Heme-binding Protein HRG-1 Is Induced by Insulin-like Growth Factor I and Associates with the Vacuolar H\(^+\)-ATPase to Control Endosomal pH and Receptor Trafficking

Endocytosis and trafficking of receptors and nutrient transporters are dependent on an acidic intra-endosomal pH that is maintained by the vacuolar H\(^+\)-ATPase (V-ATPase) proton pump. V-ATPase activity has also been associated with cancer invasiveness. Here, we report on a new V-ATPase-associated protein, which we identified in insulin-like growth factor I (IGF-I) receptor-transformed cells, and which was separately identified in Caenorhabditis elegans as HRG-1, a member of a family of heme-regulated genes. We found that HRG-1 is present in endosomes but not in lysosomes, and it is trafficked to the plasma membrane upon nutrient withdrawal in mammalian cells. Suppression of HRG-1 with small interfering RNA causes impaired endocytosis of transferrin receptor, decreased cell motility, and decreased viability of HeLa cells. HRG-1 interacts with the c subunit of the V-ATPase and enhances V-ATPase activity in isolated yeast vacuoles. Endosomal acidity and V-ATPase assembly are decreased in cells with suppressed HRG-1, whereas transferrin receptor endocytosis is enhanced in cells that overexpress HRG-1. Cellular uptake of a fluorescent heme analogue is enhanced by HRG-1 in a V-ATPase-dependent manner. Our findings indicate that HRG-1 regulates V-ATPase activity, which is essential for endosomal acidification, heme binding, and receptor trafficking in mammalian cells. Thus, HRG-1 may facilitate tumor growth and cancer progression.

Cancer cell invasiveness is associated with increased expression of components of the endocytic trafficking machinery (1, 2), and altered trafficking of growth factor receptors and integrins may have a profound effect on tumor growth and invasive potential (3, 4). Cancer cells are also highly dependent on a continuous supply of nutrients and micronutrients. These are acquired through transporters that may be either channels or receptors that deliver their cargo via endocytosis (4–6). The expression levels and trafficking of nutrient transporters are tightly regulated by growth factors, especially through activity of the insulin/IGF-I\(^2\)-activated PI3K/mammalian target of rapamycin signaling pathway (7). Both overexpression of nutrient transporters and decreased degradation in lysosomes have been linked to cellular transformation (7, 8).

An increasingly acidic luminal pH gradient from early to late endosomes is required for receptor trafficking and is maintained by the V-ATPase proton pump acting in concert with other pH regulatory channels (9, 10). The V-ATPase consists of two multisubunit domains. The \(V_c\) domain has eight subunits (A–H), which is located on the cytoplasmic side of membranes, and hydrolyzes ATP. The \(V_o\) domain has six subunits (a, d, e, c\(^\prime\), and c\(^\prime\)), which is embedded in membranes, and translocates protons. Activity of the complex is regulated in at least three ways as follows: by reversible domain assembly, which is dependent on glucose and PI3K signaling; by the RAVE regulatory protein complex in yeast; and by targeting of subunits to specific membranes (10–13). In mammals, further regulation may be attributed to subunit isoforms that target V-ATPase to specific tissues or organelles (9, 10).

The V-ATPase has been detected at the plasma membrane of cancer cells (14, 15), which is thought to favor invasiveness, although the c subunit from the \(V_o\) domain has been associated with promoting cellular transformation (16). Very little is known about the regulation of V-ATPase function in epithelial cancer cells. Nonetheless, it is reasonable to propose that it may be influenced by the insulin/IGF-I/PI3K signaling pathway, which has increased activity in many cancers. PI3K has been shown to be essential for V-ATPase assembly in renal epithelial cells (12).

In an effort to identify IGF-I-regulated proteins that are important for cancer progression, we isolated a series of genes that are differentially expressed in nontransformed cells (R\(^-\), derived from the IGF-IR knock-out mouse) and highly transformed cells (R\(^+\) and R\(^-\) cells that overexpress the IGF-IR) (17–19). Among these was a gene encoding an endosomal protein that we found to associate with the endosomal V-ATPase and to regulate V-ATPase activity in endosomes. As

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3 The abbreviations used are: IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; V-ATPase, vacuolar H\(^+\)-ATPase; siRNA, small interfering RNA; Tf, transferrin; TFR, transferrin receptor; HA, hemagglutinin; PI3K, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; HBSS, Hanks’ balanced salt solution; PIPES, 1,4-piperazinediethanesulfonic acid; ZnMP, zinc mesoporphophyrin IX.
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this study was conducted, the same gene was identified to encode a heme-binding protein necessary for heme homeostasis in *Caenorhabditis elegans* and for erythropoiesis and development in zebrafish (20). Heme transport into and within cells may involve endocytosis and trafficking of heme transporters/receptors (21–23). Overall, our data indicate that HRG-1 is the first known heme-binding protein that regulates function of the V-ATPase in endosome acidification and trafficking of receptors essential for cell metabolism.

