THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 25, pp. 17494–17502, June 20, 2008

Neogenin-mediated Hemojuvelin Shedding Occurs after Hemojuvelin Traffics to the Plasma Membrane*

Received for publication, December 26, 2007, and in revised form, April 29, 2008 Published, JBC Papers in Press, April 29, 2008, DOI 10.1074/jbc.M710527200

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***HFE2* (hemochromatosis type 2 gene) is highly expressed in skeletal muscle and liver hepatocytes. Its encoded protein, hemojuvelin (HJV), is a co-receptor for the bone morphogenetic proteins 2 and 4 (BMP2 and BMP4) and enhances the BMP-induced hepcidin expression. Hepcidin is a central iron regulatory hormone predominantly secreted from hepatocytes. HJV also binds neogenin, a membrane protein widely expressed in many tissues. Neogenin is required for the processing and release of HJV from cells. The role that neogenin plays in HJV trafficking was investigated, using HepG2 cells, a human hepatoma cell line. Knockdown of endogenous neogenin markedly suppresses HJV release but has no evident effect on HJV trafficking to the plasma membrane. The addition of a soluble neogenin ectodomain to cells markedly inhibits HJV release, indicating that the HJV shedding is not processed before trafficking to the cell surface. At the plasma membrane it undergoes endocytosis in a dynamin-independent but cholesterol-dependent manner. The additional findings that HJV release is coupled to lysosomal degradation of neogenin and that cholesterol depletion by filipin blocks both HJV endocytosis and HJV release suggest that neogenin-mediated HJV release occurs after the HJV-neogenin complex is internalized from the cell surface.**

Iron is an essential nutrient for a variety of biochemical processes. Iron uptake into the body via the intestines is controlled primarily by hepcidin (1). Hepcidin, a central iron-regulatory peptide hormone, is predominantly produced by hepatocytes, circulates in blood, and is excreted in urine (1–5). Hepatic hepcidin expression is regulated by dietary or parenteral iron loading, iron stores, erythropoietic activity, tissue hypoxia, and inflammation (1). Hepcidin deficiency resulting from the primary mutations in human *HFE* (hemochromatosis gene), *TFR2* (transferrin receptor 2 gene), hemochromatosis type 2 gene (*HFE2*), or the hepcidin gene itself (*HAMP*) is the major cause of hereditary hemochromatosis (6). This heterogeneous group of inherited iron overload disorders has a wide range of clinical severity.

HFE2 is a recently cloned gene in humans and encodes a protein termed hemojuvelin (HJV)[2] (7). Its ortholog in mice is called repulsive guidance molecule c (RGMc) because it is the third member of the RGM family to be cloned (7–10). RGMa and RGMb are expressed primarily in the developing and adult central nervous system, which do not overlap with *HFE2* expression (8–10).

The importance of HJV in iron homeostasis has been demonstrated by the observations that the homozygous or compound heterozygous mutations of *HFE2* gene cause the type 2A juvenile hemochromatosis, a particularly severe form of hereditary hemochromatosis (7, 11, 12). Disruption of both *HFE2* alleles ($Hjv^{-/-}$) in mice also causes a severe iron overload (5). The marked suppression of hepatic hepcidin expression detected in juvenile hemochromatosis patients with the *HFE2* mutation as well as in the $Hjv^{-/-}$ mice has implicated HJV as a key upstream regulator of hepatic hepcidin expression (5, 7, 13). In the liver, a recent study using lacZ as a marker indirectly showed a selective expression of HJV in periportal hepatocytes (5). The hepatocyte is, therefore, the principal site in which HJV exerts its regulatory role on hepcidin expression.

Like the other two RGM family members (RGMa and RGMb), HJV is a co-receptor for the bone morphogenetic proteins 2 and 4 (BMP2 and BMP4). HJV enhances hepatic hepcidin expression via the BMP signaling pathway (14). Neither RGMa nor RGMb appears to play a role in the regulation of hepcidin expression. BMPs are cytokines of the transforming growth factor β superfamily that exhibit multiple roles in a wide variety of processes through different signaling pathways (15, 16). BMP signaling is initiated upon ligand binding to BMP receptors, which leads to a sequential phosphorylation of receptor-activated Smads (Smad1, Smad5, and Smad8). The phosphorylated Smad1/5/8 form heteromeric complexes with Smad4. Upon formation, the complex translocates from the cytoplasm to the nucleus to regulate gene expression (17). Recent findings showing that liver-specific disruption of Smad4

* This work was supported, in whole or in part, by National Institutes of Health Grants DK080765 (to A.-S. Z.) and DK54488 (to C. A. E.). This work was also supported by funds from Amgen (to A.-S. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: HJV, hemojuvelin; BMP, bone morphogenetic protein; CM, conditioned culture medium; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; PI-PLC, Phosphatidylinositol-specific phospholipase C; RGM, repulsive guidance molecule; Tf, transferrin; TGN, *trans*-Golgi network; MEM, minimum essential medium; siRNA, small interfering RNA; MesNa, sodium 2-mercaptoethanesulfonate; tTA, tetracycline transactivator.

markedly decreases hepcidin expression and causes iron accumulation in mice (18) also support the importance of BMP signaling in iron homeostasis.

The regulation of HJV appears to be primarily at the post-transcriptional level. *HFE2* is expressed highly in both skeletal and heart muscle and at lower levels in liver (7). *HFE2* mRNA levels do not vary with iron loading in the liver of mice or with iron depletion in the skeletal muscle of rats (19, 20). The levels of serum HJV do increase in the early phase of iron deficiency in rats (20). HJV is a GPI-anchored protein (21), and *in vitro* studies demonstrated that HJV undergoes active release (shedding) from the *HFE2*-transfected cells as well as from the differentiated C2 or C2C12 cells (mouse myoblast cell lines), which robustly express endogenous HJV (20, 22, 23). In agreement with the finding in iron-deficient rats, HJV release from the cell lines is inhibited by iron-saturated Tf (holo-Tf) or non-Tf iron salts (20, 22, 24, 25). The observations that no evident defect in muscle development is observed in juvenile hemochromatosis patients and $Hjv^{-/-}$ mice rule out the possibility that HJV has a primary role in muscle development (7, 11, 12). However, skeletal muscle, accounting for ~35–40% of body weight, has the highest expression of *HFE2* mRNA (7) and is also a significant iron consumer for myoglobin synthesis with serum Tf presumably as its source of iron. The findings that soluble HJV plays a critical role in the negative regulation of hepatic hepcidin expression through BMP signaling in hepatocytes support the idea that skeletal muscle may serve as a body iron sensor as well as the major source of serum HJV to indirectly modulate hepatic hepcidin expression by regulating the HJV release into the circulation (20, 22, 26, 27).

