain. Expression of the Hrs FYVE domain alone displayed a significant inhibitory effect on citron kinase-mediated HIV-1 production. In contrast, Hrs△FYVE, an Hrs mutant in which the FYVE domain is deleted, displayed a much weaker effect on citron kinase-mediated HIV-1 production than full-length Hrs or its FYVE domain alone. Collectively, these results indicate that the FYVE domain is important for the inhibitory effect of Hrs on HIV-1 production.

Hrs and its isoform Hrs-2 play a negative role in exocytosis (Bean et al., 1997; Kwong et al., 2000). Citron kinase augments HIV-1 production by enhancing cellular exocytosis. Depletion of Hrs by RNAi facilitated citron kinase-mediated enhancement of HIV-1 production, suggesting that the inhibitory effect of Hrs on HIV-1 production occurs via endogenous citron kinase. Our results suggest that Hrs inhibits exocytosis by interacting with citron kinase, providing insight into the mechanism by which Hrs inhibits citron kinase-mediated HIV-1 production. It should be noted that although the virus production experiments were conducted in HEK 293T cells, our conclusions should be general. Indeed, it has been demonstrated in previous reports that HIV-1 uses the same budding pathways in T cells, HeLa cells, and HEK 293 cells (Ono and Freed, 2004; Dong et al., 2005; Finzi et al., 2007; Perez-Caballero et al., 2009).

In summary, our results indicate that Hrs interacts with citron kinase via its FYVE domain, and Hrs acts as an inhibitor of citron kinase-mediated HIV-1 virion production.

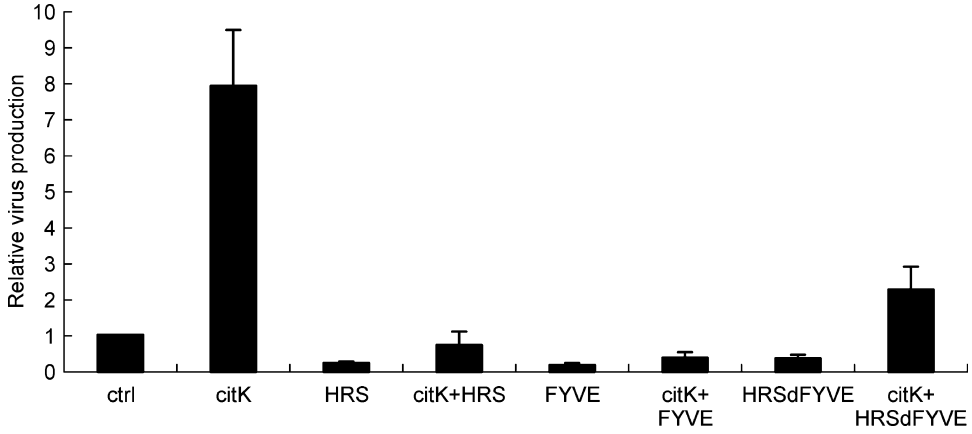

Figure 3. Overexpression of Hrs inhibits citK-mediated HIV-1production. The plasmids expressing the indicated proteins were cotransfected into HEK293T cells with the HIV-1 pseudovirus-producing constructs (pNL4-3luc and pVSVG), and pCMV-renilla, which expresses renilla luciferase and served as a control for transfection efficiency and sample handling. At 48 h post-transfection, the HIV-1 pseudovirus was harvested to infect HeLa cells. The luciferase activities were measured in the producer HEK293T cells and the recipient HeLa cells. The firefly luciferase activity in the producer cells was normalized by the renilla luciferase activity. Relative virus production was calculated as the luciferase activity in HeLa cells divided by the normalized luciferase activity in HEK293T cells. The data are means ± standard error of three independent measurements.