**MATERIALS AND METHODS**

*General Reagents and Antibodies—IGF-I was from PeproTech Inc. (Rocky Hill, NJ), Concana
tamin A, baflomycin A, nocodazole, propidium iodide, goat serum, crystal violet, leupeptin, E-64, FITC-transferrin and FITC-dextran 40 kDa (FD40), DMEM, glucose-free DMEM, sodium azide, 2-deoxy-D-glucose, nigericin, NaCl and all other salts and reagents were from Sigma unless otherwise stated. LY294002, rapamycin, and PD98059 were from Calbiochem. Alexa-488 transferrin, LysoTracker Red, and LysoSensor Green were from Molecular Probes (Eugene, OR). Antibodies used were as follows: anti-EEA1 (BD Transduction Laboratories); anti-transferrin receptor and anti-Rab11 antibody (Zymed Laboratories Inc.); anti-β-actin and Rab7 (Sigma); anti-LAMP1 and anti-HA (clone 16B12) (Covance); anti-His (Qiagen, UK); anti-c subunit (Chemicon); and anti-A1 subunit (Santa Cruz Biotechnology). Generation of mouse monoclonal V-ATPase anti-A subunit antibody was described previously (24).

**Cloning of Human HRG-1 cDNA—**cDNA encoding the human HRG-1 gene sequence was obtained from the I.M.A.G.E. Consortium. The open reading frame (nucleotides 98–538) was amplified by PCR using primers to incorporate XhoI restriction sites at the 5’ and 3’ ends and ligated into the pcDNA3-HA vector. For cloning into the yeast expression vector, the human HRG-1 coding sequence was digested with XhoI and Smal restriction enzymes and ligated into Xhol/Smal-digested pGBK-T7 vector.

**HRG-1 Polyclonal and Monoclonal Antibody Generation—**A peptide corresponding to amino acid residues 131–146, both inclusive (HRYRADFADISIL DDRPF), of the human HRG-1 protein sequence was conjugated to keyhole limpet hemocyanin for inoculation of rabbits (Davids Biotechnologie, Germany). The generated antibody was affinity-purified with immobilized peptide. Alternatively, the peptide CHRYRADFADISIL DDRPF (amino acids 130–145) conjugated to keyhole limpet hemocyanin was used to immunize BALB/c mice and subsequent generation of hybridoma cell lines producing HRG-1 monoclonal antibodies (GenScript). The generated antibody was affinity-purified with immobilized protein A. Specificity was confirmed by peptide competition assays. Both antibodies were used at a dilution of 1:250 for immunofluorescence and immunoblotting.

**Cell Culture and Transfection—**R− cells are an embryonic fibroblast cell line derived from *igf1r−/−* mice, and the R+ cell line is R− cells stably overexpressing the IGF-IR (19). R+, R−, HeLa, HEK-293T, normal rat kidney, and MCF-7 cells were maintained in DMEM supplemented in 1 mM L-glutamine, 10% fetal bovine serum (FBS), and 5 mg/ml penicillin and streptomycin antibiotics. For IGF-I stimulation, R+ and MCF-7 cells were starved of serum for 4 h before stimulation with 100 ng/ml IGF-I for the indicated times. HEK-293T cells were transfected using the calcium phosphate transfection method. MCF-7/HA-HRG-1 pools were generated by transfecting MCF-7 cells using Lipofectamine followed by selection for 3 weeks with 1 mg/ml neomycin. Protein expression was confirmed by Western blot and immunofluorescence.

**Immunofluorescence—**Cells were seeded on serum-coated 10-mm glass cover slips and allowed to attach. The cells were washed with phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde in PBS, and permeabilized with 0.1% Triton X-100 in PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2, pH 6.9) for 5 min. Prior to incubation with primary antibody, the cells were blocked with 5% goat serum in PBS. Primary antibodies were diluted in 5% goat serum/PBS, and incubations were performed for 1 h at room temperature, after which the cells were washed with PHEM and incubated with Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, Soham, Cambridgeshire, UK) together with Hoechst before examination with a Nikon T600 fluorescent microscope. Images were captured under ×100 objective using a SPOT CCD camera. Confocal sections were obtained using an Olympus Fluoview FV1000 confocal laser scanning biological microscope (software FV10-ASW version 1.6). Serial z-sections were obtained every 1 μm (usually 8–10 sections/field) to generate representative composite images, and cross hairs through zoom images correspond to z sections depicted on x and y axis, respectively.

**Antibody Uptake by Live Cells—**MCF7/HA-HRG-1 growing on coverslips cells were incubated overnight with either HA or HRG-1 antibodies diluted in complete or serum-free medium. Cells were then fixed in 4% paraformaldehyde, permeabilized, and incubated with Cy2- or Cy3-conjugated secondary antibodies together with Hoechst for 1 h. As a positive control Fo2 antibody staining, cells seeded at the same time were fixed, permeabilized, and incubated with primary antibody for 1 h followed by incubation with secondary antibodies as before.

**Analysis of Plasma Membrane Expression of HRG-1 by Flow Cytometry—**Cells were cultured in complete media and then serum-starved for 2 h with or without 20 μM nocodazole for the last 30 min of starvation. Cells were lifted with PBS/EDTA (2 mM) and washed with PBS. Cell pellets were resuspended in 100 μl of anti-HRG-1 antibody diluted in 5% goat serum, 0.1% Na Dick/PBS and incubated on ice for 1 h. Samples were washed twice with PBS and incubated with Cy2-conjugated secondary antibody for 1 h on ice. Samples were washed with PBS and resuspended in 500 μl of PBS, and fluorescence intensity was analyzed by flow cytometry.