HJV also binds neogenin, a receptor for RGMa and netrins (28, 29). Neogenin is a membrane protein and widely expressed in most tissues, including liver and skeletal muscle (30–34). The interactions of neogenin with RGMa and netrins are essential for neural development (35, 36). Our previous study showed that the interaction with neogenin is required for HJV release from muscle cells (20). The G320V mutation in HJV accounts for approximately two-thirds of cases of type 2A juvenile hemochromatosis. This mutation disrupts the interaction of HJV with neogenin, blocks HJV release, and results in the decreased HJV targeting to the cell surface and the retention of HJV in the endoplasmic reticulum (ER) (7, 20, 21, 37). These findings imply that neogenin may play a critical role in HJV intracellular trafficking. How neogenin is involved in this process remains to be elucidated.

In this study, we investigated the role of neogenin in HJV trafficking in HepG2 cells. We found that knockdown of endogenous neogenin markedly suppresses HJV release but has no evident effect on HJV trafficking to the plasma membrane. Release of HJV requires not only neogenin but also endocytosis presumably by a cholesterol-sensitive and dynamin-independent pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HepG2 cells were purchased from ATCC and maintained in MEM, 10% fetal calf serum, 1 mM pyruvate, 1× nonessential amino acids (complete medium). HepG2 cells stably expressing G320V (G320V-HepG2) were generated using Nucleofector kit V (Amaxa Biosystems) as previously described (20). The G320V mutant *HFE2* cDNA was prepared as previously reported (21). HepG2 cells stably transfected with wild type *HFE2* (HJV-HepG2) or pcDNA3 empty vector (control-HepG2) were generated previously (20). The stably transfected cells were maintained in complete medium with 800 μg/ml G418.

Knockdown of Endogenous Neogenin—Neogenin siRNA (Dharmacon) was used to knock down the endogenous neogenin in control and HJV-HepG2 cells as previously described (20). RNAiMAX reagent (Invitrogen) was used for the transfection. The negative control siRNA was the same as previously described (20). The cells were transfected with the siRNA twice on days 1 and 3 to maximize the efficacy of the knockdown. HJV in cell lysates and the conditioned medium (CM) and neogenin in cell lysates were analyzed by Western blot.

Flow Cytometry Analysis—Flow cytometry analysis was used to quantify the cell surface HJV in HJV-HepG2 cells with or without neogenin knockdown. Briefly, the cells were first detached from flasks with the cell dissociation buffer (Invitrogen). The cells were then incubated with affinity-purified rabbit anti-HJV antibody (4 μg/ml) in Hanks' buffer supplemented with 3% fetal bovine serum for 30 min at 4 °C, followed by incubation with phycoerythrin-conjugated goat anti-rabbit IgG (1:500 dilution; Caltag, Burlingame, CA) in the same buffer for 30 min at 4 °C. Flow cytometry analysis was performed on a Becton Dickinson FACSCalibur flow cytometer at the Core Facility of Oregon Health & Science University. Rabbit IgG and control-HepG2 cells were used as negative controls. The levels of cell surface HJV are expressed as arbitrary units. We used the standard deviation and the paired and two-tailed Student's *t* test to evaluate the statistical significance of the cell surface HJV in HepG2 cells with or without neogenin knockdown.

Phosphatidylinositol-specific Phospholipase C (PI-PLC) Cleavage of Cell Surface HJV—Approximately 10^6 HJV-HepG2 cells in 6-well plate were incubated in 0.5 ml of plain MEM in presence or absence of PI-PLC (Molecular Probes) at the concentration of 1 unit/ml at 37 °C in 5% CO_2 incubator or at 4 °C for the time intervals indicated in the text. The supernatants were collected, and the cell lysates were prepared using NET-Triton buffer (150 mM NaCl, 5 mM EDTA, and 10 mM Tris (pH 7.4) with 1% Triton X-100) with 1× protease inhibitor mixture (Roche Applied Science). Approximately one-third of the cell lysates or supernatants was subjected to 11% SDS-PAGE, followed by immunodetection of HJV and neogenin as described under "Immunodetection."

HJV Release—The effects of the ectodomain (extracellular domain) of neogenin, bafilomycin A (an inhibitor of the vacuolar H^+-ATPase; Sigma), dynasore (a cell-permeable inhibitor of dynamin; Sigma), and filipin (a cholesterol-binding agent; Sigma) on HJV release from HJV-HepG2 cells were examined. The ectodomain of neogenin was generated by subcloning the neogenin cDNA encoding the hydrophobic signal sequence (residues 1–33) and the ectodomain (residues 34–1103) with a C-terminal His_6 tag into the pVL1393 baculovirus transfer vector (BD Biosciences). The recombinant protein was purified from the supernatant of baculovirus-infected High 5 cells using nickel-nitrilotriacetic acid and gel filtration chromatography. HJV-HepG2 cells were cultured in the 12-well plates. After 48 h

of incubation with 60% confluence, fresh MEM, 5% fetal calf serum was changed with the addition of neogenin ectodomain at 0, 250, 500, 750, and 1,000 nM and incubated for 24 h. HJV in ~50% of cell lysate and 20% of the CM was analyzed by Western blot. For the inhibitors, the experiments were conducted when HJV-HepG2 cells were ~80% confluence. The cells were incubated in the absence or presence of bafilomycin A (100 nM), dynasore (80 and 160 μM), or filipin (1, 5, and 10 μg/ml) for the indicated time intervals where no evident cytotoxicity was detected. The whole cell lysate and 50% of CM were analyzed.

