

# The ORF3 Protein of Hepatitis E Virus Delays Degradation of Activated Growth Factor Receptors by Interacting with CIN85 and Blocking Formation of the Cbl-CIN85 Complex<sup>∇</sup>

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**Hepatitis E virus (HEV) causes an acute self-limiting disease that is endemic in developing countries. Previous studies suggested that the ORF3 protein (pORF3) of HEV is required for infection *in vivo* and is likely to modulate the host response. Our previous work showed that pORF3 localizes to early and recycling endosomes and causes a delay in the postinternalization trafficking of epidermal growth factor receptor (EGFR) to late endosomes/lysosomes. Here we report that pORF3 also delays the trafficking and degradation of activated hepatocyte growth factor receptor (c-Met) and delineate the mechanistic details of these effects. A mutant ORF3 protein, which does not localize to endosomes, also showed similar effects on growth factor receptor trafficking, making this effect independent of the endosomal localization of pORF3. The ORF3 protein was found to interact with CIN85, a multidomain adaptor protein implicated in the Cbl-mediated downregulation of receptor tyrosine kinases. This interaction competed with the formation of the growth factor receptor-Cbl-CIN85 complex, resulting in the reduced ubiquitination of CIN85 and trafficking of the growth factor receptor complex toward late endosomes/lysosomes. We propose that through its effects on growth factor receptor trafficking, pORF3 prolongs endomembrane growth factor signaling and promotes cell survival to contribute positively to viral replication and pathogenesis.**

Hepatitis E virus (HEV) is a waterborne pathogen and the causative agent of hepatitis E. It is transmitted feco-orally and causes large outbreaks as well as sporadic disease (21, 37). While this disease is endemic in developing countries of Asia, Africa, and South America, significant levels of seropositivity in residents of developed countries have also been observed, which is possibly linked to the zoonotic transmission of HEV (25, 34). It is estimated that about 2 billion people, one-third of the world's population, live in areas where HEV infection is endemic (1). Although the disease is generally self-limited, fulminant hepatitis with increased mortality has been reported for a small fraction of patients, especially pregnant women (17, 33). Recently classified as the only member of the genus *Hep- eivirus* of the family *Hepeviridae*, HEV has a 7.2-kb capped, polyadenylated, positive-sense RNA genome that contains three open reading frames (ORFs). The *orf1* gene encodes a viral nonstructural polyprotein, *orf2* codes for the viral capsid protein, and *orf3* encodes a small protein that is proposed to optimize the host cell environment for viral replication (5, 6, 16, 18, 30, 31). A role for pORF3 in the release of HEV from infected cells was also proposed recently (46). While *orf3* appears to be dispensable for HEV replication in replicon-transfected cells (9), it was required for the *in vivo* infection of monkeys experimentally infected by the intrahepatic inoculation of the viral RNA genome (10). The requirement of

pORF3 for infectivity in pigs inoculated with HEV was also demonstrated previously (15).

The *orf3* gene of HEV encodes a small protein of 123 amino acids (aa) designated the ORF3 protein (pORF3); a recent report proposed that pORF3 is translated from a bicistronic subgenomic RNA and is 9 amino acids shorter at its N-terminal end (11). Both forms of recombinant pORF3 show identical subcellular localizations and have similar functional effects (5). The ORF3 protein is phosphorylated at a single serine residue (Ser80) by the cellular extracellularly regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family (16, 47). It contains two hydrophobic domains in its N-terminal half and two proline-rich regions toward its C-terminal end. Our previous studies have shown that pORF3 binds cellular proteins containing Src homology 3 (SH3) domains through its proline-rich motif (18) and upregulates the ERK/MAPK pathway by binding and inhibiting its cognate MAPK phosphatase (16). The ORF3 protein was also found previously to attenuate the mitochondrial death pathway (31) and to upregulate the expression of glycolytic pathway enzymes through the stabilization of hypoxia-inducible factor 1 (30). Recently, we showed that pORF3 delays the movement of activated epidermal growth factor (EGF) receptors (EGFRs) into the degradative compartment and of the phosphorylated signal transducer and activator of transcription 3 (pSTAT3) protein into the nucleus (5). The former is likely to result in extended growth factor signaling from intracellular sites, and the latter is likely to result in an attenuation of the acute-phase response, a major inflammatory pathway in the liver.

Growth factor receptors control a wide variety of biological processes, including cell proliferation, differentiation, survival, and migration (44). Ligand binding leads to receptor dimer-

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ization and autophosphorylation, followed by the binding of the ubiquitin ligase Cbl (41), which promotes the ubiquitination of the activated receptor at the plasma membrane and regulates its endocytosis (12, 14, 32). In complex with the growth factor receptor (GFR), Cbl is phosphorylated at C-terminal tyrosine residues, resulting in a conformational change to an open and more stable form of the PxxxPR motif. This enhances the interaction between specific proline-arginine motifs in Cbl and the SH3 domains of CIN85, a multidomain adapter protein (20, 39). In turn, CIN85 recruits endophilins to the EGFR-Cbl complexes, which regulate EGFR endocytosis by changing the curvature of the plasma membrane (4, 39). Cbl also mediates the ubiquitination of CIN85 and other molecules in the complex, which directs the receptor-ligand complex for lysosomal degradation (13, 36, 45). Any block in CIN85 binding to Cbl or its ubiquitination is known to inhibit the degradation of the receptor (13, 39). The relatively low affinity of CIN85 for its target proteins allows the rapid exchange of proteins/peptides, depending on their local concentration and compartmentalization. Several vesicular trafficking effectors interact directly with CIN85 at different steps of endocytosis and regulate different steps of endocytic trafficking (19). Thus, CIN85 acts as a master regulator and controls distinct steps in the trafficking of growth factor receptors along endocytic pathways.

The duration and strength of activated growth factor signals are tightly regulated in the cell through various negative regulatory mechanisms. Signal termination is accomplished by endocytosis, leading to the degradation of activated receptors. Several viruses exploit endocytosis of growth factor receptors for increased survival and successful replication inside the host cell (24, 27). Previous studies showed that hepatitis viruses, which include hepatitis B virus (HBV), hepatitis C virus (HCV), and HEV, also modulate growth factor receptor endocytosis (5, 23, 26, 29). We recently showed that pORF3 localizes to early and recycling endosomes and causes a delay in the postinternalization trafficking of EGFR (5). Here we show that pORF3 similarly affects the hepatocyte growth factor (HGF) receptor (HGFR) (c-Met). We further show that pORF3 interacts with CIN85, and this interaction deregulates the formation of the Cbl-CIN85 complex that is crucial for growth factor receptor trafficking to the degradative compartments. This study provides mechanistic details of a previously reported observation (5).

#### MATERIALS AND METHODS

**Plasmids, cell lines, and antibodies.** The ORF3 (ORF3/4) and vector control (pCN) stable cell lines, monoclonal antibodies to the ORF3 protein, and the pORF3-EGFP construct and its various mutants (5, 18, 30) were all described previously. HEV replicon plasmid pSK-E2 was a kind gift from Suzanne Emerson, NIH (9); the pCDNA3-FLAG-CIN85 construct was obtained from Sachiko Kajigaya, NIH (40); and the red fluorescent protein (RFP)-tagged Rab5 construct was provided by Satyajit Mayor, National Centre for Biological Sciences, India. Antibodies to c-Met (for immunoprecipitations and flow cytometry), EGFR, ERK1, EGFP, CIN85, ubiquitin, and phospho-tyrosine were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-c-Met, c-Met, and phospho-Cbl were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Calbiochem (San Diego, CA), while Alexa dye-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR).