**Western Blotting and Immunoprecipitation—**Whole cell lysates were prepared in RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μg pepstatin, 2 mg/ml aprotinin, 1 μg NaVO4) and analyzed by Western blotting. Primary antibodies were diluted in 5% milk/TBS-Tween 20 (TBS-T) and incubated with membranes at 4°C overnight. Membranes were washed with TBS-T, incubated with horseradish peroxidase-conjugated secondary antibody.
For immunoprecipitation of His-c subunit, 800 μg of transfected HEK-293T lysates were precleared with protein G-Sepharose beads (GE Healthcare) at 4 °C for 1 h. The lysate was incubated with anti-His antibody at 4 °C overnight, and protein G beads were then added for 1 h at 4 °C with gentle rotation. Immunocomplexes were analyzed by Western blotting using anti-His and anti-HA antibodies. For immunoprecipitation of endogenous V-ATPase A subunit, HA-HRG-1-transfected HEK-293T lysates were precleared as for His-c subunit immunoprecipitations. The lysates were then incubated with the monoclonal anti-A subunit antibody (25) at 4 °C overnight, and immune complexes were analyzed by Western blotting.

**Small Interfering RNA (siRNA)—To suppress HRG-1 expression specifically,** two siRNAs targeting the human HRG-1 mRNA were obtained from Dharmacon (Lafayette, CO). The sequences of the siRNA oligonucleotides were as follows: siHRG-15'-UGGUGACCCAGUGUAUAA-3' (nucleotides 158–176 of HRG-1 coding sequence) and siHRG-125'-GCA-CGUGAUAUCAUGCA-3' (nucleotides 165–183). HeLa and MCF-7 cells were transfected with 10 nm siRNA oligonucleotides using the Oligofectamine transfection reagent (Invitrogen), as per manufacturer’s instructions. A nontargeting oligonucleotide from Ambion (Cambridgeshire, UK) was used as a negative control. Suppression of HRG-1 expression was examined 24–96 h after transfection by Western blotting with the anti-HRG-1 polyclonal antibody.

**Yeast Two-hybrid Screen—**The MATCHMAKER yeast two-hybrid system (Clontech) was used to identify HRG-1-interacting proteins. The pGBK7-HRG-1 recombinant yeast expression vector, containing the GAL4 DNA binding domain, was used as bait to screen a human fetal brain library, constructed in the pACT2 vector, containing the GAL4 DNA activation domain. Selection of colonies containing putative interacting proteins was carried out by plating onto synthetic dropout media, as per the manufacturer’s protocol. Library plasmid DNA was extracted, and the inserts were sequenced.

**Cell Viability and Migration Assays—siRNA-transfected HeLa cells were seeded at a density of 3 × 10⁴ cells/well in triplicate wells of a 24-well tissue culture plate 24 h after transfection.** Cell viability in DMEM, 10% FBS was assessed every 24 h by the uptake of propidium iodide using flow cytometry on a FACSCalibur instrument (BD Biosciences).

**Transferrin Receptor-mediated Endocytosis Assays and Transferrin-Alexa488 Binding Assay—**Twenty four hours after siRNA transfection, HeLa cells were starved of serum for 30 min before being pulsed with either 25 μg/ml transferrin Alexa Fluor488 (Molecular Probes, Eugene, OR) or transferrin-FITC (Sigma) in 10% FBS/DMEM for 15 min at 37 °C, 5% CO₂. The extent of transferrin uptake was determined by fluorescence intensity in the FL1H channel using the FACSCalibur instrument.

To assess transferrin-Alexa488 binding, cells were serum-starved for 1 h, then transferred to ice, and washed with cold 0.1% BSA/PBS. Cells were incubated with 25 μg/ml transferrin Alexa Fluor488 diluted in 0.1% BSA/PBS on ice for 90 min. The cells were washed three times with cold 0.1% BSA/PBS, trypsinized, washed once more, and resuspended in 2% FBS/PBS before FACS analysis. For Tf/TF-Alexa488 internalization assays, after the 90-min binding of Tf-Alexa488, prewarmed complete media were added to the cells, and they were left at 37 °C for the indicated periods of time. The cells were then transferred to ice, washed with cold BSA/PBS, and the noninternalized receptors removed by a 5-min acid wash (0.2 M acetic acid, 0.2 M NaCl). The cells were then washed and harvested for FACS analysis.