Expression of Wild Type and Mutant Dynamin—To block dynamin-mediated endocytosis, adenoviruses were used to infect cells to introduce wild type or K44A mutant dynamin-1 (dynamin) into HJV-HepG2 cells. The viruses were kindly provided by Dr. Sandra Schmid at the Scripps Research Institute (La Jolla, CA). The viral infections were conducted as described previously with some modifications (38). Briefly, HJV-HepG2 cells were subcultured into 12-well plates ~24 h before the infection. The cells were overlaid with 250 μl of binding medium (Hanks' salts containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes, pH 7.2) containing the mixture of tTA activator virus and wild type or K44A-dynamin-1 virus at equal ratios. To generate the cells with various levels of dynamin, different amounts of virus were added as indicated in the text. After 2 h of incubation at 37 °C to enable viral attachment and infection, binding medium was changed to complete medium. After 18 h of incubation to allow the expression of introduced dynamin, the medium was replaced with 600 μl of fresh complete medium. The cultured conditioned medium was collected to assay HJV release after an additional 24 h of incubation. The levels of dynamin in cell lysate were analyzed as described under "Immunodetection." In this study, cells infected with tTA activator virus alone were served as negative controls.

HJV Internalization—The internalization of biotinylated cell surface HJV was conducted as previously described (39–41). Briefly, HJV or control HepG2 cells in 6-well plate at ~80% confluence were biotinylated with 0.25 mg/ml Sulfo-NHS-Biotin (Pierce) at 4 °C for 20 min. The cells were then incubated at 37 °C for the time intervals indicated in the text. Biotin remaining on cell surface was stripped using MesNa (Sigma) at 4 °C. Alternatively, the cells were incubated in presence of PI-PLC (1 unit/ml) at 4 °C for 3 h to release the cell surface GPI-anchored proteins. The cells were then solubilized in NET-Triton/1× protease inhibitors mixture, followed by using streptavidin-agarose beads (Pierce) to isolate the biotinylated HJV (internalized fraction). The total cell surface HJV was isolated with strepavidin-agarose from biotinylated cells labeled at 4 °C.

^{125}I-Tf Uptake—^{125}I-Tf uptake was used to detect the effect of dynasore and filipin on *TfR1*-mediated Tf uptake. The rate of ^{125}I-Tf uptake was determined as described previously (42) with the following modifications. HepG2 cells in 6-well plates were first preincubated in 1 ml of uptake medium (MEM, 2 mg/ml ovalbumin, 20 mM Hepes, pH 7.2) with dynasore (80 and 160 μM) or filipin (10 μg/ml) for 30 min at 37 °C. ^{125}Tf uptake was then initiated by changing to the uptake medium containing 50 nM ^{125}Tf as well as the same concentrations of dynasore or filipin. The inclusion of 1 mg/ml unlabeled Tf was used as the control for nonspecific uptake. After an 8-min incubation at 37 °C, externally bound Tf was stripped by acid wash. The radioactivity remaining within cells was counted. The amount of specific uptake in the presence of inhibitors was expressed as the percentage of corresponding controls. The standard deviation and the paired and two-tailed Student's *t* test were used to evaluate the statistical significance between the groups with and without inhibitors.

Immunodetection—Cell lysate protein and CM were subjected to SDS-PAGE under reducing conditions, followed by transfer onto nitrocellulose membrane. The membranes were probed with affinity-purified rabbit anti-HJV antibody (0.22 μg/ml), rabbit anti-neogenin antibody (0.4 μg/ml; Santa Cruz Biotechnology), mouse anti-dynamin (1:4000; Upstate), or mouse anti-β-actin antibody (1:10,000; Chemicon International), followed by immunodetection using corresponding horseradish peroxidase-conjugated secondary antibody (Chemicon International, Temecula, CA). The bands were exposed to x-ray film by chemiluminescence (Super Signal; Pierce).

RESULTS

Knockdown of Endogenous Neogenin Has No Effect on HJV Trafficking to the Plasma Membrane but Blocks HJV Release—Our previous studies demonstrate that HJV interacts with neogenin in HEK293 cells and that interaction with neogenin is required for HJV release in C2C12 cells, a mouse myoblast cell line (20, 21). Recent studies indicate that the G320V mutant form of HJV that does not bind neogenin remains predominantly in the ER and does not traffic efficiently to the plasma membrane (21, 37). These observations suggest a critical role of neogenin in HJV trafficking. To gain insight into the function of neogenin in this process, we first examined its role in HJV trafficking to the plasma membrane in HepG2 cells. HepG2 cells are of a relatively well differentiated human hepatoma cell line that expresses many hepatocyte-specific genes including Tf, hepcidin, *TfR2*, and ceruloplasmin (data not shown). The endogenously expressed neogenin is readily detectable by Western blot (Fig. 1*A*). We used siRNA to knock down the neogenin in HepG2 cells stably transfected with HJV (HJV-HepG2) and then quantified the levels of cell surface HJV by flow cytometry analysis. In comparison with the control siRNA, siRNA specific to human neogenin was able to eliminate detectable neogenin (Fig. 1*A*). Flow cytometry analysis revealed a mild but not statistically significant increase ($p = 0.36$) (Fig. 1*C*). Similar results were obtained by immunofluorescent analysis of nonpermeabilized cells (data not shown). To confirm the lack of change in cell surface HJV with the knockdown of neogenin, we examined the amount of HJV released by PI-PLC, which cleaves the phosphodiester bond of GPI-linked proteins. Similar quantities of HJV were released upon PI-PLC cleavage whether or not neogenin was present in the cells (Fig. 1*A*). Consistent with the previous reports showing complex processing of HJV (21, 23), three distinct HJV bands migrating at approximately 50, 33, and 15 kDa were detected in the supernatants of PI-PLC digestion (Fig. 1*A*, *bottom panel*). Down-regulation of neogenin does not alter this profile. Taken together, these results indicate that neogenin is not required for HJV trafficking to the plasma membrane.