**Transfections.** Transient transfections with the indicated expression vectors were carried out with Huh7, ORF3/4, or pCN cells by using Eugene-6 reagent

(Roche, Mannheim, Germany) according to the supplier's protocol. Cells were typically transfected at about 50% confluence by using 2  $\mu$ g plasmid per 60-mm dish. For cotransfection experiments, equal amounts of different plasmids totaling 3  $\mu$ g were used per 60-mm dish. For transfections in larger dishes, the DNA amounts were increased proportionately.

**Flow cytometry.** Transfected Huh7 cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) to remove any unbound ligand. The cells were then chilled on ice to stop membrane trafficking, harvested, fixed using 2% paraformaldehyde, resuspended in PBS containing 1% bovine serum albumin (BSA), and blocked for 45 min on ice. For total c-Met detection, the cells were permeabilized with 100% methanol at  $-20^{\circ}\text{C}$  before blocking. The blocked cells were labeled with anti-c-Met antibodies followed by an Alexa594-conjugated secondary antibody. The cells were washed with ice-cold PBS and analyzed by flow cytometry (CyanADP; DakoCytomation, Denmark). For each sample, 10,000 cells were analyzed, and the enhanced green fluorescent protein (EGFP)-expressing cells were gated for estimating the levels of cell surface and total c-Met.

**Immunoprecipitation and Western blotting.** Cells were lysed with a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and a protease inhibitor cocktail (Roche, Mannheim, Germany). The clarified supernatant was quantified for protein concentration by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). For immunoprecipitation, 1 mg of total proteins in 500  $\mu$ l of lysis buffer was incubated with 20  $\mu$ l of protein A-agarose beads (GE Healthcare, Uppsala, Sweden) for 1 h at 4 $^{\circ}\text{C}$ . The precleared lysate was then incubated with 2  $\mu$ g of the antibody overnight at 4 $^{\circ}\text{C}$  followed by 20  $\mu$ l of protein A-agarose beads for 2 h at 4 $^{\circ}\text{C}$ . After five washes in lysis buffer, the beads were boiled in Laemmli buffer, and the proteins were separated by SDS-PAGE. For Western blotting, proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Hybond ECL; GE Healthcare), and the membrane was blocked with Tris-buffered saline (TBS) containing 5% Blotto (Bio-Rad, Hercules, CA) for 1 to 2 h at room temperature (RT) and washed with TBST (TBS containing 0.1% Tween 20). The membrane was then incubated overnight at 4 $^{\circ}\text{C}$  with the primary antibody appropriately diluted in TBST-5% BSA, washed three times for 10 min each with TBST, and incubated with HRP-linked secondary antibodies diluted in TBST-5% Blotto for 1 h at RT. After washing as described above, chemiluminescent detection of proteins was carried out by using the Phototope detection system (Cell Signaling Technology, Beverly, MA) according to the supplier's protocol.

**Microscopy.** To study the localization of pORF3 and its mutants, Huh7 cells were transiently cotransfected with the appropriate expression vectors. After 48 h, the cells were fixed by using 2% paraformaldehyde for 20 min and imaged by using a confocal microscope (Nikon A-1R). For endogenous CIN85 and HEV replicon-expressed ORF3 staining, cells were fixed with 2% paraformaldehyde for 20 min, permeabilized at RT with 0.4% Triton X-100 for 15 min, and stained by using the appropriate primary and secondary antibodies.

**Replicon transfection and ORF3 expression.** Plasmid pSK-E2 was linearized at a unique BglII site located immediately downstream of the HEV poly(A) tract, and capped transcripts were synthesized as previously described (9). Transcription mixtures were cooled on ice and then mixed with a liposome mixture consisting of 25  $\mu$ l of DMRIE-C (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol; Invitrogen, Carlsbad, CA) in 1 ml of Opti-MEM. This mixture was added to a T25 flask containing washed S10-3 cells at about 40% confluence. S10-3 cells are a subclone of the Huh7 cell line and were a kind gift of Suzanne Emerson (NIH). The flasks were incubated at 34.5 $^{\circ}\text{C}$  for 6 h, an additional 1 ml of Opti-MEM was added, and incubation was continued overnight. The cells were then trypsinized, split into two T25 flasks, and incubated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). On day 5 posttransfection, cells were again trypsinized and plated for immunostaining, which was performed on day 8 posttransfection.

**MTT assay.** pCN and ORF3/4 cells were transiently transfected with either the pCDNA3-FLAG-CIN85 expression vector or the empty vector as a control. After 48 h, the cells were washed, and 200  $\mu$ g/ml MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] was added for 45 min. After the removal of the medium, dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance was read at 540 nm. The readings were converted into percentage values, taking the control value as 100%.

#### RESULTS

**ORF3-expressing cells show delayed degradation of the hepatocyte growth factor receptor.** Our previous study showed that pORF3 delayed the degradation of the activated epidermal growth factor receptor (EGFR). This effect could either be

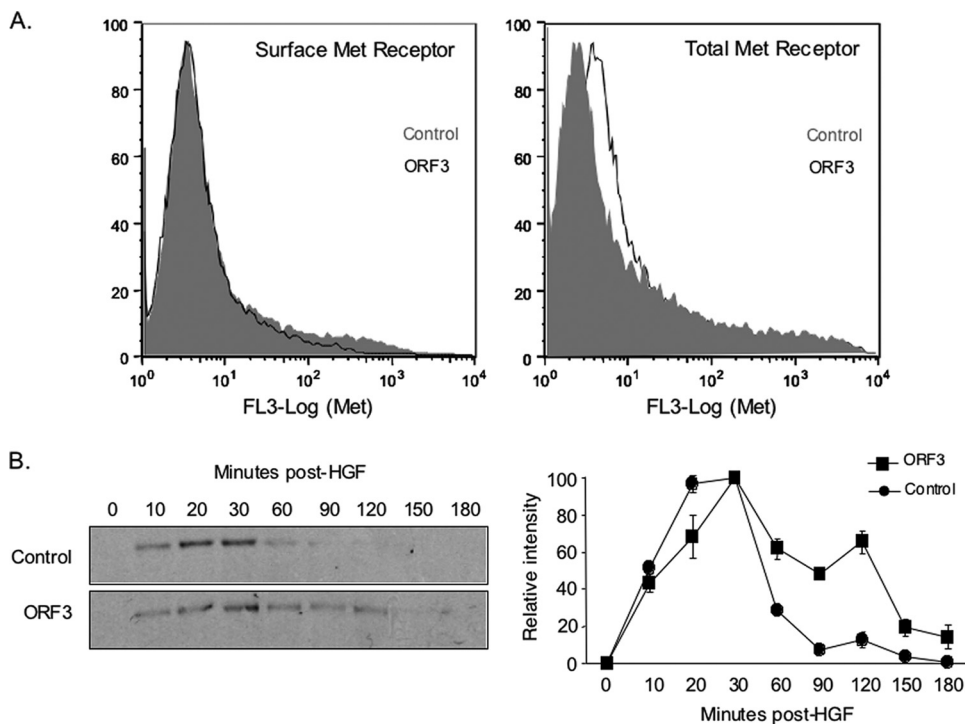


FIG. 1. The ORF3 protein delays degradation of the activated hepatocyte growth factor receptor, c-Met. (A) Flow cytometric analysis of surface or total c-Met in Huh7 cells transfected with either pEGFP-N1 (gray area) or pEGFP-ORF3 (black line). Single-parameter histograms are shown for EGFP-positive cells. (B) Huh7 cells were transfected as described above and 36 h later were serum starved for 12 h. The cells were then pulsed with 50 ng/ml HGF for the indicated times. Cell lysates containing equal amounts of total protein were Western blotted with anti-phospho-Met antibodies. The graph on the right shows decay curves, taking the highest intensity (at 30 min post-HGF treatment) to be 100%. The gel and decay curves are representative of three separate experiments.