**Endosomal pH Estimation—**We followed the protocol described by Nilsson et al. (26), with some modifications. Twenty four hours after siRNA transfection, MCF-7 or HeLa cells were seeded in 6-well plates. The next day, the media were replaced with complete media containing 0.5 mg/ml FITC-dextran 40 kDa (FD40), with or without 100 nM of either concanamycin A or bafilomycin A, and incubated at 37 °C with 5% CO₂ for 3 h. The samples were then washed twice with PBS, trypsinized, transferred to FACS tubes, centrifuged and then washed with HBSS (Invitrogen) and centrifuged again. Cell pellets were kept on ice and resuspended in HBSS immediately before analysis by FACS. The mean fluorescence detected in FL1 and FL2 channels was quantified, and the FL1/FL2 ratio was calculated for each sample. To create the calibration curve, cells were loaded with FD40 and treated as described above, but the cell pellets were resuspended in a series of citric acid/potassium phosphate (C/K) buffers based on McIlvaine tables, with pH ranging from 4.0 to 6.0, and containing 50 mM sodium azide, 50 mM 2-deoxy-D-glucose, and 10 μM nigericin to equilibrate the intracellular pH with the pH of the C/K buffers. The cells were incubated for 10 min on ice, and the FL1 and FL2 mean fluorescence values were quantified. The FL1/FL2 ratio of these samples was then correlated with the pH value of the solutions used to equilibrate the cells, and the resulting equation was used to estimate the endosomal pH value of the samples.
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analyzed in a FLEXstation II fluorimeter (Molecular Devices). Afterward, the plates were stained with crystal violet and scanned in the Odyssey Infrared system to estimate cell number. The fluorescence data were then normalized to the cell staining data to control for cell density.

**V-ATPase Activity Assays**—The yeast strain YPH500 was transformed with human HRG-1 in the pGBK-T7 vector (HRG-1) or the pGBK-T7 empty vector. Vacuolar membranes were isolated as described previously (27) followed by SDS-PAGE and Western blotting using the monoclonal antibodies 10D7 (24) against subunit a, 8B1-F3 (24) against subunit A, or antibody against HRG-1. ATPase activity was measured using a coupled spectrophotometric assay in the presence or absence of 1 μM concanamycin, as described previously (27). ATP-dependent proton transport was measured using the fluorescence probe 9-amino-6-chloro-2-methoxyacridine in the presence or absence of 1 μM concanamycin, as described previously (27).

**V-ATPase Assembly Assay**—siNeg- and siHRG-1-transfected MCF7 cells were maintained in complete media for 24 h post-transfection, starved from serum and glucose for 4 h, and then stimulated with 10 mM glucose for 15 min. The cells were transferred to ice, and the cytosolic fraction was purified. Cells were resuspended in cold STE buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.6) supplemented with protease and phosphatase inhibitors and lysed mechanically by passing them through a 26-gauge needle. The lysates were centrifuged at 12000 × g for 10 min at 4 °C to obtain the post-nuclear supernatant, containing cytosol and cellular membranes. Equal volumes of post-nuclear supernatant were centrifuged at 100,000 × g for 1 h to produce the S-100/cytosolic fraction and a membrane pellet. Equal volumes of cytosolic fractions were loaded to a protein gel, and the amount of A1 present in these fractions was quantified by Western blotting. Actin was used as a loading control for the cytosolic fraction and the A1/actin ratio was used as measurement of the amount of A1 present in the cytosol of the cells.

**ZnMP Uptake Assays**—We performed these assays as described previously (20) with some modifications. Control cells and cells overexpressing the HRG-1 protein were grown in complete media for 24 h post-transfection, starved from serum and glucose for 4 h, and then stimulated with 10 mM glucose for 15 min. The cells were transferred to ice, and the cytosolic fraction was purified. Cells were resuspended in cold STE buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.6) supplemented with protease and phosphatase inhibitors and lysed mechanically by passing them through a 26-gauge needle. The lysates were centrifuged at 12000 × g for 10 min at 4 °C to obtain the post-nuclear supernatant, containing cytosol and cellular membranes. Equal volumes of post-nuclear supernatant were centrifuged at 100,000 × g for 1 h to produce the S-100/cytosolic fraction and a membrane pellet. Equal volumes of cytosolic fractions were loaded to a protein gel, and the amount of A1 present in these fractions was quantified by Western blotting. Actin was used as a loading control for the cytosolic fraction and the A1/actin ratio was used as measurement of the amount of A1 present in the cytosol of the cells.

**Membrane Sedimentation Assays**—siNeg- and siHRG-1-transfected MCF7 cells were maintained in complete media for 24 h post-transfection, starved from serum and glucose for 4 h, and then stimulated with 10 mM glucose for 15 min. The cells were transferred to ice, and the cytosolic fraction was purified. Cells were resuspended in cold STE buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.6) supplemented with protease and phosphatase inhibitors and lysed mechanically by passing them through a 26-gauge needle. The lysates were centrifuged at 12000 × g for 10 min at 4 °C to obtain the post-nuclear supernatant, containing cytosol and cellular membranes. Equal volumes of post-nuclear supernatant were centrifuged at 100,000 × g for 1 h to produce the S-100/cytosolic fraction and a membrane pellet. Equal volumes of cytosolic fractions were loaded to a protein gel, and the amount of A1 present in these fractions was quantified by Western blotting. Actin was used as a loading control for the cytosolic fraction and the A1/actin ratio was used as measurement of the amount of A1 present in the cytosol of the cells.

**Hemin-Agarose Pulldown Assays**—The hemin-agarose pull-down assay was performed as described by Rajagopal et al. (20) using MCF-7/H4-HRG-1 cells lysed in MS buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES) at pH 5.0, 6.0, or 7.5.