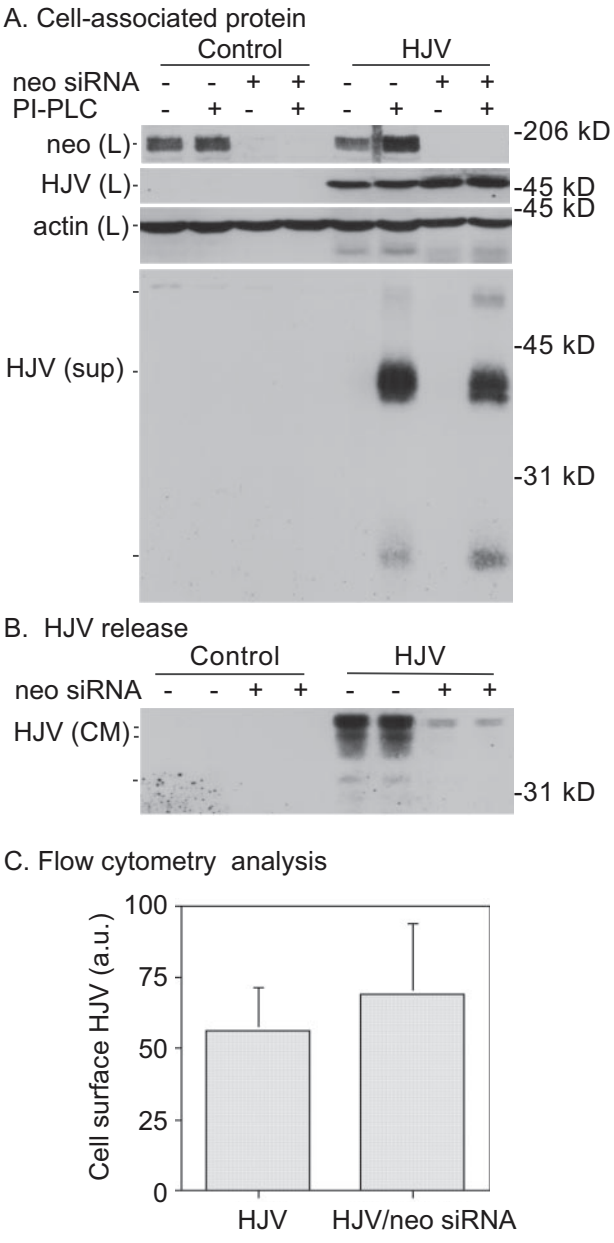

FIGURE 1. **Knockdown of neogenin blocks HJV release but does not affect HJV cell surface expression.** We used siRNA specific to human neogenin and Lipofectamine RNAiMax to knockdown the endogenous neogenin in control and HJV-HepG2 cells as described under "Experimental Procedures." Scrambled siRNA was used as a parallel negative control. *A*, cell-associated protein. PI-PLC digestion was used to examine the effect of neogenin knockdown on the dynamic HJV expression on cell surface. The cells from above were incubated in the presence or absence of PI-PLC at the concentration of 1 unit/ml for 2 h in 37 °C CO_2 incubator. HJV, neogenin (*neo*), and actin in cell lysates (*L*) and the HJV in supernatant (*HJV (sup)*) were detected by Western blot analysis. *B*, HJV release. At 24 h after the second transfection of cells with siRNA, the cells were pooled and subcultured into 12-well plates with complete medium containing 10% fetal calf serum. Approximately 48 h later, 100 μl of the CM was subjected to SDS-PAGE and Western blot analysis for HJV (*HJV (CM)*). *C*, flow cytometry analysis of the cell surface HJV. Flow cytometry analysis of cell surface HJV was analyzed as described under "Experimental Procedures" and expressed as arbitrary units (*a.u.*). The averages of four individual analyses and the standard deviations were presented. All of the other experiments were repeated at least three times with consistent results.

Because neogenin did not affect the biosynthetic pathway of HJV, the effect of neogenin on the shedding of HJV was examined. Detection of HJV in the CM by Western blot analysis

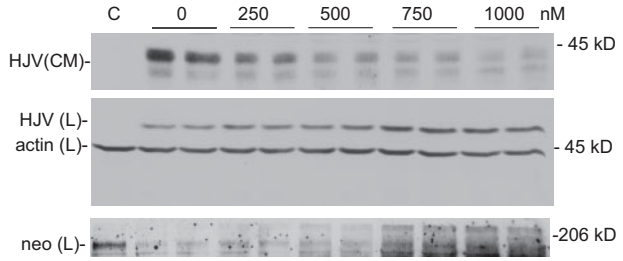

FIGURE 2. **The ectodomain of neogenin blocks HJV release.** *HFE2* stably transfected (HJV) and empty vector transfected control (*C*)-HepG2 cells in 12-well plate were incubated in the absence or presence of the soluble ectodomain of neogenin at 0–1000 nM for 24 h. Approximate half of the cell lysate (*L*) and 20% of the CM was subjected to Western blot analysis for HJV and neogenin (*neo*). Actin was also probed in the lysate as a protein loading control. The experiments were repeated three times with consistent results.

revealed a marked decrease of released HJV when neogenin was depleted (Fig. 1*B*). The multiple HJV bands migrating at ~37 kDa in the conditioned medium are in agreement with the previous observations (20, 23), implying the existence of multiple cleavage sites (Fig. 1*B*). These results suggest that both myocytes and hepatocytes share similar pathways of neogenin-mediated HJV release. Importantly, these results also imply that neogenin influences the shedding of HJV after it traffics to the cell surface.

Soluble Neogenin Inhibits HJV Release—Neogenin is a type I transmembrane protein consisting of an extracellular ectodomain, a transmembrane domain, and a cytoplasmic domain (33). The binding of neogenin to HJV has been mapped to the ectodomain of neogenin with a binding affinity of ~500 nM (43). We used purified ectodomain of neogenin to competitively disrupt the HJV-neogenin interaction at the cell surface and then examined the effect of this disruption on HJV release. In this study, we directly added ectodomain of neogenin to the culture medium of HJV-HepG2 cells. After 24 h of incubation, HJV in the cultured conditioned medium was analyzed by Western blot. In comparison with the parallel controls showing active HJV release, the dose response to neogenin ectodomain indicates that inhibition of HJV release was achieved over a similar concentration range as the binding affinity (Fig. 2, *top panel*). A similar inhibition of HJV release was observed when neogenin expression was knocked down using neogenin siRNA (Fig. 1*B*). Endogenous neogenin levels increase slightly upon treatment with the ectodomain fragment (Fig. 2, *bottom panel*), indicating that the binding of soluble neogenin to HJV could inhibit the down-regulation of endogenous neogenin by HJV. Together with the neogenin knockdown studies, these results indicate that the neogenin fragment competes with the endogenous neogenin for binding to HJV. The interaction of HJV with full-length neogenin is essential for both the processing and release of HJV as well as for the down-regulation of neogenin after HJV reaches the cell surface.