EGFR specific or applicable to other activated growth factor receptors as well. The hepatocyte growth factor (HGF) receptor, c-Met, is relevant, as it is expressed on hepatocytes and guides their survival and proliferation. Huh7 cells were transfected to express the ORF3-EGFP fusion protein or EGFP only as a control. At 48 h posttransfection, the steady-state levels of surface or total c-Met were quantitated by antibody labeling and flow cytometry of cells that were gated for EGFP expression. While there was no change in the surface expression of c-Met, its total levels were elevated in ORF3-expressing cells (Fig. 1A). Like EGFR, following stimulation with HGF, c-Met is also phosphorylated, endocytosed, and eventually de-

livered to the lysosomal compartment for degradation and signal cessation. To compare the decay kinetics of phosphorylated c-Met in ORF3-expressing versus control cells, the transfected cells were serum starved and then stimulated with HGF for different times, as indicated. The cells were harvested at various times poststimulation, and the lysates were Western blotted with anti-phospho-c-Met antibodies. In control as well as ORF3-expressing cells, c-Met phosphorylation reached a peak 30 min following HGF stimulation. Subsequently, the rate of the loss of the phospho-c-Met signal was slower in ORF3-expressing cells than in control cells (Fig. 1B). To check the effect of delayed degradation on the total levels of EGFR

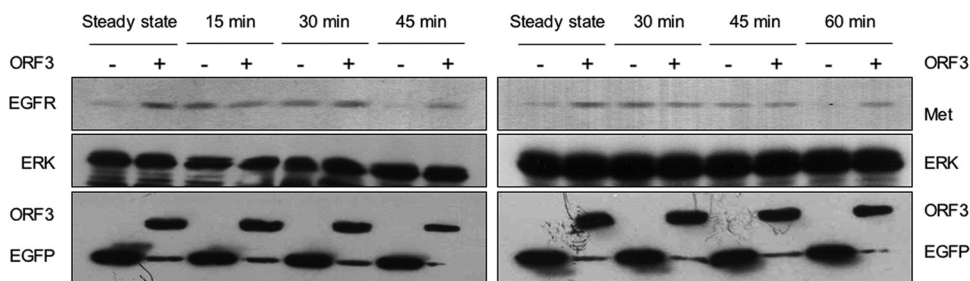


FIG. 2. The ORF3 protein also affects total growth factor receptor levels in cells. Huh7 cells were transfected with plasmid pORF3-EGFP (+) or pEGFP-N1 (-). After 36 h, six sets of cells were serum starved, and two sets were kept in complete medium. Three sets of serum-starved cells were pulsed with 100 ng/ml EGF, and the other three sets were pulsed with 50 ng/ml HGF for the indicated times. Cell lysates were prepared, and equal amounts of total protein were Western blotted with anti-EGFR (left) or anti-c-Met (right) antibodies. Normalized lysates were also Western blotted with anti-ERK1 and anti-EGFP antibodies for loading and expression controls, respectively.



