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Colocalization of Ferroportin-1 With Hephaestin on the Basolateral Membrane of Human Intestinal Absorptive Cells

Okhee Han* and Eun-Young Kim
Department of Nutritional Sciences, Pennsylvania State University, University Park, Pennsylvania 16802

Abstract An iron exporter ferroportin-1 (FPN-1) and a multi-copper oxidase hephaestin (Heph) are predicted to be expressed on the basolateral membrane of the enterocyte and involved in the processes of iron export across the basolateral membrane of the enterocyte. However, it is not clear where these proteins are exactly located in the intestinal absorptive cell. We examined cellular localization of FPN-1 and Heph in the intestinal absorptive cells using the fully differentiated Caco-2 cells. Confocal microscope study showed that FPN-1 and Heph are located on the basolateral membrane and they are associated with the transferrin receptor (TfR) in fully differentiated Caco-2 cells grown on microporous membrane inserts. However, Heph protein was not detected in the crypt cell-like proliferating Caco-2 cell. In stably transfected human intestinal absorptive cells expressing human FPN-1 modified by the addition of GFP at the C-terminus, we show that FPN-1-GFP is located on the basolateral membrane and it is associated with Heph suggesting the possibility that FPN-1 might associate and interact with Heph in the process of iron exit across the basolateral membrane of intestinal absorptive cell. J. Cell. Biochem. 101: 1000–1010, 2007. © 2007 Wiley-Liss, Inc.

Key words: intestine; ferroportin-1; hephaestin; transferrin receptor; cellular localization

The molecular mechanism of intestinal iron absorption has been recently proposed based on the possible roles of the newly identified genes, such as ferroportin-1 (FPN-1) and hephaestin (Heph), divalent metal transporter-1 (DMT-1) and ferrireductase duodenal cytochrom *b* (Dcytb1). The vectorial passage of iron through the enterocyte entails three steps: uptake across the brush membrane, intracellular translocation across the cytosol, and release of iron across the lateral membrane of enterocyte into the circulation [Hentze et al., 2004].

The transporter responsible for the export of iron across membranes was identified in three different laboratories and is variously referred to as FPN-1 [Donovan et al., 2000], Ireg1 [McKie et al., 2000], and MPT1 [Abboud and Haile, 2000]. When expressed in Xenopus oocytes, it stimulates the release of iron in the presence of ceruloplasmin (Cp) [McKie et al., 2000], supporting its physiological role in iron export. The transporter, FPN-1 has been suggested to be a major iron exporter in small intestine. Although FPN-1 protein was detected on the basolateral membrane of enterocytes in mice [Donovan et al., 2005], the exact function of FPN-1 during the process of iron export from intestinal enterocytes into the circulation unknown.

The Heph was identified as the one responsible for sex-linked anemia (*sla*) in mice [Vulpe et al., 1999]. The *sla* mice develop moderate to severe microcytic hypochromic anemia due to impaired intestinal iron absorption [Pinkerton, 1968; Bannerman, 1976]. However, iron uptake across the brush border membrane in *sla* mice is normal indicating there is a defect in an 'exit' step of iron absorption. Thus, Heph could possibly be the factor limiting iron transfer across the basolateral membrane of enterocyte. Heph was proposed to have a multi-copper oxidase activity due to its homology with Cp, and to be located on the basolateral membrane in intestinal enterocytes because it has a single

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*Correspondence to: Okhee Han, Department of Nutritional Sciences, S-126 Henderson Building South, Pennsylvania State University, University Park, PA 16802. E-mail: ouh1@psu.edu

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predicted transmembrane domain. The previous study showed that Heph protein was located on the basolateral membrane of small intestine in mice [Kuo et al., 2004]. Further detailed studies are needed to characterize its function on exit of iron from the intestinal absorptive cell into the circulation.

Based on molecular genetic studies in yeast cells [De Silva et al., 1995; Stearman et al., 1996; Singh et al., 2006], it was recently proposed that FPN-1 and Heph may be located on the basolateral membrane of enterocytes, and they may interact in the processes of iron export across the basolateral membrane of intestinal absorptive cells, but it remains to be explored. It is not clear where these proteins are exactly located, and how they are involved in the process of iron exit in intestinal enterocytes. Therefore, we initially investigated cellular localization of FPN-1 and Heph proteins in intestinal absorptive cells utilizing a human intestinal absorptive cell model, Caco-2 cell. We also studied cellular localization of Heph and FPN-1-GFP in human intestinal absorptive cells expressing GFP tagged human FPN-1 protein.

Since it is unable to separate the roles of multiple factors in the processes of iron absorption due to the complexity of the experimental animal models, investigators have been used long-term enterocyte cell culture models to investigate mechanisms of intestinal iron metabolism. Caco-2 cell monolayers have been widely used to identify molecular and cellular mechanisms of iron absorption as well as factors influencing iron bioavailability [Nunez et al., 1994; Han et al., 1995; Glahn et al., 1996; Tallkvist et al., 2001]. Upon confluence, Caco-2 cells spontaneously differentiate to display many of the morphological and functional features of mature small intestinal enterocytes and have been widely used as a model of normal human intestinal epithelium [Louvard et al., 1992; Peterson et al., 1993; Bissonnette et al., 1994; Han et al., 1998].