HJV Release Is Correlated with Neogenin Degradation—We observed that expression of HJV in HepG2 cells resulted in lower levels of endogenous neogenin (20). To further evaluate the role of HJV in the down-regulation of neogenin, we first compared the effect of wild type and G320V HJV expression on the level of neogenin. The G320V mutant, which does not bind neogenin, was used as a negative control. Wild type HJV, but

not the G320V mutant, markedly decreased neogenin protein levels and undergoes active release (Fig. 3*A*). A similar effect obtained in at least three individual stably transfected clones for each construct ruled out a clonal effect (data not shown). Alternatively, we used PI-PLC, which can release cell surface HJV into the medium, to determine the effect of HJV on neogenin levels. PI-PLC was directly added into the culture medium of HepG2 cells expressing HJV or G320V HJV at 37 °C, and the cells were incubated for 3 h. Under these conditions, PI-PLC is expected to release HJV. If the full-length HJV-neogenin complex is required for internalization and neogenin degradation, treatment of HJV-HepG2 cells with PI-PLC should release HJV from cell and elevate the cellular neogenin level. Consistent with this hypothesis, PI-PLC released HJV and resulted in a marked increase of neogenin after a 3-h incubation, as compared with the corresponding controls with no PI-PLC (Fig. 3*B*). Importantly, an increase of neogenin was not detected in the empty vector-transfected control or in G320V HepG2 cells where the G320 HJV mutant is located mainly in the ER (Fig. 3*B*) or in HJV-HepG2 cells at 4 °C (data not shown). These results suggest that HJV and neogenin internalize prior to neogenin degradation.

The increased neogenin degradation could result from the following two possibilities, the release of HJV/neogenin as a complex and/or the sorting to and degradation of neogenin in lysosomes. No shed neogenin could be detected in the concentrated conditioned medium from HJV-HepG2 cells by Western blot analysis under conditions where cellular neogenin decreased (data not shown). To determine whether neogenin is degraded in lysosomes, we treated HJV-HepG2 cells with bafilomycin A, an inhibitor of the vacuolar H^+-ATPase (44), which dissipates the pH gradient in the intracellular organelles and thereby blocks protein degradation in lysosomes. After a 4-h incubation with 100 nM bafilomycin A, a significant increase of cellular neogenin in HJV-HepG2 cells was apparent (Fig. 3*C*). Interestingly, HJV release does not seem to be altered. These results suggest that lysosome is the site of neogenin degradation and that HJV shedding does not depend on low pH. In addition, the mild increase of cellular HJV in presence of bafilomycin A (Fig. 3*C*) also implies that HJV release might not be the sole pathway for cellular HJV turnover. Rather, a certain portion of HJV may also be degraded in lysosomes. Taken together, the above observations imply that the interaction of neogenin with HJV triggers its internalization from plasma membrane for either cleavage or lysosomal degradation.

HJV Release Is Inhibited by Cholesterol Depletion but Not by Dynamin Inhibitors—Plasma membrane proteins can be internalized by at least four known pathways, three of which require dynamin for fission of the invaginated vesicles from the plasma membrane (45). To determine whether HJV release from cells was dependent on its endocytosis, we examined whether the disruption of dynamin function affects HJV release from HJV-HepG2 cells. Initially, dynasore, a cell-permeable inhibitor specific for the dynamin GTPase (46), was used to inhibit endocytosis. Unexpectedly, no evident effect was detected when cells were incubated in the presence of dynasore at the recommended concentrations (80 and 160 μM) (Fig. 4*A*). To test whether these cells were sensitive to dynasore, the effect of