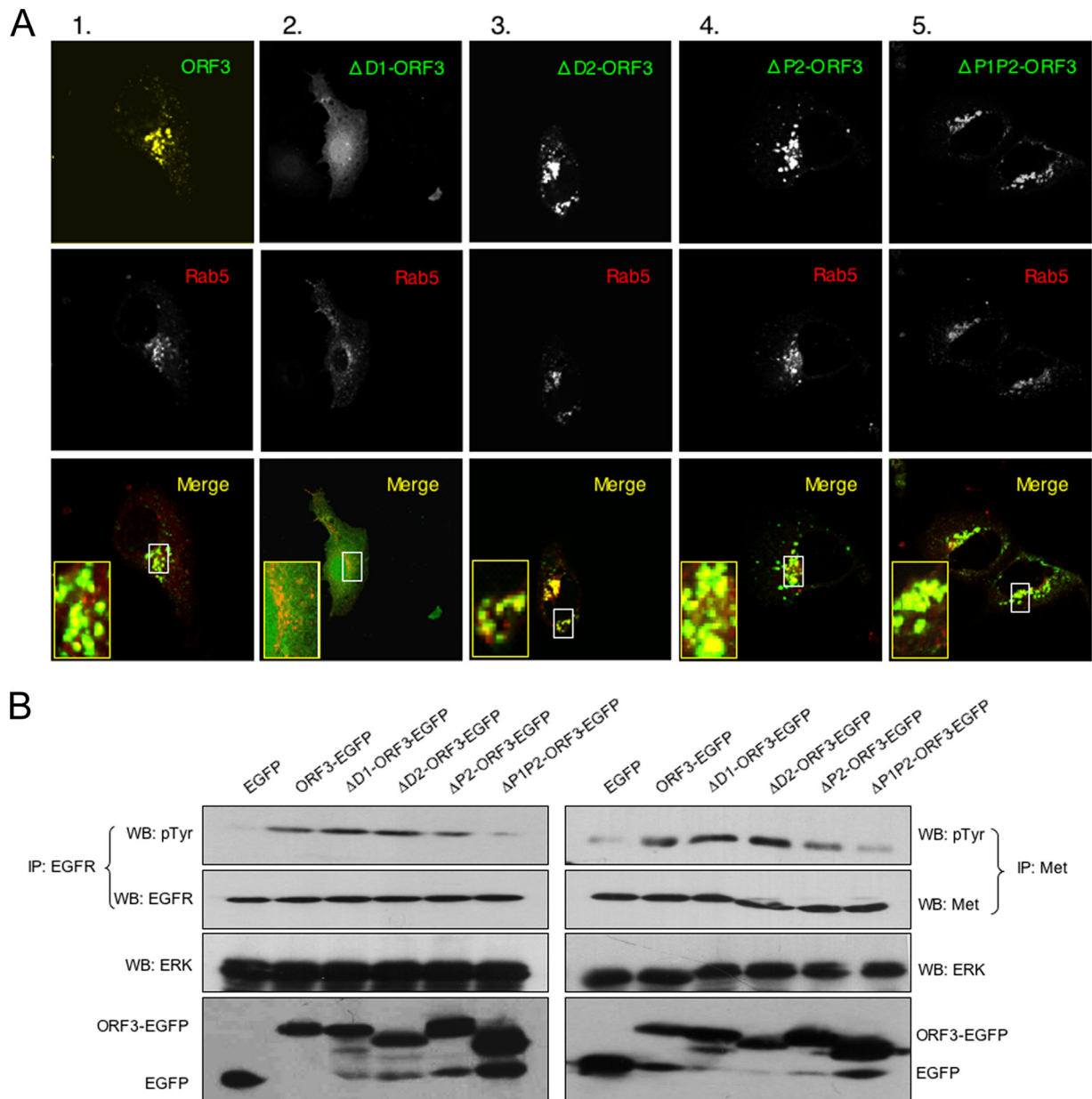


FIG. 3. The endosomal localization of pORF3 is not responsible for its effect on EGFR degradation. (A) Huh7 cells were cotransfected with pRab5-RFP and either pORF3-EGFP or expression vectors for mutant ORF3-EGFP fusion proteins. The fixed cells were then imaged directly for fluorescent proteins by confocal microscopy. The pORF3 and Rab5 signals were pseudocolored green and red, respectively. The merged pictures of single confocal planes are shown. In all cases, representative images that were observed for at least 70% of coexpressing cells are shown. (B) Huh7 cells were transfected to express EGFP or the wild-type or mutant ORF3-EGFP fusion protein in two sets. After 36 h, the cells were serum starved for 12 h. One set was pulsed with 100 ng/ml EGF for 30 min, and another set was pulsed with 50 ng/ml HGF for 45 min. Cell lysates containing equal amounts of total protein were immunoprecipitated (IP) with anti-EGFR (left) or anti-c-Met (right) and then Western blotted (WB) with anti-pTyr and either anti-EGFR (left) or anti-c-Met (right) antibodies. Normalized lysates were also Western blotted with anti-ERK1 and anti-green fluorescent protein (GFP) antibodies for loading and expression controls, respectively.

and c-Met, we transfected Huh7 cells to express either the ORF3-EGFP fusion protein or EGFP as a control. After 36 h, the cells were serum starved overnight and stimulated with either EGF or HGF for different times, as indicated. One set was also prepared without serum starvation to check for steady-state levels, which showed higher levels of EGFR and c-Met in ORF3-expressing cells (Fig. 2). For EGF-stimulated cells, a clear difference in EGFR levels between ORF3-ex-

pressing and control cells was visible after 45 min of stimulation; the c-Met levels were significantly different after 60 min of HGF stimulation (Fig. 2). Together, these results show that analogous to its effects on EGFR, the ORF3 protein delayed the degradation of c-Met.

**The endosomal localization of pORF3 is not responsible for its effects on receptor endocytosis.** The ORF3 protein contains two hydrophobic domains (D1 and D2) in its N-terminal half

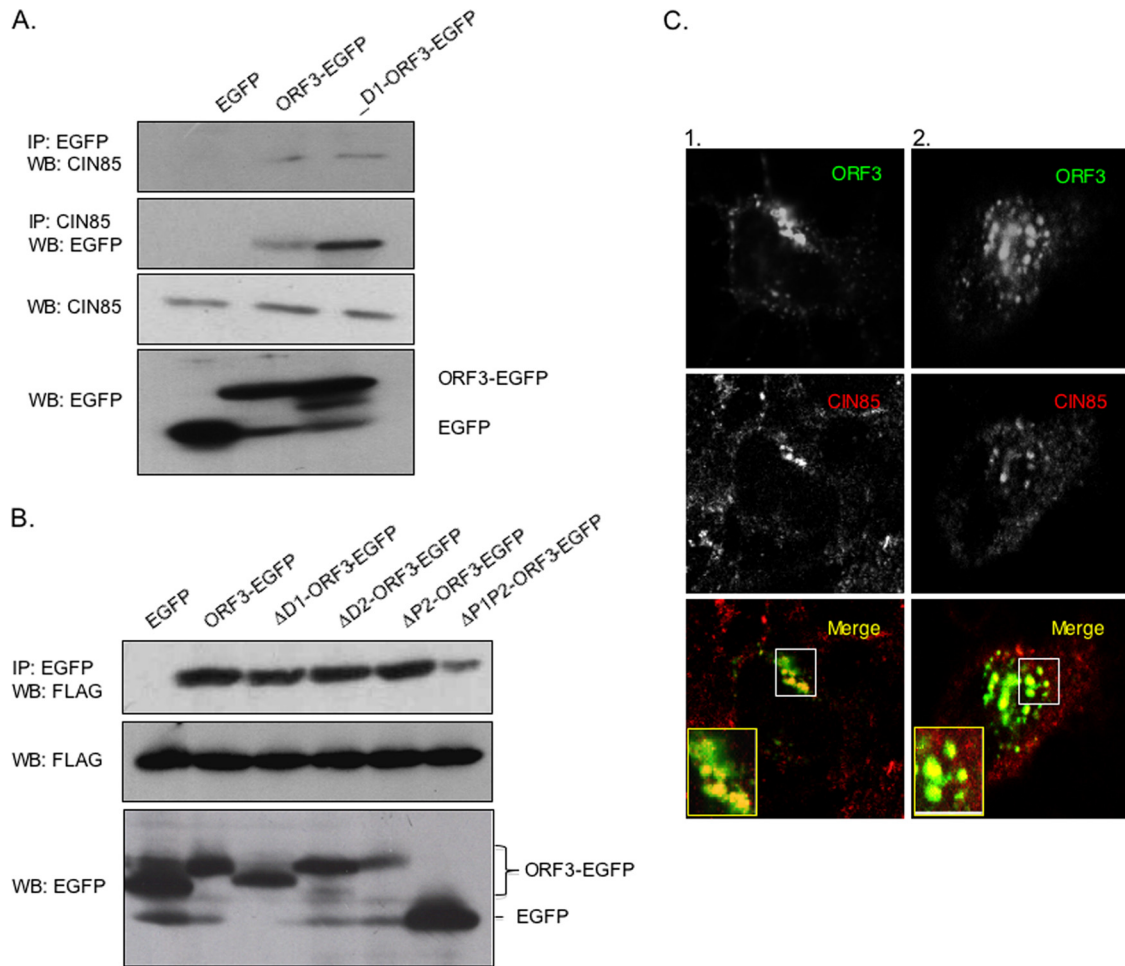


FIG. 4. The ORF3 protein interacts with CIN85. (A) Huh7 cells were transfected to express EGFP (control) or the ORF3-EGFP or  $\Delta$ D1-ORF3-EGFP fusion protein. After 48 h, cell lysates were prepared and immunoprecipitated with either anti-EGFP or anti-CIN85 antibodies, followed by Western blotting with anti-CIN85 or anti-EGFP antibodies, respectively. Normalized lysates were also Western blotted with anti-EGFP and anti-CIN85 antibodies, which served as expression controls for the expressed proteins. (B) Huh7 cells were cotransfected with pcDNA3-FLAG-CIN85 and either pEGFP-N1 (control) or pORF3-EGFP. After 48 h, cell lysates were prepared, and equal amounts of total protein were immunoprecipitated with anti-GFP, followed by Western blotting with anti-FLAG antibodies. Cell lysates were also Western blotted with anti-FLAG and anti-GFP antibodies to serve as expression controls. (C) Huh7 cells were transfected with the pORF3-EGFP expression vector (1), or S10-3 cells were transfected with *in vitro*-synthesized capped replicon transcript (2), as described in Materials and Methods. The cells were fixed and stained for CIN85 using a rabbit anti-CIN85 antibody and for pORF3 with a monoclonal anti-ORF3 antibody (2). The stained cells were imaged by confocal microscopy. The pORF3 and CIN85 signals were pseudocolored green and red, respectively. The merged pictures of single confocal planes are shown. Details are also shown for the boxed regions. In all cases, representative images that were observed for at least 70% of coexpressing cells are shown.