We used 300 nmol of hemin-agarose (Sigma) per assay (equivalent to 50 μl of hemin-agarose slurry) and 50 μl of Sepharose 4B slurry as negative control, with 300 μg of lysate.

**RESULTS**

**HRG-1 mRNA and Protein Are Induced by IGF-I**—The mouse slc48a1/hrg-1 gene (NCBI accession number NM_026353) is expressed as two transcripts of 2.5 and 1.6 kb that are more abundant in R1 cells than in R2 cells (Fig. 1A). The 2.5-kb variant has a larger 3′-untranslated region, but both species are predicted to encode a 16.5-kDa protein. The hrg-1 1.6-kb mRNA variant is more predominantly expressed in adult mouse tissues than the 2.5-kb variant, with the highest expression in liver and kidney, followed by heart and brain (Fig. 1B). Immunofluorescence microscopy analysis using an antibody raised against the C terminus of the protein demonstrated that HRG-1 expression is higher in R1 than in R2 cells (Fig. 1C). The hrg-1 1.6-kb mRNA (Fig. 1D) and HRG-1 protein (Fig. 1E) are induced in response to IGF-I stimulation of R2 cells. Induction of mRNA was not greatly altered by inhibition of PI3K or extracellular signal-regulated kinase (ERK) signaling pathways (data not shown), suggesting it is not entirely due to IGF-I-mediated activation of these pathways.

**HRG-1 Is Located in Endosomes and Is Trafficked to the Plasma Membrane**—The subcellular location of HRG-1 was investigated by immunofluorescence using the anti-HRG-1 antibody. HRG-1 is expressed in several cell lines with a punctated cytoplasmic distribution resembling endosomes (supple-
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HRG-1 protein sequence with MemBrain software (28) suggests two full transmembrane helices and two half-transmembrane helices. However, taken together with the data indicating orientation of the N and C termini on opposite sides of the membrane, we propose that the protein may contain one transmembrane helices and three half-transmembrane helices. These data demonstrate that HRG-1 is present at the plasma membrane and that the C terminus of the protein (HRG-1 antibody epitope) resides at the external face of the plasma membrane and in the endosome lumen; the N terminus (HA antibody epitope) is located on the cytosolic side.

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The colocalization of HRG-1 with endosomal compartments was investigated by confocal microscopy in MCF-7 cells expressing HA-tagged HRG-1. HA-HRG-1 strongly colocalizes with the TfR and partially colocalizes with EEA1 (early endosomal autoantigen-1), Rab11, and Rab7 (Fig. 2A). This indicates that HRG-1 is located in early, recycling, and late endosomes. However, HA-HRG-1 does not colocalize with LAMP-1, which implies no significant expression in lysosomes.

The distribution of HRG-1 throughout the endosomal compartments prompted us to investigate whether HRG-1 is trafficked to the plasma membrane. Using the polyclonal anti-HRG-1 antibody, HRG-1 was detected by flow cytometry at the plasma membrane of live HeLa cells (Fig. 2B, black line). Serum starvation increased HRG-1 expression at the plasma membrane (Fig. 2B, gray line), but this translocation was prevented by nocodazole, which disrupts microtubule polymerization necessary for endosome trafficking (Fig. 2B, dashed line). These results indicate that the C terminus of HRG-1 is located on the extracellular/lumenal side of the membrane and that HRG-1 traffics to the membrane upon nutrient withdrawal (Fig. 2C).

To further investigate the relative orientation of the N and C termini of HRG-1 on cell membranes, we incubated live MCF-7/HA-HRG-1 cells with either anti-HRG-1 antibody (epitope on C terminus) or anti-HA antibody (epitope on N terminus) in complete media or under serum starvation conditions. As can be seen in Fig. 2D, live MCF-7/HA-HRG-1 cells internalize the HRG-1 antibody in both conditions, but there is no visible internalization of the HA antibody. Staining of permeabilized cells showed that both antibodies recognized HA-HRG-1 protein. These data demonstrate that HRG-1 is present at the plasma membrane and that the C terminus of the protein (HRG-1 antibody epitope) resides at the external face of the plasma membrane and in the endosome lumen; the N terminus (HA antibody epitope) is located on the cytosolic side.