The aims of the current study were to characterize the cellular localization of FPN-1 and Heph in a human intestinal absorptive cell model. Our results demonstrate that: (1) FPN-1 and Heph are expressed on the basolateral membrane and they are colocalized with transferrin receptor (TfR) in the intestinal absorptive cell model, and (2) Heph protein is colocalized with FPN-1-GFP on the basolateral membrane in the intestinal absorptive cells expressing GFP tagged FPN-1.

MATERIALS AND METHODS

Reagents

Tissue culture media and Hanks' balanced salts solution (HBSS), glutamine, non-essential amino acids, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Monoclonal mouse anti-human TfR was obtained from Zymed (San Francisco, CA). Nitrocellulose membranes, enhanced chemiluminescence (ECL) kits for Western blotting protein detection, the peroxidase-coupled sheep anti-mouse and donkey anti-rabbit antibodies were purchased from PerkinElmer (Boston, MA). Alexa Fluor 448 conjugated goat anti-rabbit (or -mouse) IgG, Alexa Fluor 546 conjugated goat anti-rabbit (or - mouse) IgG and Alexa Fluor 594 conjugated wheat germ agglutinin (WGA) were purchased from Molecular Probes (Eugene, OR). Unless otherwise noted, all other reagents were purchased from Sigma Chemical (St Louis, MO), Invitrogen, VWR (West Chester, PA), or Bio-Rad (Hercules, CA).

Production of FPN-1 and Heph Antibodies

Peptide fragments of FPN-1 and Heph were synthesized with an additional cysteine residue for conjugation to keyhole limpet hemocyanin (KLH) at the COOH terminal end (Zymed). Sequences were verified by amino acid analysis and mass spectroscopy. KLH-conjugated peptides were injected into New Zealand White rabbits (1 mg peptide/rabbit) for polyclonal antibody production. Immunized rabbit serum was purified by Affi-gel (Bio-Rad) using the immunized peptides. The FPN-1 peptide was synthesized according to the predicted amino acid sequence obtained from the human FPN-1 cDNA corresponding to 18 amino acids (KQLNLHKDTEPKPLEGTH) of the protein in the internal loop between putative transmembrane regions four and five [Devalia et al., 2002]. Heph peptide was synthesized according to the predicted amino acid sequence obtained from the human Heph cDNA corresponding to the C-terminal 15 amino acids (QHRQRKLRRN RRSIL) predicted to be on the cytoplasmic surface of the membrane [Vulpe et al., 1999; Syed et al., 2002]. Comparison of both peptide

sequences with public sequence databases using BLAST identified only FPN-1 and Heph sequences.

The specific binding activity of the antibodies was verified by incubating with peptide. Briefly, Caco-2 proteins were resolved and transferred as described below. After the blots were blocked with 5% non-fat powdered milk, they were incubated with affinity purified FPN-1 (1:2,000) or Heph (1:2,000) antibodies in the presence (lanes 3,6) or absence (lanes 2,5) of the corresponding peptides, followed by incubation with secondary antibodies. Both FPN-1 and Heph peptides used were in 50-fold excess over the antibodies used. The blots were visualized using ECL after incubation with secondary antibody. As a negative control, the membranes were incubated with preimmune sera (lanes 1,4) prior to incubation with secondary antibodies (Fig. 1A). Three FPN-1 bands were detected in Caco-2 cell lysates (Fig. 1A, lane 2) and all bands were blocked by the presence of a molar excess of FPN-1 peptide (Fig. 1A, lane 3). Usually, only the top band is detected from Caco-2 cell lysates in other experiments as shown in Figure 1B and the additional immunoreactive bands mostly represent either degradation products or premature proteins as reported by other investigators [Knutson et al., 2005; Goncalves et al., 2006].