and two proline-rich regions (P1 and P2) in its C-terminal half. To determine which domain of the ORF3 protein is responsible for its endosomal localization, Huh7 cells were cotransfected with the Rab5-RFP expression plasmid along with expression plasmids for wild-type ORF3 or its mutants in domains D1 (deletion of aa 15 to 31), D2 (deletion of aa 37 to 62), P2 (aa 104 to 113; all Pro changed to Ala), and P1 plus P2 (deletion of aa 74 to 123). The cells were then imaged by confocal microscopy. Except for D1-deleted ORF3 ( $\Delta$ D1-ORF3), all other mutants showed a punctate distribution and colocalization with Rab5, a marker of early endosomes (Fig. 3A). The  $\Delta$ D1-ORF3 mutant, however, was distributed evenly in the cell and showed no colocalization with Rab5 (Fig. 3A2). This clearly demonstrated that hydrophobic domain 1 (D1) of

the ORF3 protein is responsible for its endosomal localization. We then tested whether the endosomal localization of pORF3 was responsible for its effect on EGFR phosphorylation, a modification that marks the activated receptor for degradation. Huh7 cells were transfected to express either wild-type ORF3-EGFP, mutant ORF3-EGFP fusion proteins, or EGFP only as a control. At 36 h posttransfection, cells were serum starved overnight and then stimulated with either EGF for 30 min or HGF for 45 min. The cells were harvested, and the lysates were immunoprecipitated with anti-EGFR or anti-Met antibodies followed by Western blotting with anti-pTyr antibodies. There was a consistent increase in phospho-EGFR and phospho-c-Met levels in cells expressing either the wild-type or mutant ORF3 proteins compared to control cells expressing only

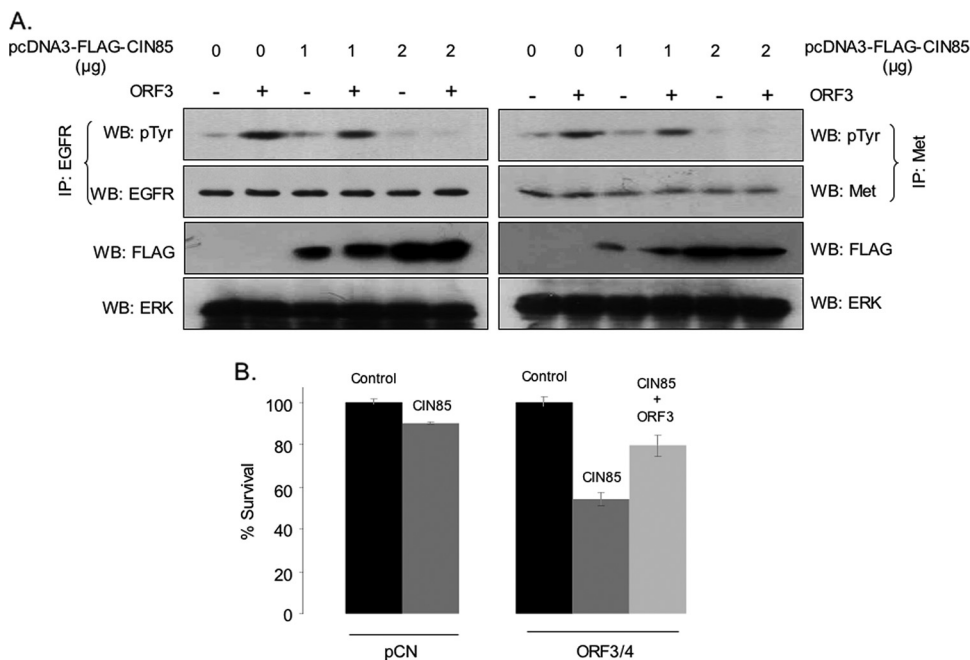


FIG. 5. Overexpression of CIN85 reverses the effect of pORF3 on growth factor endocytosis and cell survival. Control and ORF3-expressing stable cell lines were transfected with different amounts of the pcDNA3-FLAG-CIN85 expression vector as indicated and a vector control to keep the total amount of plasmid constant. After 36 h, cells were serum starved overnight and then pulsed with either 100 ng/ml EGF for 30 min or 50 ng/ml HGF for 45 min. Cell lysates were prepared, and equal amounts of total protein were immunoprecipitated by using anti-EGFR (left) or anti-Met (right) and then Western blotted with anti-pTyr and anti-EGFR or anti-Met antibodies, as shown. Normalized lysates were also Western blotted with anti-FLAG and anti-ERK1 antibodies for CIN85 expression and loading controls, respectively. (B) pCN and ORF3/4 cells were transiently transfected with the pcDNA3-FLAG-CIN85 expression vector and control empty vector. After 48 h, the cells were washed, and an MTT assay was carried out as described in Materials and Methods. The readings were converted into percent survival, taking the control as 100%.

EGFP (Fig. 3B). Compared to wild-type pORF3, its hydrophobic domain mutants  $\Delta$ D1 and  $\Delta$ D2 showed no decrease, while the proline-rich-region mutants  $\Delta$ P2 and  $\Delta$ P1P2 showed  $\sim$ 20% and  $\sim$ 50 to 60% reductions, respectively, in phospho-EGFR and phospho-c-Met levels. However, none of these mutants totally abrogated the stabilizing effects of pORF3 on activated growth factor receptors. Thus, while the  $\Delta$ D1-ORF3 protein does not localize to early endosomes like the wild-type ORF3 protein, it shows similar effects on GFR phosphorylation. Alternatively, the  $\Delta$ P2-ORF3 and  $\Delta$ P1P2-ORF3 proteins localize to early endosomes but show reduced phospho-GFR levels. This finding supports the idea that the localization of pORF3 to endosomes is not responsible for its effect on GFR trafficking, and this function is probably linked to the proline-rich regions of pORF3.

**The ORF3 protein interacts with CIN85, a regulator of growth factor receptor endocytosis.** The CIN85 protein is a major regulator of growth factor receptor endocytosis, which functions by binding Cbl and several other adapter proteins (19, 35, 39, 40). The ubiquitination of activated growth factor receptors and CIN85 by Cbl directs the complex for lysosomal degradation. Since the Cbl-CIN85 interaction is through proline-rich regions in the former and SH3 domains in the latter, and since pORF3 contains proline-rich regions, one of which was previously shown to bind SH3 domains (18), we wondered if pORF3 functions by binding CIN85 and competing it away from the Cbl-CIN85 complex. To test for a direct interaction between pORF3 and CIN85, we transfected Huh7 cells to

express either the ORF3-EGFP or the  $\Delta$ D1-ORF3-EGFP fusion protein using pEGFP as a control. The cell lysates were immunoprecipitated with anti-EGFP or anti-CIN85 antibodies followed by Western blotting with anti-CIN85 or anti-EGFP antibodies, respectively. As shown in Fig. 4A, anti-EGFP antibodies precipitated endogenous CIN85 from Huh7 cells expressing the ORF3-EGFP or  $\Delta$ D1-ORF3-EGFP fusion protein but not from cells expressing EGFP. Similarly, anti-CIN85 antibodies precipitated the ORF3-EGFP or  $\Delta$ D1-ORF3-EGFP fusion protein but not EGFP (Fig. 4A), confirming the specific interaction between these proteins. We also carried out a coimmunoprecipitation assay using lysates from Huh7 cells cotransfected with vectors expressing wild-type or mutant ORF3-EGFP fusions and an expression vector for FLAG-tagged CIN85. As described above, cell lysates were immunoprecipitated with anti-EGFP antibodies and then Western blotted with anti-FLAG antibodies. The results presented in Fig. 4B show that FLAG-tagged CIN85 was pulled down from cells expressing wild-type or mutant pORF3 but not from control cells. As described above, this showed that the hydrophobic domains (D1 and D2) of the ORF3 protein were not required for its binding to CIN85. It further showed that sequences outside the proline-rich domains (P1 and P2) may also be involved in the binding of pORF3 to CIN85. We also checked the localization of pORF3 and CIN85 in Huh7 cells and found that the two proteins colocalize (Fig. 4C). Whether the ORF3-EGFP fusion protein was expressed from a plasmid vector (Fig. 4C1) or the native ORF3 protein was expressed from the transfected HEV