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hairs through zoom images correspond to z sections depicted on the x and y axis, respectively. Original magnification is \( \times 60 \), and bars represent 5 or 10 \( \mu \text{m} \) (zoom images). B, HRG-1 expression at the plasma membrane was detected by flow cytometry with anti-HRG-1 antibody on HeLa cells that were maintained in complete media (CM, black line), serum-starved (SS, dark gray line) for 2 h, or serum-starved for 1.5 h and incubated with 20 \( \mu \text{M} \) nocodazole (Noc, dashed line) for a further 30 min in serum-free media. Control cells stained with secondary antibody alone are indicated by solid gray histogram. C, schematic representation of the predicted transmembrane topology of the HRG-1 protein taking into consideration the orientation of the N and C termini. The epitope (peptide) used to raise anti-HRG-1 antibodies is indicated in green. D, anti-HRG-1 antibody becomes internalized. MCF-7/HA-HRG-1 cells were incubated with either HRG-1 (green) or HA (red) antibodies overnight, in complete media (CM) or serum-free media (SS), and then fixed permeabilized and stained with secondary antibodies. The pictures show a typical example of HRG-1 staining due to HRG-1 antibody uptake by live cells. As positive control for antibody staining, cells were fixed, permeabilized, and stained with HA and HRG-1 antibodies (bottom panels).
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HRG-1 Is Required for Receptor Endocytosis, Cell Migration, and Survival—As HRG-1 is an endosomal protein, we next investigated whether HRG-1 expression is important for endocytosis, and thus cell survival or migration, by suppressing its expression with siRNA in HeLa cells. Two hrG-1-specific siRNAs, siHRG1 and siHRG2, effectively suppress expression of HRG-1 in HeLa cells compared with control siRNA (Fig. 3A). HeLa cultures are viable at 48 h post-transfection but exhibit substantial cell death by 72 and 96 h (Fig. 3B, C, and D). This correlated with a reduction of ~15 and 30%, respectively, in Tf-Alexa488 binding to the cells (Fig. 3E), which indicates decreased plasma membrane TfR levels. Similar effects on TfR endocytosis and expression were observed in MCF-7 cells with suppressed HRG-1 expression (supplemental Fig. S3).

HRG-1 Associates with the V-ATPase and Enhances Its Activity in Yeast—To investigate HRG-1 function in the endocytic pathway, we searched for HRG-1-interacting proteins using a yeast two-hybrid screen. The most frequently isolated clone from a human library encoded the 16-kDa c subunit of the V-ATPase proton pump and endocytosis was measured in sInRNA-transfected HeLa cells 24 h post-transfection and following 4 h of serum starvation. Cell suspensions were loaded into the upper wells of Boyden Transwell chambers and were left to migrate for 4 h. The amount of Tf-Alexa488 uptake was quantified by flow cytometry. The data are presented as the percentage of Tf-Alexa488 uptake relative to control siNeg levels (100%). Results are representative of three independent experiments.

Endocytosis was assessed by measuring the uptake of fluorescently labeled transferrin (Tf-Alexa488) via the constitutively recycled TfR. Uptake of Tf-Alexa488 was reduced by ~20 and 40%, respectively, in siHRG1 and siHRG2-transfected HeLa cells when compared with controls (Fig. 3D). This correlated with a reduction of ~15 and 30%, respectively, in Tf-Alexa488 binding to the cells (Fig. 3E), which indicates decreased plasma membrane TfR levels. Similar effects on TfR endocytosis and expression were observed in MCF-7 cells with suppressed HRG-1 expression (supplemental Fig. S3).
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We next investigated whether HRG-1 expression is necessary for V-ATPase to function as a regulator of endosomal and lysosomal pH in mammalian cells. Early endosomal pH was assessed using transferrin-FITC (pH-dependent fluorescence) and transferrin-Alexa488 (pH-independent fluorescence) following uptake in parallel cultures for 15 min. An increase in Tf-FITC/Tf-Alexa488 fluorescence ratio indicates increased early endosomal pH, which reflects decreased V-ATPase activity. In siHRG1 and siHRG2 HeLa cells, the FITC/Alexa488 ratio was 2- and 2.5-fold higher, respectively, than in control cells (Fig. 5A), indicating that suppression of HRG-1 decreases the acidity of early endosomes. Average endosomal pH was calculated after uptake of FITC-dextran (FD40) for 3 h by using a calibration curve generated from cells prebuffered to defined pH values (supplemental Fig. S4). The endosomal pH of siHRG-1/HeLa cells increased from 0.2 to 0.5 pH units, comparable with the pH increase elicited by the V-ATPase inhibitors bafilomycin or concanamycin A (Fig. 5B). As HRG-1 does not colocalize with the lysosomal marker LAMP1 (Fig. 2A), we wanted to determine whether HRG-1 suppression affects lysosomal pH using the acidotropic lysosome dyes LysoTracker Red and LysoSensor Green. No significant difference in fluorescence was observed in cells with HRG-1 suppressed compared with controls (Fig. 5A). The c subunit is known to interact with proteins other than V-ATPase components (29, 30), so we also tested whether HRG-1 associates with the V-ATPase holoenzyme. HA-HRG-1 could be coimmunoprecipitated with endogenous c subunit (cytosolic V0 domain) (Fig. 4C), indicating that HRG-1 associates with the assembled V-ATPase holoenzyme.

To investigate whether HRG-1 influences V-ATPase activity, we expressed it in yeast, which lack an HRG-1 orthologue. Vacuolar membranes purified from yeast overexpressing HRG-1 contain subunit A (V0 domain), subunit A (V0 domain), and HRG-1 (Fig. 4D). HRG-1-expressing vacuoles displayed an increase of 40% in concanamycin-sensitive ATPase activity (Fig. 4D, gray bars) and of 35% in concanamycin-sensitive proton transport compared with vector-expressing vacuoles (Fig. 4D, white bars). This demonstrates that HRG-1 is targeted to yeast vacuoles and that it enhances V-ATPase function.