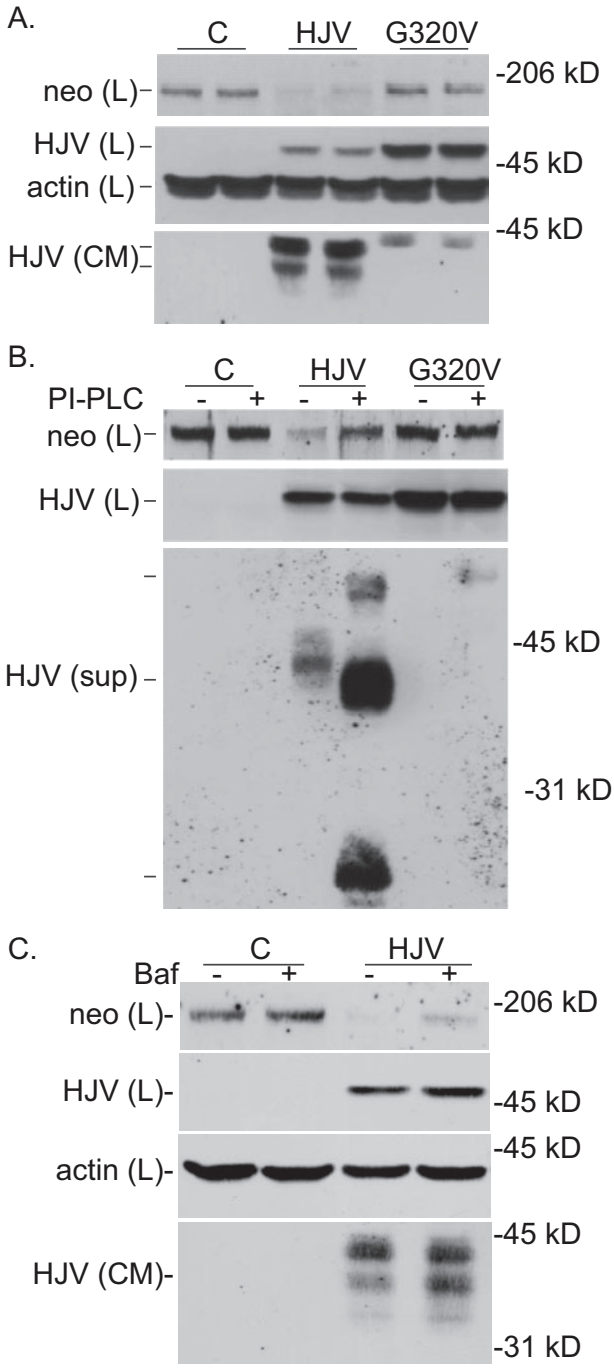

FIGURE 3. **HJV release is correlated with neogenin degradation.** *A*, expression of HJV, but not G320V mutant, decreases the level of neogenin protein in HepG2 cells. Cell lysate from 2×10^5 cells and CM from 0.8×10^5 cells of overnight culture were subjected to SDS-PAGE, followed by immunodetection of the HJV in both cell lysates (*HJV (L)*) and CM (*HJV (CM)*), and neogenin (*neo (L)*) and actin in the lysate. *B*, PI-PLC digestions. HJV, G320V, and control-HepG2 cells in 12-well plates at approximate 70% confluence were incubated in 300 μl of MEM with or without the addition of PI-PLC (1 unit/ml) for 3 h at 37 °C CO_2 incubator. Approximately half of the cell lysate or supernatant were subjected to SDS-PAGE, followed by immunodetection of HJV in both cell lysate (*L*) and supernatant (*sup*), and neogenin (*neo*) and actin in the lysate. *C*, bafilomycin A increases cellular neogenin. Control (*C*) and HJV-HepG2 cells in 12-well plate were incubated for 4 h in complete medium with or without the addition of 100 nM bafilomycin A (*Baf*). Neogenin, HJV, and actin in cell lysate (*L*) and HJV in approximately one-third of CM were detected by Western blot using the specific antibodies. All of the experiments were repeated at least three times with consistent results.

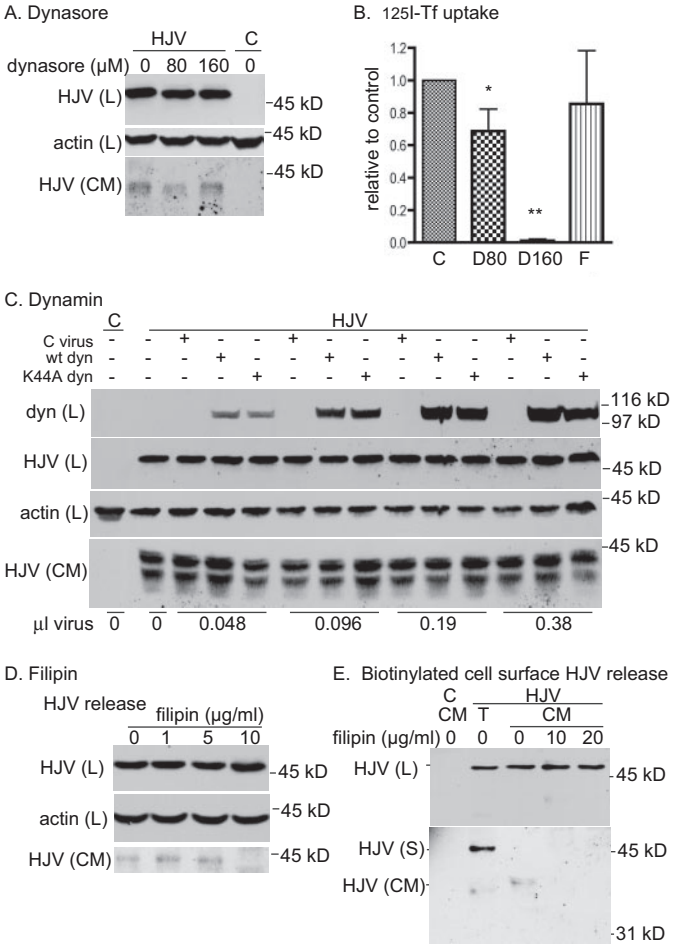

FIGURE 4. **Depletion of cholesterol inhibits HJV release.** *A*, dynasore does not inhibit HJV release. HJV-HepG2 cells in 12-well plates were incubated in the presence of 0, 80, and 160 μM of dynasore for 2 h at 37 °C. HJV in both cell lysate (*L*) and 50% of CM was detected by Western blot. *B*, ^{125}I-Tf-uptake. HepG2 cells in 6-well plates with approximate 80% confluence were first pre-incubated in absence or presence of dynasore (80 and 160 μM; *D80* and *D160*, respectively) and filipin (10 μg/ml) (*F*) for 30 min at 37 °C. ^{125}I-Tf uptake was initiated by incubating the cells in the presence of 50 nM ^{125}I-Tf and the same concentrations of inhibitors. After 8 min of incubation at 37 °C, membrane-bound ^{125}I-Tf was removed by acid wash. The ^{125}I radioactivity was counted. The inclusion of 1 mg/ml unlabeled cold Tf was used as the nonspecific uptake control. The rates of specific uptake in the presence of inhibitors were expressed as the percentage of the corresponding controls. The results are from four individual experiments. *, $p = 0.0281$; **, $p < 0.0001$. *C*, wild type and K44A mutant dynamin. HJV-HepG2 cells in 12-well plates were infected with the mixture of tTA activator virus and different amounts of adenovirus containing either wild type or K44A mutant dynamin (0, 0.048, 0.096, 0.19, and 0.38 μl of stock virus/well). tTA activator virus alone was used as a negative control. The cells were first incubated for ~18 h to allow the expression of introduced dynamin. Afterward, ~20% of the CM collected from the incubation between 18 and 42 h post-infection was detected for HJV by Western blot. In addition, dynamin (*dyn*), HJV, and actin in the cell lysate (*L*) at 42 h post-infection were also analyzed. *D*, filipin inhibits HJV release. HJV-HepG2 cells in 12-well plates were incubated in the presence of 0, 1, 5, and 10 μg/ml of filipin for 2 h at 37 °C. HJV in both cell lysate (*L*) and 50% of CM was detected by Western blot. *E*, filipin inhibits the biotinylated cell surface HJV release. Cell surface proteins in HJV-HepG2 cells were biotinylated at 4 °C, followed by incubation in the presence of 0, 10, and 20 μg/ml of filipin at 37 °C for 2 h. The biotinylated HJV that was released into the medium (*HJV (CM)*), as well as the total biotinylated cell surface HJV (T) (*HJV (S)*), was isolated using streptavidin-agarose beads and subjected to Western blot (lower panel). The total HJV in cell lysate (*L*) was also detected (*upper panel*). All of the experiments were repeated at least three times with consistent results. In each experiment, the lysate and CM from control-HepG2 cells (*C*) were included as a negative control for HJV.