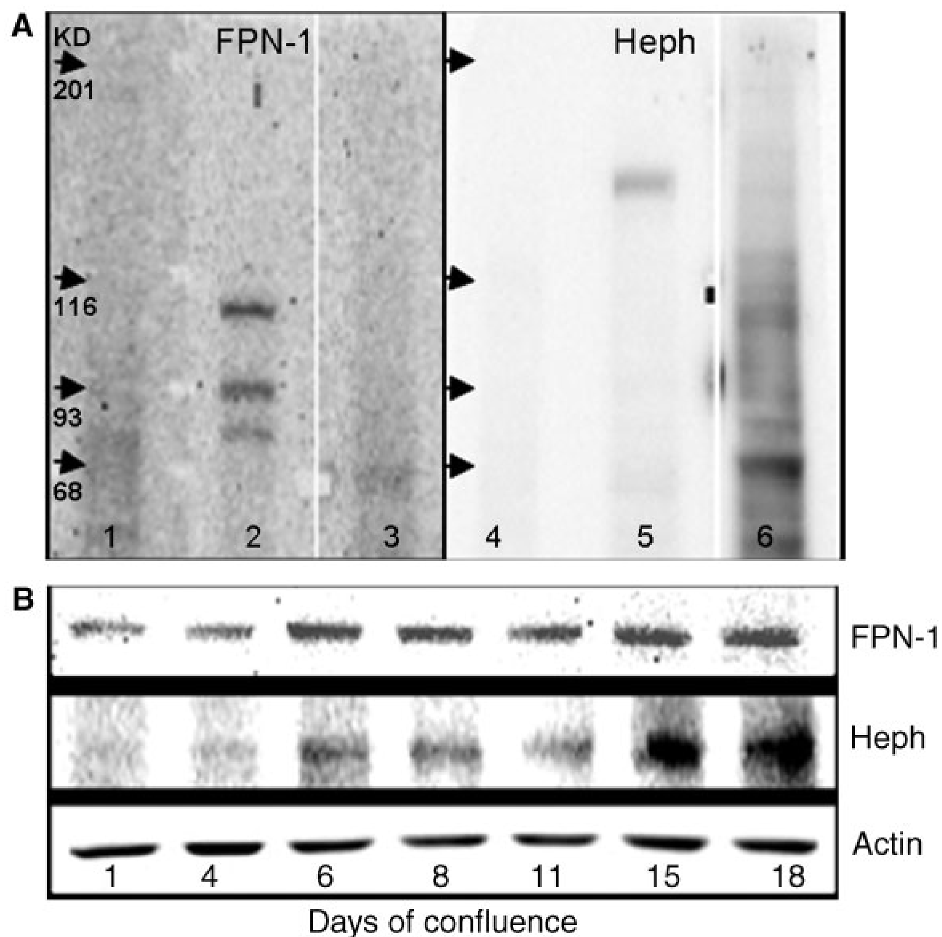

Fig. 1. Expression of FPN-1 and Heph proteins in human intestinal Caco-2 cells. **A**: Verification of specific binding activities of FPN-1 and Heph antibodies. Caco-2 cell proteins were separated by SDS–PAGE and transferred onto the nitrocellulose membrane. The membranes were incubated with FPN-1 or Heph antibody in the presence (**lane 3**,**6**) or absence (**lane 2**,**5**) of the corresponding peptides, followed by incubation with secondary antibodies. The visualized antigens by enhanced chemiluminescence (ECL) were detected using ChemiDoc XRS system (Bio-Rad, Hercules, CA). As a negative control, the membranes were incubated with preimmune sera (**lane 1**,**4**) prior to incubation with secondary antibodies. **B**: Expression of FPN-1 and Heph protein during cellular differentiation of Caco-2 cells. Cells were collected during cell differentiation from 1 d post-confluence to 18 d post-confluence and homogenized. Total protein was resolved by 7.5% SDS–PAGE and electroblotted to the nitrocellulose membrane prior to Western blotting for FPN-1, Heph, or β-actin protein.

Plasmid Construct

Human FPN-1 coding sequence was modified by in-frame addition of a GFP tag at the C-terminus. This was carried out by PCR amplification from human FPN-1 cDNA (RZPD, Germany) using oligonucleotide FPN-1 5′ (5′-GTCGC**CTGCAG**TCATGACCAGG-3′) and FPN-1 3′ (5′-GTTAAA**CTGCAG**CAAACAACA-CATG-3′) containing the PST I restriction sites (Boldfase indicate PST I restriction site). The PCR product was digested with PST I and inserted into the corresponding sites of a pEGFP vector (BD Biosciences Clontech, Palo Alto, CA).

Cell Culture and Transfection

Cell culture. The human Caco-2 cells were cultured as previously described [Han et al., 1995; Han and Wessling-Resnick, 2002]. For experiments, 50,000 cells/cm^2 in a volume of 1.5 ml complete Dulbecco's Modified Essential Medium (DMEM) were seeded on 3 μm microporous membrane inserts (4.9 cm^2). The basolateral (bottom) chamber contained 2.5 ml complete DMEM. The complete culture medium contained DMEM supplemented with 25 mM glucose, 2 mM glutamine, 100 μM MEM nonessential amino acids, 100 U/L penicillin G, 100 mg/L streptomycin, and 10% FBS. The culture medium was changed every 2 days, and cells were used after 17 days post-confluence for experiments. Cells are fully differentiated at 17 days after confluence in normal cell culture conditions [Louvard et al., 1992; Han et al., 1995; Han and Wessling-Resnick, 2002].

Transfection. Cells were seeded in a 6-well plate for the transfection. At 80% confluence, cells were transfected with 1 μg of FPN-1-pEGFP construct using Lipofectamin LTX (Invitrogen), following the manufacturer's standard method. After 48 h of incubation, Geneticin (Invitrogen, G418; 800 μg/ml) was added into the culture medium to select the transfected cells. Stably transfected cells were selected after 3 weeks, and maintained in the medium containing G418.

Western Blot Analysis

Western blot analyses were performed to determine FPN-1 and Heph protein levels in Caco-2 cells. Protein samples extracted from the same cells were used for all Western blot analyses. Cell lyses (40 μg) were solubilized in Laemmli buffer, boiled for 5 min, and separated by a 7.5% SDS–PAGE. Proteins were transferred by electroblotting to nitrocellulose membranes, which were then blocked by 5% non-fat powdered milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) at room temperature for 1 h. The membranes were then incubated for 2 h at room temperature with an affinity purified FPN-1 (or Heph) antibody (1:2,000) in TBS containing 0.05% Tween 20 (TBST). The membranes were washed for several times with TBST and then incubated for 1 h at room temperature with peroxidase-linked goat anti-rabbit IgG (1:3,000). The visualized antigens by ECL were detected ChemiDoc XRS system (Bio-Rad).