**HRG-1 Suppression Decreases V-ATPase Activity and Alters Endosomal pH with Consequences for Cell Survival and Transferrin Receptor Trafficking**—We next investigated whether HRG-1 expression is necessary for V-ATPase to function as a regulator of endosomal and lysosomal pH in mammalian cells. Early endosomal pH was assessed using transferrin-FITC (pH-dependent fluorescence) and transferrin-Alexa488 (pH-independent fluorescence) following uptake in parallel cultures for 15 min. An increase in Tf-FITC/Tf-Alexa488 fluorescence ratio indicates increased early endosomal pH, which reflects decreased V-ATPase activity. In siHRG1 and siHRG2 HeLa cells, the FITC/Alexa488 ratio was 2- and 2.5-fold higher, respectively, than in control cells (Fig. 5A), indicating that suppression of HRG-1 decreases the acidity of early endosomes. Average endosomal pH was calculated after uptake of FITC-dextran (FD40) for 3 h by using a calibration curve generated from cells prebuffered to defined pH values (supplemental Fig. S4). The endosomal pH of siHRG-1/HeLa cells increased from 0.2 to 0.5 pH units, comparable with the pH increase elicited by the V-ATPase inhibitors bafilomycin or concanamycin A (Fig. 5B). As HRG-1 does not colocalize with the lysosomal marker LAMP1 (Fig. 2A), we wanted to determine whether HRG-1 suppression affects lysosomal pH using the acidotropic lysosome dyes LysoTracker Red and LysoSensor Green. No significant difference in fluorescence was observed in cells with HRG-1 suppressed compared with controls (Fig. 5A and supplemental Fig. S5), which indicates that lysosomal pH is not altered by suppression of HRG-1. A similar decrease in endosomal acidity was observed in siHRG-1/HeLa cells (supplemental Fig. S6).

Next we addressed the mechanism by which HRG-1 regulates V-ATPase activity. Because HRG-1 is not an integral component of the V-ATPase, we hypothesized that it may modulate V-ATPase holoenzyme assembly. V-ATPase disassembles when glucose and serum are withdrawn, and it re-assembles in complete media (CM), starved of serum and glucose for 4 h (SGF), or serum/glucose-starved, and then stimulated with 10 mM glucose for 15 min (Gluc). The cytosolic fraction of the cells was purified, and the presence of A1 was quantified by Western blot using actin as loading control. Data are presented as relative levels of cytosolic A1 in each of the conditions analyzed. This experiment is representative of three independent experiments with similar results.
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Assembles after glucose re-addition in a PI3K-dependent manner (12), so we asked if HRG-1 influences glucose-induced re-assembly of the V-ATPase. We quantified the amount of free cytosolic A1 subunit (V1 domain) as a measure of disassembled V-ATPase (method validation shown in supplemental Fig. S7). Cells with suppressed HRG-1 cultured in complete medium consistently had higher levels of cytosolic A1 subunit compared with controls, which indicates decreased V-ATPase assembly. Moreover, glucose re-addition did not reduce cytosolic A1 levels in serum- and glucose-starved siHRG2 cells (Fig. 5C). Therefore, glucose-induced re-assembly of the V-ATPase is impaired when HRG-1 is suppressed. Taken together, our data indicate that HRG-1 participates in V-ATPase activity regulation but only in the endocytic compartments in which HRG-1 is expressed (endosomes but not lysosomes).

Overexpression of HRG-1 Enhances TfR Internalization—Our findings that HRG-1 is expressed at high levels in transformed cells and cancer cell lines and that it enhances V-ATPase function in yeast suggest it may modulate endocytosis of receptors in mammalian cells. To test this, MCF-7 cell lines overexpressing HA-HRG-1 were assessed for endocytosis. In MCF-7/HA-HRG-1 cells total cellular levels of TfR protein were 20% higher than in controls (Fig. 6A), which suggests reduced degradation of TfR. Internalization rates of Tf-Alexa488 at 5 and 10 min were enhanced by ~15% in HA-HRG-1 cells compared with controls, although cell surface TfR expression was equivalent (Fig. 6B). Our results indicate that HRG-1 enhances transferrin uptake via receptor endocytosis.

HRG-1 Enhances Heme Transport in a V-ATPase-dependent Manner—After we had already characterized HRG-1 function in V-ATPase regulation, we found that the same gene was described by Rajagopal et al. (20) as a heme-binding protein (HRG-1) essential for erythropoiesis in zebrafish. Heme trafficking is a pH-dependent process, so we first investigated heme uptake in HeLa cells with suppressed HRG-1. We found that siHRG2/HeLa cells display ~30% less uptake of the fluorescent heme analogue ZnMP than control cells (Fig. 7A). Because ZnMP fluorescence intensity depends on pH (31), we performed ZnMP uptake assays and measured ZnMP fluorescence after equilibrating the cells to pH 5.5. The proportion of change between siNeg and siHRG2 cells was the same as in cells without pH equilibration (Fig. 7B), indicating that the differences in ZnMP fluorescence in siHRG2 cells were due to decreased uptake and not an artifact due to altered endosomal pH.