dynasore on ^{125}I-Tf uptake was measured because transferrin receptor-mediated Tf uptake is a dynamin-dependent process. Consistent with the previous observations (46), dynasore significantly inhibited and completely blocks ^{125}Tf internalization at 80 and 160 μM, respectively (Fig. 4*B*). These results suggest that HJV release is independent of dynamin.

To further substantiate this observation, we next examined the effects of the K44A mutant dynamin. The K44A mutation disrupts the dynamin GTPase activity. It is widely used as a dominant-negative to block the dynamin-dependent endocytosis (38). HJV-HepG2 cells were infected with four different concentrations of adenovirus encoding either wild type or K44A mutant dynamin. Consistent with the dynasore results, no evident inhibition of HJV release was detected when cells were infected with the virus encoding the K44A dynamin mutant (Fig. 4*C*). These results again support that dynamin-dependent endocytosis is not involved in HJV release.

HJV is a GPI-anchored protein (21). Depletion of cholesterol by filipin, a sterol-binding agent that binds to cholesterol, disrupts both the endocytosis and exocytosis of GPI-anchored proteins (45, 47–49). Therefore, we examined the effect of filipin on HJV release from HJV-HepG2 cells. Filipin was added directly to the culture medium at the concentrations ranging from 0 to 10 μg/ml. The cells were incubated for 2 h at 37 °C. A marked decrease of HJV in the conditioned medium was evident in the presence of 10 μg/ml of filipin (Fig. 4*D*). Longer periods of incubation were not pursued because of the cytotoxicity of filipin. Alternatively, the effects of filipin on the release of biotinylated HJV were measured. Consistent with the above finding, filipin was also found to markedly suppress the release of HJV, which was derived from the cell surface (Fig. 4*E*). Further analysis revealed that filipin does not significantly alter the ^{125}Tf internalization compared with the control ($p = 0.4997$) (Fig. 4*B*). The relatively large variability in Tf uptake may result from the effect of cholesterol depletion on clathrin-coated pit internalization. Previous studies showed that the acute cholesterol depletion markedly reduces the rate of internalization of *TfR1* by more than 85% (50). These results suggest that HJV release depends on cholesterol and possibly on cholesterol-dependent HJV endocytosis.

Endocytosis of Hemojuvelin—To elucidate whether cell surface HJV undergoes endocytosis, cell surface proteins were labeled at 4 °C with a cleavable form of biotin. Less than 10% of the total HJV was biotinylated, suggesting that the majority of HJV is localized intracellularly (Fig. 5*A*). To detect internalized HJV, the cells were warmed to 37 °C for 10 min. Any biotin remaining on cell surface was stripped by the nonpermeable reducing agent, MesNa. The internalized HJV was then isolated using streptavidin-agarose beads. Controls showed that MesNa could strip most of the biotin coupled to HJV in HJV-HepG2 cells left at 4 °C (Fig. 5*A*). Internalization was a rapid process. The internalized HJV was detectable by Western blot after 5 min of incubation and reaches a plateau after 10 min of incubation (Fig. 5*A* and data not shown). Similar results were also obtained by using PI-PLC to strip the cell surface HJV (data not shown). These findings indicate that HJV undergoes endocytosis in HepG2 cells.

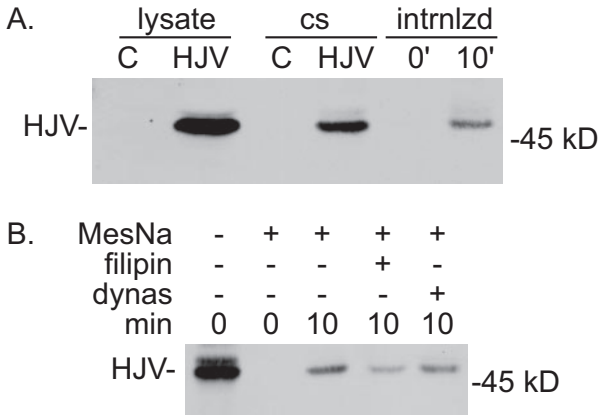

FIGURE 5. **HJV undergoes endocytosis.** *A*, internalization of biotinylated cell surface HJV. Cell surface proteins in control (*C*) and HJV-HepG2 cells were biotinylated at 4 °C, followed by incubation at 37 °C for 0 and 10 min (*0′* and *10′*). After stripping the biotin remaining on cell surface, the internalized HJV (*intrnlzd*) was isolated using streptavidin-agarose beads and subjected to Western blot. Approximately 10% of lysate from the cells without biotinylation (lysate) and the total biotinylated cell surface HJV without incubation and stripping (*cs*) were included for the analysis. *B*, filipin inhibits HJV internalization. The experiments were conducted essentially the same as described in *A*, except that the biotinylated cells were first preincubated in the absence or presence of 10 μg/ml filipin or 160 μM dynasore (*dynas*) at 4 °C for 30 min before the incubation at 37 °C.