Immunofluorescence Analysis

The fully differentiated Caco-2 cells grown on microporous membrane inserts were fixed with 2% formaldehyde and permeabilized with 0.3% Triton X-100 for 30 min. Caco-2 cells were then incubated with indicated primary antibodies (anti-FPN-1; 1:200, anti-Heph; 1:200, anti-TfR; 1:500) with 1% BSA in PBS for 2 h at room temperature. After several washes, Caco-2 cells were then incubated with either Alexa Fluor 546 (red) conjugated goat anti-rabbit (or -mouse) IgG or Alexa Fluor 488 conjugated goat anti-rabbit (or -mouse) IgG for 1 h at room temperature. To stain the plasma cell membrane, the fixed cells were incubated with Alexa Fluor 594 conjugated WGA for 10 min at room temperature. The inserts were placed on glass slides and mounted. Since the inserts were placed on the microscope stage with their bases on top of a glass slide, the direction of the laser beam was basal to apical. The optical sections had the same basal to apical direction. To determine the cellular location of proteins, the fluorescence image was taken at every 1.0 μm from the basal end to the apical side. The images for XY view were taken at 5 μm from the basal side. To analyze fluorescence intensity in the basal to apical axis from cells, the lateral view (*xz*) is made up by the addition of consecutive pixels in the *y*-axis. Cells were analyzed at 63× magnification on a laser scanning confocal microscope (Fluoview 1000 Confocal Laser Scanning Microscope, Olympus).

RESULTS

Expression of FPN-1 and Heph in Human Intestinal Absorptive Cells

The recent studies have suggested the possibility that FPN-1 and Heph proteins are located on the basolateral membrane of intestinal absorptive cells and involved in iron export across the basolateral membrane of enterocytes [Vulpe et al., 1999; Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000; Kuo et al., 2004; Donovan et al., 2005]. To determine cellular distribution of FPN-1 and Heph protein in the intestinal absorptive cells, the fully differentiated Caco-2 cells grown on microporous membrane inserts were fixed and incubated with an affinity purified FPN-1 or Heph antibody. The antibodies specificity was confirmed by peptide competition (Fig. 1A). The staining of FPN-1 and Heph protein was also blocked by FPN-1 and Heph peptide, respectively (data not shown). Our individual localization studies indicate that FPN-1 and Heph proteins are located on the cell membrane of intestinal absorptive cells as seen for TfR staining. To determine whether FPN-1 and Heph proteins are located on the basolateral membrane of intestinal absorptive cells, the fully differentiated Caco-2 cells grown on microporous inserts were fixed and stained for basolateral membrane with Alexa Fluor 594 conjugated WGA by adding the Alexa Fluor 594 conjugated WGA in the bottom chambers after fixing the live cells. The cells were then permeabilized