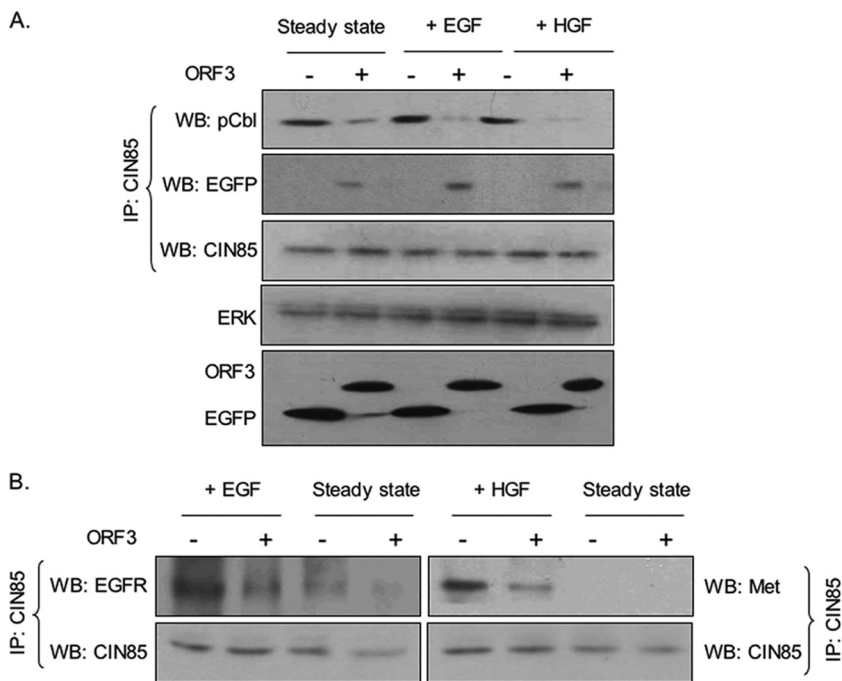


FIG. 6. The ORF3 protein reduces formation of the growth factor receptor-Cbl-CIN85 complex. (A) Huh7 cells were transfected with plasmid pORF3-EGFP (+) or pEGFP-N1 (-). After 36 h, two sets of cells were serum starved, and one set was kept in complete medium. One set of serum-starved cells was pulsed with 100 ng/ml EGF for 30 min, and the other was pulsed with 50 ng/ml HGF for 45 min. Cell lysates were prepared, and equal amounts of total protein were immunoprecipitated with anti-CIN85 antibodies followed by Western blotting with anti-pCbl, anti-EGFP, or anti-CIN85 antibodies. Normalized lysates were also Western blotted with anti-ERK1 and anti-EGFP antibodies as loading and expression controls, respectively. (B) The anti-CIN85 immunoprecipitates from A were Western blotted with anti-EGFR antibodies (for EGF-stimulated lysates) or anti-c-Met antibodies (for HGF-stimulated lysates). The immunoprecipitates from non-serum-starved cells (steady state) were Western blotted with both antibodies. Immunoprecipitates were also Western blotted with anti-CIN85 antibodies.

replicon (Fig. 4C2), the proteins showed significant overlap with endogenous CIN85 in Huh7 cells. Together, these results confirm that pORF3 interacts directly with CIN85.

**Overexpression of CIN85 reverses the effects of pORF3 on growth factor receptors and cell survival.** Since pORF3 interacts directly with CIN85, this interaction might be responsible for delayed receptor degradation in ORF3-expressing cells. To test this directly, we took cells that stably expressed pORF3 (ORF3/4 cells) and controls (pCN cells) and transfected different subsets with 0, 1, 2, and 3  $\mu$ g of the pcDNA3-FLAG-CIN85 expression vector. After 36 h, the cells were stimulated with either EGF for 30 min or HGF for 45 min and harvested, and the lysates were immunoprecipitated with anti-EGFR or anti-c-Met antibodies, followed by Western blotting with anti-pTyr antibodies. The steady-state levels of phosphorylated (activated) EGFR or c-Met were higher in ORF3-expressing cells than in control cells (Fig. 5A). However, when CIN85 was overexpressed in these cells, the stabilizing effects of pORF3 on activated EGFR and c-Met were progressively lost in cells transfected with 1 or 2  $\mu$ g of the pcDNA3-FLAG-CIN85 expression vector (Fig. 5A), while transfection with 3  $\mu$ g of the pcDNA3-FLAG-CIN85 expression vector resulted in severe cell death in the ORF3-expressing cells (not shown). These results support our hypothesis that the direct interaction of pORF3 and CIN85 is responsible for the former's effects on growth factor receptors. Since the overexpression of CIN85 is likely to reverse the effects of pORF3 on growth factor signal-

ing, we checked the effects of pORF3 on cell survival in the presence and absence of CIN85. We took the stable cell lines ORF3/4 and pCN (as a control) and transfected one subset with 2  $\mu$ g of the pcDNA3-FLAG-CIN85 expression vector and the other with an empty control vector. Another set of ORF3/4 cells was cotransfected with 2  $\mu$ g each of pcDNA3-FLAG-CIN85 and pORF3-EGFP. After 48 h, cell viability was evaluated by using a colorimetric assay based on MTT staining. The ORF3/4 cells that also expressed CIN85 showed about 50% cell death, but when more ORF3 was transiently expressed in these cells, there was only about 20% cell death (Fig. 5B). These results suggest that the effects of pORF3 on the growth factor pathway are also responsible for its pro-survival effects on cells.

**The ORF3 protein blocks formation of the Cbl-CIN85 complex.** The ORF3 protein is likely to attenuate growth factor receptor (GFR) degradation by interacting with CIN85 in two possible ways. It could trap the GFR-Cbl-CIN85 complex in early or recycling endosomes by delaying its trafficking to the late endosomal/lysosomal compartment, or it could interfere with the formation of the Cbl-CIN85 complex. To test this, we transfected Huh7 cells to express either the ORF3-EGFP fusion protein or EGFP as a control. After 36 h, the cells were serum starved overnight and then stimulated with either EGF or HGF for 30 min or 45 min, respectively. One set of transfected cells was left without serum starvation to assess steady-state levels. The lysates were immunoprecipitated with anti-



CIN85 antibodies, followed by Western blotting with anti-phospho-Cbl antibodies. In ORF3-expressing cells stimulated with either EGF or HGF, or cells kept in serum-containing medium, there was a reduced level of binding of CIN85 with phospho-Cbl (Fig. 6A). Upon densitometric quantitation, this reduction was ~60 to 75% in ORF3-expressing cells compared to control cells, suggesting that pORF3 interferes with the formation of the Cbl-CIN85 complex. We also used the same anti-CIN85 immunoprecipitated lysate to assess the levels of growth factor receptors in the CIN85 complex. As expected, reduced levels of EGFR and c-Met were observed in association with CIN85 (Fig. 6B). These results confirm that pORF3 competes with Cbl to form a complex with CIN85 and that this competition leads to reduced levels of the growth factor receptors in the GFR-Cbl-CIN85 complex; the latter is essential for the endocytosis and trafficking of activated GFRs to the degradative compartment.