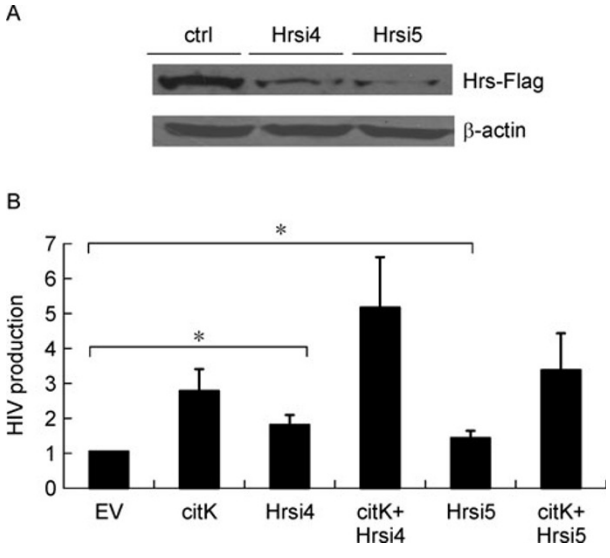

Figure 4. Depletion of Hrs increases HIV-1 production and facilitates citK-mediated HIV-1 production. The HIV vector-producing constructs (pNL4-3luc and pVSVG) were cotransfected into HEK293T cells with the plasmid expressing citron kinase, the plasmid expressing shRNA directed against Hrs (Hrsi4 and Hrsi5), and pCMV-renilla, which expressed renilla luciferase and served as a control for transfection efficiency and sample handling. At 48 h post-transfection, the HIV-1 pseudovirus was harvested to infect recipient HeLa cells. The luciferase activities were measured in the producer HEK293T cells and the recipient HeLa cells. The firefly luciferase activity in the producer cells was normalized by the renilla luciferase activity. Relative virus production was calculated as the luciferase activity in HeLa cells divided by the normalized luciferase activity in HEK293T cells. The data are means ± standard error of three independent measurements. *$p < 0.05$.

MATERIALS AND METHODS

Plasmids and cell lines

The plasmids pCMV-VSVG, pNL4-3-luc, and pCMV-renilla have previously been described (Mai and Gao, 2010). Plasmids pCAG, pCAG/citron kinase, pKU-Hrs, and pKU-HrsdFYVE have also been described previously (Loomis et al., 2006; Bouamr et al., 2007). To generate shRNAs directed against Hrs, oligonucleotides were annealed and cloned into pSuper.retro-puro using the *Bam*HI and *Hin*dIII sites. The sequences of the oligos are listed below: Hrs RNAi-4: 5′-GATCCCCAAGTGGAGGTAAACGTCCGTATTCAAGAGATACGGACGTTTACCTCCACTTTTTTTA-3′ (forward) and 5′-AGCTTAAAAAAAGTGGAGGTAAACGTCCGTATCTCTTGAATACGGACGTTTACCTCCACTTGGG-3′ (reverse); Hrs RNAi-5: 5′-GATCCCCAGGTAAACGTCCGTAACAATTCAAGAGATTGTTACGGACGTTTACCTTTTTTA-3′ (forward) and 5′-AGCTTAAAAAAGGTAAACGTCCGTAACAATCTCTTGAATTGTTACGGACGTTTACCTGGG-3′(reverse).

All the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Transfections were performed by either calcium phosphate precipitation or polyethylenimine transfection.

Immunofluorescence microscopy

HeLa cells were grown on coverslips, fixed in 4% paraformaldehyde, permeabilized in 0.4% Triton X-100, and incubated with primary and secondary antibodies. The primary antibody was either 1:500 anti-myc (mouse monoclonal, Sigma) or 1:200 anti-citron (rabbit polyclonal). The secondary antibody was TRITC-conjugated anti-mouse IgG, FITC-conjugated anti-mouse IgG, or FITC-conjugated goat anti-rabbit IgG (Sigma). The coverslips were mounted onto slides, and fluorescence images were acquired under an Olympus FV500 confocal fluorescence microscope.

Co-immunoprecipitation assays

Transfected HEK293T cells were lysed 48 h post-transfection with CoIP buffer (50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.5% NP-40) supplemented with a protease inhibitor cocktail. The lysates were clarified by centrifugation at 4°C for 10 min at 13,000 rpm. The supernatant was mixed with protein G plus-agarose (Santa Cruz Biotechnology) and the indicated antibodies and then incubated at 4°C for 2 h. The resins were washed 3–4 times with CoIP buffer, and the bound proteins were detected by Western blotting.

Infection assays

HEK293T cells were seeded in 6-well plates and transfected with pNL4-3luc and pCMV-VSVG to generate HIV-1 vector NL4-3-luc. At 48 h post-transfection, the supernatants were harvested. HeLa cells were seeded in 12-well plates and infected with the NL4-3-luc virus for 3 h, and then the infection medium was replaced with fresh medium. At 48 h post-infection, the cells were lysed, and luciferase activities were measured.

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ABBREVIATIONS

ESCRT, endosomal sorting complexes required for transport; ctrl, control plasmid; EV, empty vector; citK, citron kinase; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; HIV-1, human immunodeficiency virus type 1; FYVE domain, (Fab-1, YOTB, Vac1 and EEA1) domain; Hrsi4, shRNA directed against Hrs number 4; Hrsi5, shRNA directed against Hrs number 5; MVB, multivesicular bodies

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