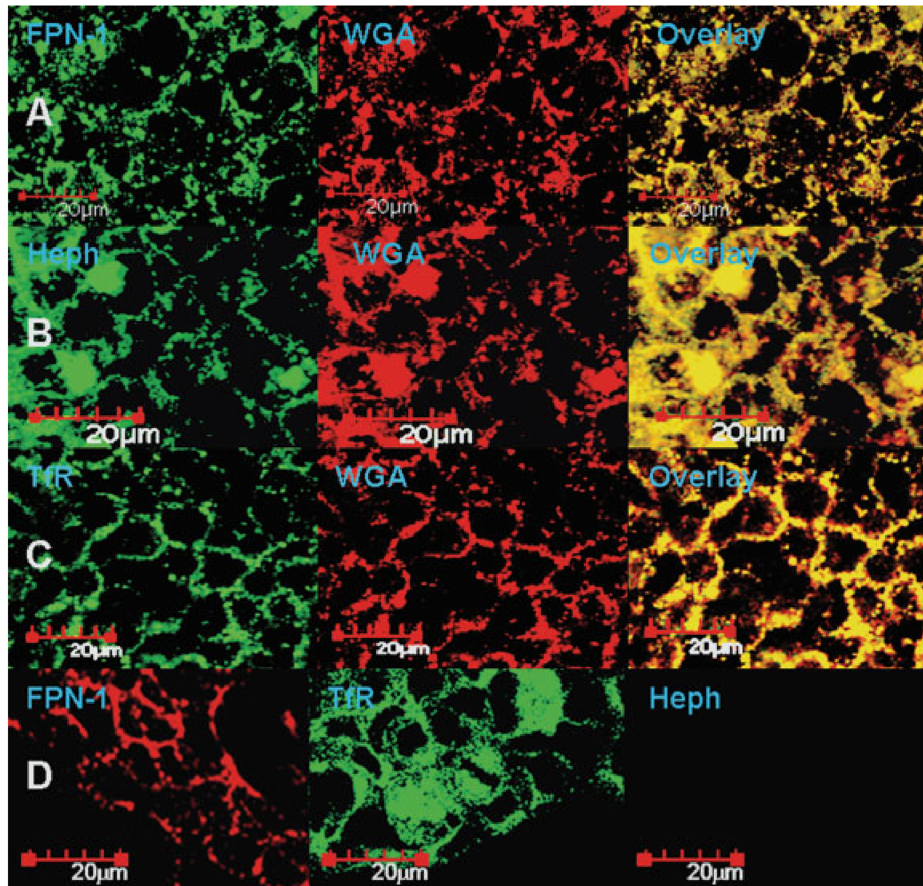

Fig. 2. Cellular localization of FPN-1 and Heph in human intestinal Caco-2 cells. The fully differentiated Caco-2 cells grown on microporous membrane inserts first were fixed and then incubated with Alexa 594 conjugated WGA in the bottom chamber to stain the basolateral membrane of cells. The cells were then permeabilized and examined following incubation with antibodies against the indicated proteins. **A–C**: Localization of FPN-1, Heph, and TfR on the basolateral membrane of intestinal absorptive cells. A: FPN-1, (B) Heph, (C) TfR. **D**: Location of FPN-1 and TfR in Proliferating Caco-2 cells. **E**,**F**: Integration of fluorescence intensity in the basal to apical axis from cells. To determine the cellular location of FPN-1 and TfR, the fluorescence image was taken at every 1.0 μm from the basal end to the apical side. The images for XY view were taken at 5 μm from the basal side. To analyze fluorescence intensity in the basal to apical axis from cells, the lateral view (*xz*) is made up by the addition of 30 consecutive pixels in the *y*-axis. TM: view of cells, AP: apical, BL: basolateral.

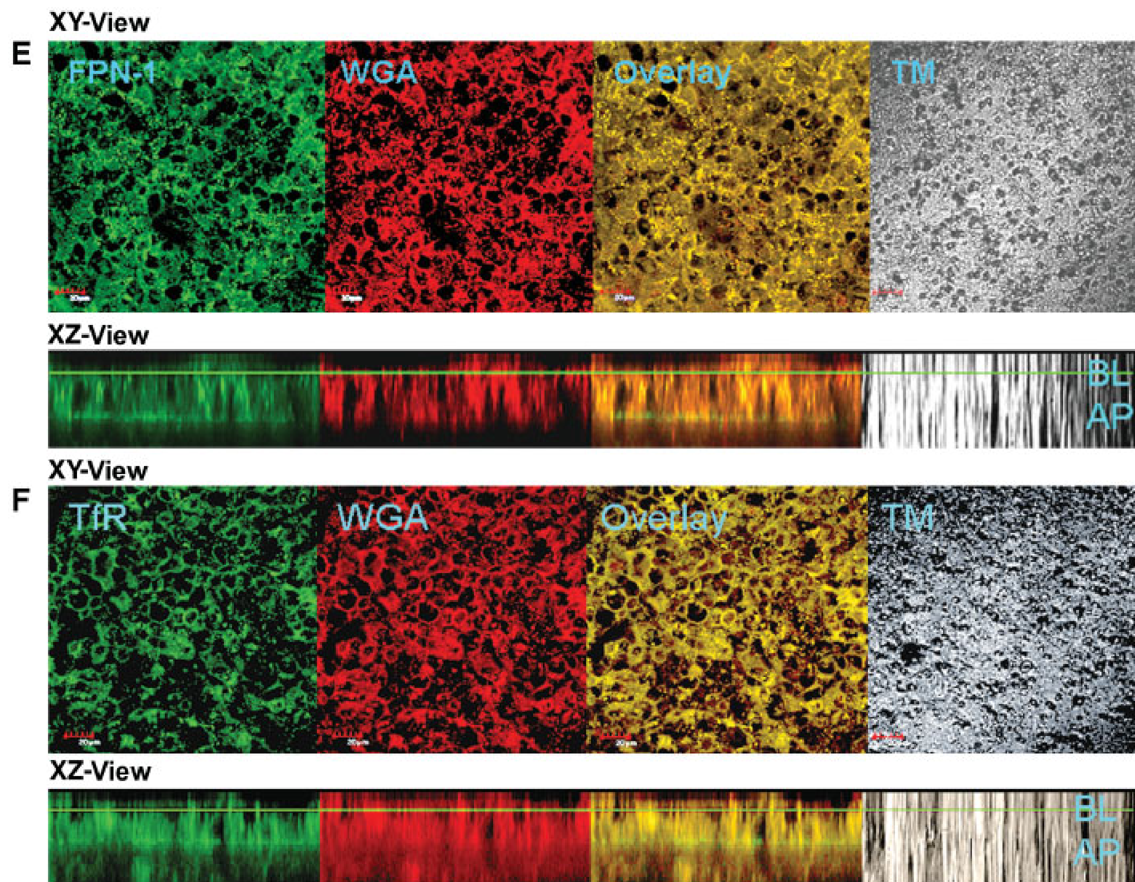

Fig. 2. (*Continued*)

and stained for FPN-1 protein. As shown in Figure 2A, the staining of FPN-1 and the cell membrane is in green and red, respectively. As shown in Figure 2A, when the green fluorescent image (FPN-1 staining) was overlayed with the red fluorescent image (the cell membrane staining), the staining shows in yellow indicating localization of FPN-1 protein on the cell membrane of intestinal absorptive cells. Similarly, the confocal microscope studies show that Heph and TfR proteins are also located on the cell membrane in human intestinal absorptive cells (Fig. 2B,C). In contrast, the crypt cell-like proliferating cells do not express Heph protein. As shown in Figure 2D, the staining of FPN-1 and TfR was found in proliferating cells but not Heph. Similarly, as shown by the Western blot (Fig. 1B), Heph protein was not detected in protein samples extracted from proliferating Caco-2 cells.