#### Reduced ubiquitination of CIN85 in ORF3-expressing cells.

The Cbl-induced ubiquitination of growth factor receptors takes place at the plasma membrane and provides the initial signal for receptor endocytosis. Cbl also remains associated with the ligand-bound EGFR in early and late endosomes. In addition to activated receptors, receptor-associated proteins like CIN85 are also ubiquitinated by Cbl, which acts as a gating receptor for ubiquitinated cargo, thus amplifying the ubiquitin network. Any block in CIN85 ubiquitination leads to a reduced degradation of the activated receptor. Our results thus far show that pORF3 interacts with CIN85 and competes with the formation of the Cbl-CIN85 complex. It is thus expected that pORF3 will also result in a reduced ubiquitination of CIN85. To test this, Huh7 cells were transfected to express either the ORF3-EGFP fusion protein or EGFP as a control. After 36 h, the cells were serum starved overnight and stimulated with either EGF or HGF for 30 or 45 min, respectively. One set was also prepared without serum starvation to check for equilibrium levels. The cell lysates were immunoprecipitated with anti-CIN85 antibodies and then Western blotted with anti-ubiquitin antibodies. Following stimulation with either EGF or HGF, there was a ubiquitination of CIN85, which was significantly lower in cells expressing the ORF3 protein. Compared to controls, we observed about 10% or 60% CIN85 ubiquitination in ORF3-expressing cells stimulated with EGF or HGF, respectively (Fig. 7A). Similarly, ORF3-expressing cells not starved of serum (steady state) also showed about 50% CIN85 ubiquitination compared to control cells (Fig. 7A). This was also observed for the steady state, where cells were neither starved of serum nor subsequently stimulated with the growth factors. There was no change in the total levels of the CIN85 protein between ORF3-expressing and control cells. Growth factor receptors are ubiquitinated by the Cbl ubiquitin ligase after binding to it. To check for any effect of pORF3 on the ubiquitination of EGFR and c-Met, we transfected Huh7 cells to express either the ORF3-EGFP fusion protein or EGFP as a control. After 36 h, the cells were serum starved overnight and stimulated with either EGF or HGF for 30 or 45 min, respectively. The cell lysates were immunoprecipitated with anti-EGFR and anti-c-Met antibodies and then Western blotted with anti-ubiquitin antibodies. As expected, we did not find any significant change in the ubiquitination of EGFR and c-Met in ORF3-expressing cells (Fig. 7B).

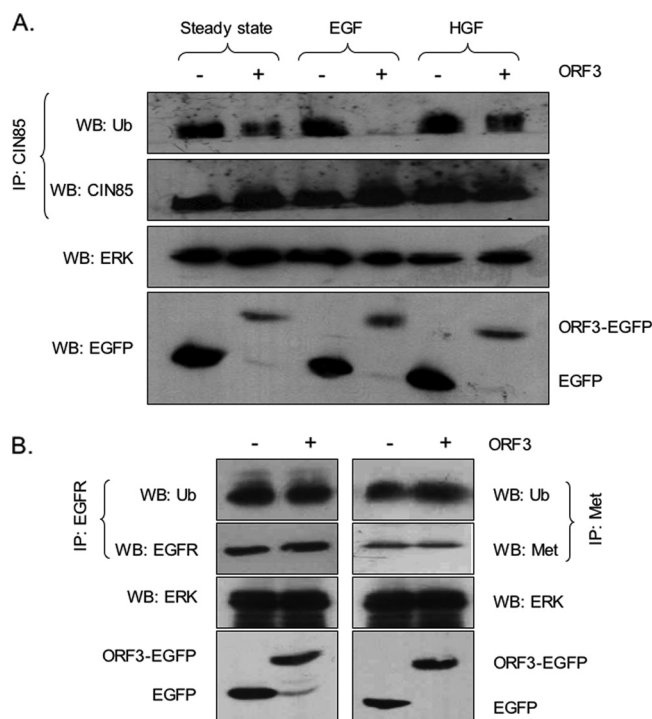


FIG. 7. The ORF3 protein reduces ubiquitination of CIN85. Huh7 cells were transfected with plasmid pORF3-EGFP (+) or pEGFPN1 (-) and treated as described in the legend of Fig. 5. Cell lysates containing equal amounts of total protein were immunoprecipitated with anti-CIN85 antibodies and then Western blotted with anti-ubiquitin (Ub) or anti-CIN85 antibodies. Normalized lysates were also Western blotted with anti-ERK1 and anti-GFP antibodies for loading and expression controls, respectively. Huh7 cells were transfected with plasmid pORF3-EGFP (+) or pEGFP-N1 (-). After 36 h, cells were serum starved and were pulsed with either 100 ng/ml EGF for 30 min or 50 ng/ml HGF for 45 min. Cell lysates were prepared, and equal amounts of total protein were immunoprecipitated with anti-EGFR (left) or anti-c-Met (right) antibodies followed by Western blotting with anti-ubiquitin and either anti-EGFR or anti-c-Met antibodies. Normalized lysates were also Western blotted with anti-ERK1 and anti-GFP antibodies for loading and expression controls, respectively.

## DISCUSSION

The controlled processing of information is critically important for the existence and development of biological systems. The loss of synchrony and interplay of signals between components of signaling networks, which are caused by several pathogens, contribute to their pathogenesis. Viruses are small and contain limited genetic information, making it imperative that viral proteins to be multifunctional.

HEV is a small virus that causes an acute self-limiting infection, even in the face of a robust antiviral immune response (7, 42). Previous studies carried out by us and others have shown that the HEV ORF3 protein regulates cellular processes and has three broad roles. The first role is the promotion of cell survival through the activation of the ERK/MAPK signaling pathway (16), attenuation of the intrinsic death pathway (31), regulation of energy homeostasis (30), and increased growth factor signaling, possibly from endomembrane sites (5). The second role is the downregulation of host immune responses through the reduced expression of acute-phase pro-