We also assessed ZnMP uptake in cells overexpressing MCF-7/HA-HRG-1. These cells exhibited nearly 2-fold increases in ZnMP uptake compared with control cells (Fig. 7C), which is in agreement with published data for HRG proteins (20). To determine whether the increase in ZnMP uptake in HA-HRG-1 cells was linked with the effects of HRG-1 on V-ATPase activity, the ZnMP uptake assays were performed in the presence of the V-ATPase inhibitor bafilomycin A. In untreated cells, HA-HRG-1 expression increased ZnMP uptake by 2-fold, but pretreatment with bafilomycin A reduced this substantially (Fig. 7D). These results indicate that function of HRG-1 in promoting heme transport is dependent on V-ATPase activity.

We next investigated how the subcellular location and trafficking of HRG-1 may affect heme binding. HRG-1 binding to heme was previously shown to be stronger at pH 6.0 than at pH 8.5, whereas binding of heme to other HRG proteins was pH-independent (20). We performed hemin-agarose pulldown assays at pH 5.0, 6.0, and 7.5, representing average pH in late endosomes, early/recycling endosomes, and extracellular media, respectively. We found that both HA-HRG-1 and endogenous HRG-1 bound the strongest to heme at pH 5.0 (Fig. 7E), which is consistent with a role for HRG-1 in heme transport in late endosomes rather than at the cell surface.
DISCUSSION

There is considerable evidence to indicate that the endocytic machinery and trafficking of nutrients is deregulated in cancer cells to support the transformed phenotype (reviewed in Refs. 6, 8). Here, we have shown that the endosomal protein HRG-1/SLC48A1 is a new IGF-I-regulated protein that may enhance the proliferative and invasive phenotype of cancer cells through its effects on endosomal acidification and receptor trafficking. HRG-1 was also previously described as a heme-binding protein (20) with conserved function. Thus, HRG-1 has a unique role in regulating pH-dependent endocytic trafficking and micronutrient uptake necessary for cellular metabolism. We initially identified HRG-1 because it had increased expression in cells transformed due to IGF-IR overexpression. As IGF-I signaling facilitates the growth, migration, and invasiveness of many tumors (32–34), we propose that HRG-1 is a micronutrient (heme) transporter that may enhance tumor growth and cancer progression. When HRG-1 is suppressed, V-ATPase activity is decreased; endosomal acidity is reduced, and receptor endocytosis is impaired. In contrast, overexpression of HRG-1 enhances receptor endocytosis. Thus, increased HRG-1 expression could have a profound effect on the growth and migratory potential of cancer cells by regulating the endocytosis and degradation of receptors that promote signaling for survival, growth, and migration.

The tissue distribution of HRG-1 mRNA (high in liver and kidney) is consistent with a dual role for HRG-1 in regulating V-ATPase activity as well as in heme intracellular transport. Both liver and kidney are known sites of heme transport, and HRG-1, HCP-1, and ABCB6 (other heme transporters) mRNAs are all expressed in those tissues (35, 36). In the kidney, the V-ATPase also plays a major role in acidification and bicarbonate absorption by nephrons (37).

The HRG protein family has four orthologues (HRG1–4) in C. elegans with different cellular locations and abilities to transport heme (20). It is interesting that HRG-1 is not present in yeast and that only the HRG-1 orthologue is present in mammalian cells. This suggests that the protein may have evolved for a broader function in mammalian cells involving the integration of heme transport with endosome acidification. Indeed, we have shown that HRG-1 not only binds heme and regulates its uptake, but we also have shown that HRG-1 has a direct effect on V-ATPase activity and glucose-induced re-assembly.
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of the holoenzyme. Moreover, the dynamic interaction observed with the c subunit and trafficking to the plasma membrane suggests a role for HRG-1 in assembly or targeting of the V₀ domain to specific membranes. Regulation of V-ATPase assembly is not completely understood but has previously been shown to involve several different proteins, including the protein complex RAVE that regulates assembly of the V₁ domain in yeast (13), and chaperones for V₀ domain assembly (9).

Our observations that HRG-1 is associated with early and late endosomes but not lysosomes and that HRG-1 suppression impairs endosome but not lysosome acidification support the specific role proposed by others for V-ATPase activity in trafficking from early to late endosomes (29). Inhibition of V-ATPase with bafilomycin or loss of a pH-dependent interaction of the V-ATPase with Arf6 and ARNO can specifically impair trafficking from early to late endosomes, which subsequently leads to impaired endocytosis (29). Here, we have shown that the defects in V-ATPase activity due to HRG-1 suppression also lead to impaired TfR endocytosis. The fact that HRG-1 is trafficked to the plasma membrane also suggests a possible role in chaperoning heme or transferrin-associated iron uptake by cells. This is supported by our observations on ZnMP uptake and TfR trafficking in cells with HRG-1 suppressed or overexpressed.

As HRG-1 regulates endosomal pH, it also has the potential to influence in a number of ways iron and heme availability within cells. HRG-1 suppression may diminish the internalization of iron through the TfR, and it could also affect the ability of the cells to acquire nutrients, to mediate signaling in response to growth factor receptor activation, and to internalize and traffic integrins and other proteins, which is necessary for cell survival, migration, and proliferation.

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