Next, we further localized FPN-1 protein by integrating fluorescence intensity of FPN-1 staining in the basal to apical axis (the *z*-axis) of cells. To analyze fluorescence intensity in the basal to apical axis from cells, the lateral view (*xz*) is made up by the addition of consecutive pixels in the *y*-axis. As described above, when the green fluorescent image (FPN-1) was overlayed with the red fluorescent image (the plasma membrane), the image shows in yellow indicating localization of FPN-1 on the cell membrane of the fully differentiated Caco-2 cells (Fig. 2E, XY view). The integration of fluorescence intensity in the basal to apical axis (the *z*-axis) of cells shows that FPN-1 is primarily located on the basolateral membrane in fully differentiated Caco-2 cells (Fig. 2E) as shown for TfR protein (Fig. 2F). The images for XY view were taken at the position indicated by the green line in XZ view. Similarly, our optical analysis data show that Heph is also located on the basolateral membrane in fully differentiated Caco-2 cells (data not shown).

Colocalization of FPN-1 and Heph With TfR in Human Intestinal Cells

Since FPN-1 and Heph proteins are located on the basolateral membrane of intestinal absorp-

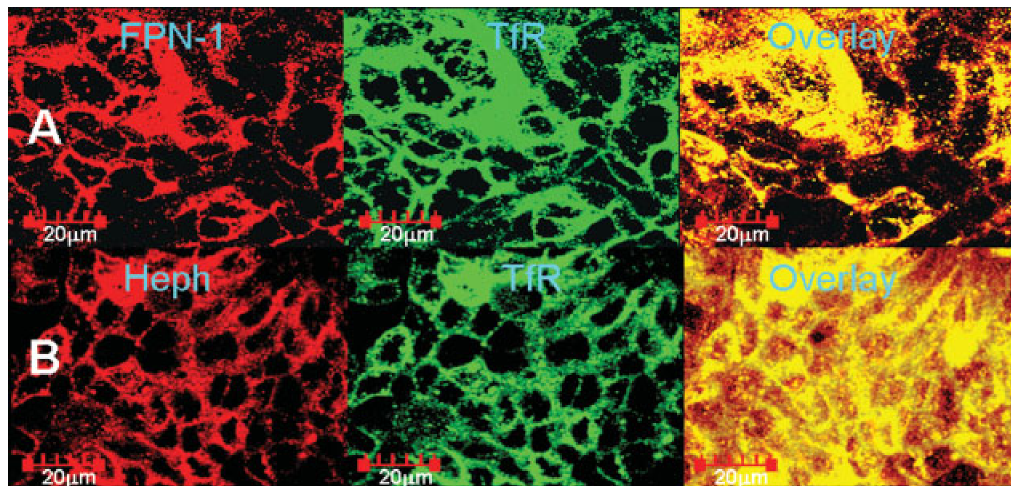

Fig. 3. Co localization of FPN-1 and Heph with TfR in fully differentiated Caco-2 cells. Colocalization of FPN-1 and Heph with TfR in fully differentiated Caco-2 cells was shown by colabeling the live cultures with FPN-1 antibody (or Heph antibody) and TfR antibody. **A**: Colocalization of FPN-1 with TfR. FPN-1 staining is represented in red and TfR is shown in green. Yellow in overlay indicates colocalization. **B**: Colocalization of Heph with TfR. Heph staining is represented in red and TfR is shown in green in fully differentiated Caco-2 cells. Yellow in overlay indicates colocalization of Heph with TfR.

tive cells as seen for TfR (Fig. 2), we next determined whether FPN-1 and Heph proteins are colocalized with TfR in intestinal absorptive cells. The human intestinal absorptive cells were fixed and then coincubated with the primary antibodies of FPN-1 (or Heph) and TfR and proteins were localized by incubating with secondary antibodies conjugated with Alexa Fluor 546 (FPN-1 or Heph) and Alexa Fluor 488 (TfR). The immunostaining of FPN-1 and TfR proteins is primarily detected on the basolateral membrane of cells as shown above (Fig. 2). As shown in Figure 3A, when the red fluorescent image (FPN-1 staining) was overlayed with the green fluorescent image (TfR staining), the staining shows in yellow indicating colocalization of FPN-1 protein with TfR protein in human intestinal absorptive cells. Similarly, Heph is also colocalized with TfR in fully differentiated Caco-2 cells (Fig. 3B).