HJV Endocytosis Is via Dynamin-independent but Possibly Cholesterol-dependent Pathway—To further characterize the pathway of HJV endocytosis, we examined the effect of filipin and dynasore on the internalization of biotinylated cell surface HJV in HJV-HepG2 cells. HJV internalization was markedly inhibited when cells were incubated in the presence of 10 μg/ml filipin (Fig. 5*B*). However, no evident inhibition was detected when cells were incubated in the presence of dynasore at concentrations of 80 and 160 μM (Fig. 5*B*; data not shown). These results suggest that HJV endocytosis is via the dynamin-independent and cholesterol-dependent pathway, although the definitive role of cholesterol in this process still remains to be further characterized by other strategies.

DISCUSSION

The role of neogenin in HJV trafficking in HepG2 cells was investigated. Our results show that knockdown of endogenous neogenin markedly suppresses HJV release but has no evident effect on HJV trafficking to the plasma membrane. The marked inhibition of HJV release by soluble neogenin suggests that the neogenin-regulated HJV release occurs after HJV traffics to the plasma membrane. Furthermore, our data also indicate that HJV shedding occurs after HJV is internalized.

HJV is a GPI-anchored protein (21) and, like other GPI-linked proteins, is co-translationally translocated into the ER where they are linked to a GPI anchor before trafficking to the cell surface (51). In this study, we excluded the possibility that HJV release occurs in its biosynthetic pathway. The observation that neogenin knockdown does not alter the HJV trafficking to the cell surface but does block the HJV release supports this assumption. In addition, these findings also have an important implication with respect to the possible defects of G320V mutation in HJV. The G320V mutation is the most frequently detected mutation in type 2A juvenile hemochromatosis, accounting for approximately two-thirds of cases (7). G320V mutation abolishes the HJV-induced hepcidin expression (14). Early studies demonstrated that it disrupts the interaction of HJV with neogenin and that G320V mutant HJV has a decreased targeting onto cell surface, is slightly shed, and is largely retained in the ER (7, 20, 21, 23, 37).[3] Because neogenin is not required for HJV trafficking to the plasma membrane, the primary defect of the G320V mutation appears to be misfolding, which consequently blocks its exit from the ER, the subsequent trafficking to the plasma membrane, as well as lack of the interaction with neogenin.

HJV release takes place after it traffics to the plasma membrane and binds neogenin. The finding that HJV can efficiently traffic to the plasma membrane in the absence of neogenin but does not undergo release rules out the possibility that neogenin acts as a chaperone to traffic HJV to the cell surface. Rather, the association with neogenin is a prerequisite for shedding, because soluble neogenin ectodomain can competitively inhibit HJV release, similar to neogenin knockdown. The finding that the addition of soluble neogenin ectodomain does not result in the depletion of endogenously expressed neogenin in cells but rather a slight increase in endogenous neogenin suggests that the inhibition of HJV release is caused by competitively disrupting the interaction of HJV with endogenous neogenin. These observations therefore implicate a critical role of neogenin transmembrane and cytoplasmic domains in the process of HJV release.

Recent studies indicate that the HJV cleavage is mediated by the proprotein convertase furin (24, 25). Furin is predominantly localized in the TGN and cycles between the TGN and the plasma membrane (52). Like transmembrane proteins, furin is synthesized in the ER, and like other pro-enzymes, it is activated until it reaches its destination, which is the TGN in the case of furin (52). This specific localization of furin and its trafficking between the plasma membrane and the TGN and our data, therefore, do not favor the findings in a recent report stating that the furin-mediated HJV shedding occurs in the ER (25). On the basis of our results, the endocytic compartments are most likely the sites of HJV shedding. First, HJV release is coupled to the increased neogenin degradation in lysosome. Second, HJV undergoes endocytosis, and blockage of HJV internalization inhibits HJV release.

Previous studies indicate that the endocytosis of many GPI-anchored proteins is through dynamin-independent but cholesterol-dependent pathway, because GPI-anchored proteins are present at the surface in cholesterol-dependent nanoscale clusters (45, 47–49). Our results showing that cholesterol inhibitor, but not dynamin inhibitor, is able to decrease HJV internalization tend to support that the endocytosis of HJV follows this pathway. These observations also imply that HJV endocytosis may depend on its GPI anchor. However, this does not exclude other possibilities because neogenin seems to play the critical role in this process. Neogenin has a cytoplasmic domain of 388 amino acids (33). The role of neogenin cytoplasmic domain in this process remains to be determined.

[3] A.-S. Zhang and C. A. Enns, unpublished observations.

On the basis of our data, we propose a model for the neogenin-required and furin-mediated HJV shedding. The association of HJV with neogenin on plasma membrane triggers the internalization of both proteins as a complex. The complex is then retrieved into a compartment, in which HJV is exposed to furin for cleavage and the subsequent release, whereas neogenin is sorted for degradation in lysosome. The fraction of HJV that is unable to associate with neogenin would be targeted for degradation.

Soluble HJV plays a critical role in the inhibition of hepatic hepcidin expression through the BMP signaling (22, 24, 26, 27). Studies in transfected cells indicate that holo-Tf and/or non-Tf iron inhibits the shedding of HJV (20, 22, 37). Animal studies suggest that serum HJV could be derived from both skeletal muscle and liver hepatocytes (20). These observations, therefore, support that serum HJV plays a critical role in the regulation of iron homeostasis. The findings in the present study showing that soluble neogenin competitively blocks HJV release and HJV release requires HJV endocytosis imply that the regulation of HJV release by holo-Tf might take place at the cell surface, after internalization or during the retrograde transport. Thus holo-Tf would disrupt HJV-neogenin complex similar to soluble neogenin and thereby modulate HJV release. However, the mechanism by which holo-Tf interferes with HJV release still remains to be elucidated.

In summary, this study demonstrates that neogenin is not required for HJV trafficking from the ER to the plasma membrane, but the HJV-neogenin interaction at the cell surface is essential for HJV release. Together with the specific localization of furin, the findings that HJV endocytosis is required for its release suggest that neogenin-dependent retrograde trafficking of HJV to furin positive compartments is necessary for HJV shedding. How this process is regulated by iron still remains to be explored and will be the subject of future research.

Acknowledgments—We thank Julia Maxson, Maja Chloupkova, Juxing Chen, and Junwei Gao in Caroline Enns' laboratory for critical reading of the manuscript and valuable comments.

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