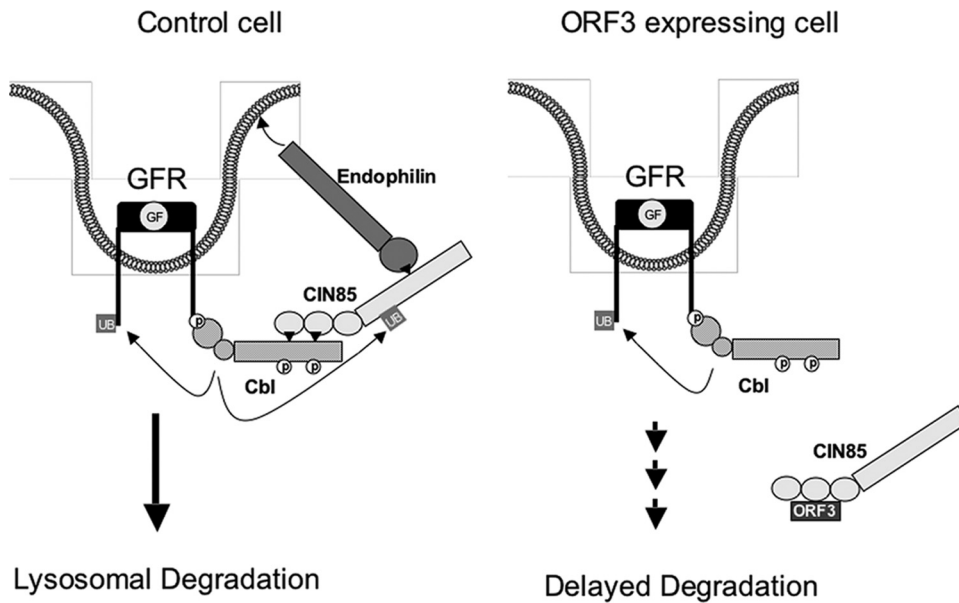


FIG. 8. Model for ORF3 protein-mediated regulation of growth factor receptor endocytosis. Following its ligation by growth factor (GF), the growth factor receptor (GFR) is phosphorylated at Tyr residues in its cytoplasmic domain. The pTyr residues bind Cbl, which in turn binds the multidomain adaptor protein CIN85 through proline-arginine-rich sequences in Cbl and the SH3 domains in CIN85. The CIN85 protein separately binds endophilin, which recruits the GFR-Cbl-CIN85 complex to clathrin-coated vesicles. The RING finger domain of Cbl recruits ubiquitin-conjugating enzymes and ubiquitinates GFR as well as CIN85, which regulates GFR trafficking toward late endosomes/lysosomes. The ORF3 protein binds CIN85, directly competes with the binding of Cbl to CIN85, and leads to a reduced ubiquitination of CIN85. This delays the trafficking of GFR to the degradative compartments.

teins (5) and increased secretion of the immunosuppressive  $\alpha$ 1-microglobulin protein (43). Recent studies have also suggested the requirement of pORF3 for virion morphogenesis and its release from infected cells (46). Thus, pORF3 appears to be a multitasking regulatory protein that helps in the establishment, propagation, and progression of HEV infection. Indeed, the ORF3 protein was required for viral infection following the intrahepatic inoculation of HEV genomic RNA directly into monkey liver (10) or pigs (15) but was dispensable for the propagation of a viral replicon in transfected cells *in vitro* (9).

Endocytosis is essential for the entry and productive infection of several viruses. Many viruses have also been found to incorporate components of the endocytic machinery in their survival and replication strategies (24, 27). We recently observed that pORF3 localizes to early and recycling endosomes and modulates the intracellular trafficking of activated EGFR (5). In the present study, we found pORF3 to have similar effects on c-Met, the cellular receptor for hepatocyte growth factor, which is critical for the survival and proliferation of hepatocytes (2, 3, 28, 38). These cells are believed to be the primary site of HEV infection and replication. A recent report demonstrated similar effects of the hepatitis C virus (HCV) NS5A protein on EGFR endocytosis (23). Hepatitis B virus (HBV) was also shown previously to activate growth factor receptor expression (26). Thus, this appears to be a general mechanism that may be utilized by hepatotropic viruses to promote the survival of infected cells, which would be required for efficient viral replication.

We also address the molecular mechanism responsible for the effects of pORF3 on growth factor receptors. An ORF3

protein deleted of its N-terminal hydrophobic domain 1 (D1) did not localize to early endosomes but still showed increased steady-state levels of phosphorylated EGFR and c-Met, which is indicative of its reduced degradation. This suggested that the endosomal localization of pORF3 was not required for its effect on growth factor receptor endocytosis and trafficking. Our earlier work showed that the ORF3 protein does not alter the endocytic trafficking of either the fluid phase or transferrin receptor but affects only the trafficking of EGFR (5).

The trafficking of growth factor receptors is regulated by a complex network of protein-protein and protein-lipid interactions as well as by protein posttranslational modifications, such as phosphorylation and ubiquitination. The ligand-induced ubiquitination of growth factor receptors has been linked to their internalization and endocytosis (14). The Cbl family of ubiquitin ligases plays pivotal roles in these processes. The Cbl protein can bind directly to phosphorylated growth factor receptors via its tyrosine kinase-binding (TKB) domain, while its RING finger domain recruits ubiquitin-conjugating enzymes (e.g., E2 and Ubc) and mediates the transfer of ubiquitin to activated receptors (8). Cbl also recruits several endocytic regulatory proteins, including CIN85-endophilin complexes, to the activated growth factor receptors (8, 35). The binding of CIN85 to Cbl is enhanced by the growth factor-induced tyrosine phosphorylation of Cbl that leads to a conformational change in the distal carboxyl termini of Cbl (20, 39). The proline-rich region of CIN85 also constitutively associates with endophilins, recruiting the Cbl-growth factor receptor complexes to clathrin-coated vesicles. CIN85 and several other proteins in the complex are also ubiquitinated by Cbl upon growth factor stimulation, which is required to direct the re-

ceptor-ligand complex for degradation (13, 36, 45). Several vesicular trafficking effectors interact directly with CIN85 at different steps of endocytosis (19). Thus, CIN85 acts as a molecular scaffold and controls distinct steps in the endocytic trafficking of growth factor receptors.

Some viruses such as herpes simplex virus (HSV) modulate growth factor endocytosis by regulating the formation of the Cbl-CIN85 complex. HSV protein 0 forms a complex with CIN85 and Cbl and mediates the ligand-independent degradation of EGFR from the cell surface; this precludes signaling to the infected cells through receptor tyrosine kinases (22). The CIN85 protein binds Cbl through SH3 domains in the former and proline-arginine-rich motifs in the latter (18). The HEV ORF3 protein is also proline rich and was shown previously to bind many SH3 domain-containing proteins (18). Here we show that pORF3 interacts directly with CIN85 and competes with the Cbl-growth factor receptor complex for its binding. The C-terminal amino acid sequence of pORF3, <sup>117</sup>PQLGPR<sup>122</sup>, is an exact match to the consensus Cbl sequence that binds the CIN85 SH3 domain (20). Following the deletion of this sequence, the ΔP1P2 mutant still showed residual binding (Fig. 4B). This could be due either to the overexpression of pORF3 or to the presence of other proline-rich motifs in pORF3 outside the P1 and P2 regions and a rather plastic SH3 binding motif in CIN85 (20). In support of the pORF3-CIN85 interaction, we also found pORF3, either overexpressed from a plasmid vector or expressed from the HEV replicon, to colocalize with CIN85. By competing with Cbl for binding to CIN85, the ORF3 protein also reduces the ubiquitination of CIN85 as well as its postinternalization interaction with other molecules, which are required for the lysosomal degradation of the receptor. These events lead to the prolonged intracellular survival of activated growth factor receptors as also endomembrane survival signaling in ORF3-expressing cells. This is likely to create a favorable environment for viral replication.

Collectively, our data provide a novel example of a viral protein that blocks growth factor receptor degradation by interacting with the multidomain adapter protein CIN85. A mechanistic model for this also emerges from our study. The ORF3 protein interacts with CIN85 and competes with the formation of the growth factor receptor-Cbl-CIN85 complex following growth factor activation. This reduced complex formation results in a reduced ubiquitination of CIN85, which results in a delayed degradation of activated receptors (Fig. 8). These effects would promote the survival of infected cells and are likely to aid in the replication and pathogenesis of these small hepatotropic viruses.

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