Colocalization of Heph With FPN-1-GFP on the Basolateral Membrane in Intestinal Absorptive Cells Expressing GFP Tagged FPN-1

The findings of the colocalization of FPN-1 and Heph with TfR on the basolateral membrane suggest the possibility that FPN-1 might be colocalized with Heph on the basolateral membrane in human intestinal absorptive cells. To determine whether FPN-1 and Heph proteins are colocalized on the basolateral membrane of intestinal absorptive cells as predicted, Caco-2 cells were stably transfected with FPN-1-GFP construct. We initially determined whether GFP-tagged FPN-1 is located on the basolateral membrane of enterocytes. The fully differentiated Caco-2 cells grown on microporous membrane inserts were fixed and the cells were incubated with Alexa 594 conjugated WGA in the bottom chamber. As shown in Figure 4A, when the green fluorescent image (GFP-tagged FPN-1 protein) was overlayed with the red fluorescent image (basolateral membrane staining), the staining showed in yellow indicating localization of FPN-1-GFP on the basolateral membrane of intestinal absorptive cells. Next, to test whether FPN-1-GFP protein is colocalized with Heph protein, the intestinal absorptive cells expressing GFP-tagged FPN-1 were fixed, permeabilized, and then incubated with the primary antibody of Heph. The staining of Heph protein was primarily found on the basolateral membrane of cells. As shown in Figure 4B, when the red fluorescent image (Heph staining) was overlayed with the green fluorescent image (GFP-tagged FPN-1), most of the staining appears in yellow indicating colocalization of Heph protein with GFP-tagged FPN-1 protein on the basolateral membrane in human intestinal absorptive cells.

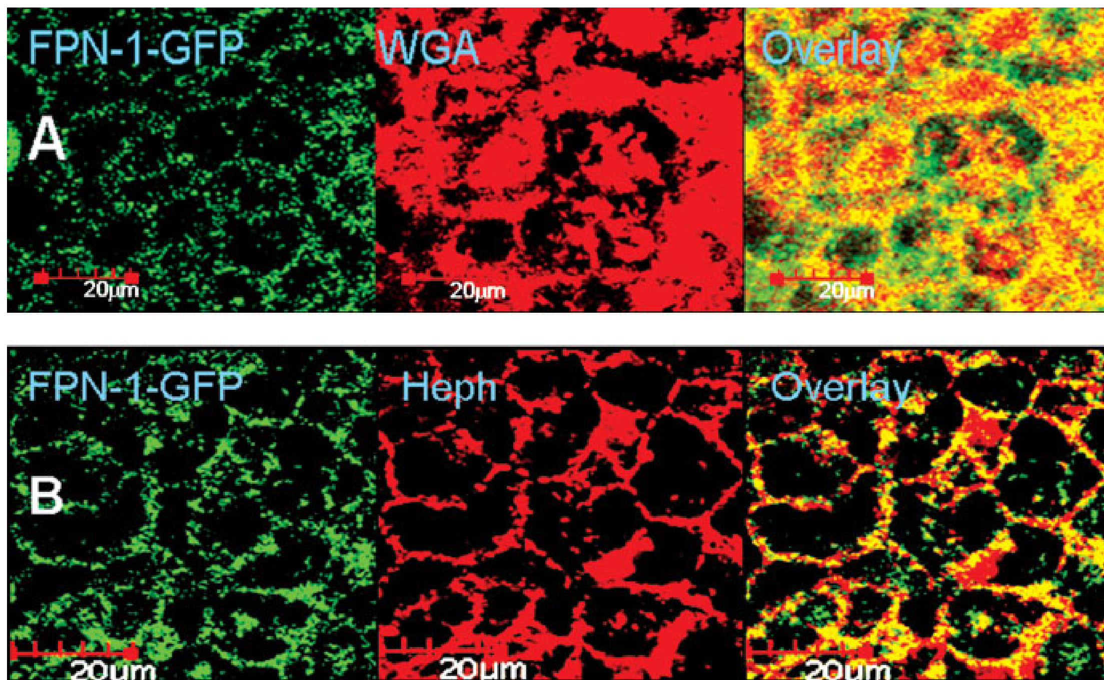

Fig. 4. Colocation of Heph with FPN-1-GFP on the basolateral membrane of intestinal absorptive cells. **A**: Location of FPN-1-GFP on the basolateral membrane of intestinal absorptive cells. The fully differentiated Caco-2 cells expressing FPN-1-GFP were fixed and incubated with Alexa 594 conjugated WGA in the bottom chamber. Yellow in overlay indicates location of FPN-1-GFP on the basolateral membrane. **B**: Colocalization of Heph with FPN-1-GFP on the basolateral membrane of intestinal absorptive cells. The fully differentiated Caco-2 cells expressing FPN-1-GFP grown on microporous membrane inserts were fixed, permeabilized, and then incubated with the primary antibody against Heph. Yellow in overlay indicates colocalization of FPN-1-GFP with Heph.

DISCUSSION

Our findings indicate that both FPN-1 and Heph proteins are primarily located on the basolateral membrane of intestinal cells, which support their proposed roles in iron exit across the basolateral membrane of enterocyte. Furthermore, confocal microscope data demonstrate that Heph is colocalized with FPN-1-GFP on the basolateral membrane in human intestinal absorptive cells. These observations support the possibility that FPN-1 and Heph may interact in the processes of iron exit across the basolateral membrane of intestinal absorptive enterocytes as shown previously in astrocytes [Jeong and David, 2003] and yeast cells [De Silva et al., 1995; Stearman et al., 1996; Singh et al., 2006].

FPN-1 protein is an important iron transporter responsible for export of iron from the intestinal enterocyte as well as from iron storage cells, such macrophages and hepatocytes. The recent elegant study by Donovan et al. [2005] showed that FPN-1 deficiency causes iron deficient anemia in FPN-$1^{-/+}$ and in conditional FPN-1 knockout mice. In their intestinal specific FPN-1 knockout mice, lack of FPN-1 protein in the intestine resulted in anemia due to iron deficiency. The immunochemistry data showed that FPN-1 was primarily located on the basolateral membrane side of intestine in wild type mice [Donovan et al., 2005; Canonne-Hergaux et al., 2006]. It is expected that an active form of FPN-1 protein is located on the plasma membrane of non-intestinal cells. Numerous clinical studies reported that mutations of FPN-1 cause the defective iron homeostasis in patients [Montosi et al., 2001; Njajou et al., 2001]. Defective cellular location of FPN-1 in patients with FPN-1 mutants might cause defective intestinal iron absorption as well as iron release from cells. Mutations of FPN-1 can cause accumulation of FPN-1 protein in the endoplasmic reticulum, and no expression of FPN-1 protein on the plasma membrane of cells [Liu et al., 2005; De

Domenico et al., 2006; Goncalves et al., 2006]. As shown in the current study, FPN-1 protein is primarily located on the basolateral membrane, which suggests that FPN-1 might normally function in fully differentiated intestinal Caco-2 cells.

Once ferrous ion is exported across the cell membrane, oxidation of Fe^{2+} to Fe^{3+} by Cp is required for the release of iron from the tissues into the circulation [Osaki et al., 1971]. However, aceruloplasminemia in mice leaded to iron accumulation in many tissues but not in the intestine [Harris et al., 1999] suggesting that intestinal epithelium utilizes another ferroxidase to convert Fe^{2+} to Fe^{3+}. In *sla* mice, the mutation of Heph results in iron deficiency anemia due to defective intestinal iron absorption. The truncated mutation of Heph results in iron deficiency anemia possibly due to mislocalization of Heph in *sla* mice because Heph protein was not detected on the basolateral membrane of small intestine in *sla* mice [Chen et al., 2004; Kuo et al., 2004]. Confocal microscope data from the current experiments show that Heph protein is primarily located on the basolateral membrane of fully differentiated human intestinal cells. Similarly, Nittis and Gitlin [2004] previously reported that Heph protein is localized on the cell membrane of intestinal T84 cells. Together, these observations support the proposed role of Heph protein in intestinal iron absorption.

The current studies demonstrate that Heph protein is colocalized with GFP-FPN-1 protein on the basolateral membrane of intestinal absorptive cells suggesting the possible interactions of FPN-1 with Heph in the processes of iron export across the basolateral membrane of enterocytes. Molecular genetic studies in yeast cells have showed that iron transport across cell surface requires both iron transporter (Ftr1) and an associated multi-copper oxidase (Fet3) [De Silva et al., 1995; Stearman et al., 1996; Singh et al., 2006]. Ftr1 is needed for Fet3 biosynthesis and localization to the plasma membrane [Stearman et al., 1996]. The requirement that both Ftr1 and Fet3 proteins must be simultaneously synthesized for proper processing suggests that these proteins might function as a complex on the cell surface. Iron export in mammalian cells requires the iron transporter FPN-1, and recent studies have revealed a direct association between FPN-1 and the glycosylphosphatidylinositol (GPI)-anchored multi-copper oxidase Cp during iron movement from mouse astrocytes [Jeong and David, 2003] suggesting a possibility that FPN-1 and Heph are required for the exit of iron across the intestinal basolateral membrane as associated.

Our immunostaining results also demonstrate that FPN-1 and Heph are colocated with TfR on the basolateral membrane in polarized enterocytes. Since FPN-1 and Heph are involved in iron export process and TfR is involved in iron import into the cell, the colocalization of FPN-1 and Heph with TfR on the basolateral membrane of the enterocyte is unexpected finding. We speculate that the colocalization of FPN-1 and Heph with TfR might allow the newly exported ferric ion to efficiently associate with the recycling apo-transferrin released from TfR. FPN-1 is also colocalized with TfR on the plasma membrane in HepG2 cells (Han et al., unpublished data).

Together, these data demonstrate that human intestinal absorptive cells express FPN-1 and Heph protein on the basolateral membrane and they are colocated with TfR in human intestinal Ceco-2 cells. Furthermore, Heph is colocalized with GFP-tagged FPN-1 protein on the basolateral membrane of human intestinal absorptive cells expressing FPN-1-GFP. The data in this study reveal the possibility that FPN-1 protein associates with Heph protein and its interaction may be a crucial factor in the process of iron transport. Future experiments will focus on characterizing functions of FPN-1 and Heph proteins in the processes of intestinal iron absorption as well as interactions of these two proteins during iron transport across the intestinal absorptive cells.

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