Human hephaestin expression is not limited to enterocytes of the gastrointestinal tract but is also found in the antrum, the enteric nervous system, and pancreatic β-cells

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Hudson DM, Curtis SB, Smith VC, Griffiths TA, Wong AY, Scudamore CH, Buchan AM, MacGillivray RT. Human hephaestin expression is not limited to enterocytes of the gastrointestinal tract but is also found in the antrum, the enteric nervous system, and pancreatic β-cells. Am J Physiol Gastrointest Liver Physiol 298: G425–G432, 2010. First published December 17, 2009; doi:10.1152/ajpgi.00453.2009.—Hephaestin (Hp) is a membrane protein with ferroxidase activity that converts Fe(II) to Fe(III) during the absorption of nutritional iron in the gut. Using anti-peptide antibodies to predicted immunogenic regions of rodent Hp, previous immunocytochemical studies in rat, mouse, and human gut tissues localized Hp to the basolateral membranes of the duodenal enterocytes where the Hp was predicted to aid in the transfer of Fe(III) to transferrin in the blood. We used a recombinant form of human Hp to obtain a high-titer polyclonal antibody to Hp. This antibody was used to identify the intracellular location of Hp in human gut tissue. Our immunocytochemical studies confirmed the previous localization of Hp in human enterocytes. However, we also localized Hp to the entire length of the gastrointestinal tract, the antral portion of the stomach, and to the enteric nervous system (both the myenteric and submucous plexi). Hp was also localized to human pancreatic β-cells. In addition to its expression in the same cells as Hp, ferroportin was also localized to the ductal cells of the exocrine pancreas. The localization of the ferroxidase Hp to the neuronal plexi and the pancreatic β-cells suggests a role for the enzymatic function of Hp in the protection of these specialized cell types from oxidative damage.

Absorbed dietary iron must be transported across the enterocyte, perhaps by transcytosis (31) or by a currently unidentified iron chaperone, and exported out of the cell and into blood. The integral membrane protein ferroportin 1 (Fpn1) is located on the basolateral enterocyte membrane and exports ferrous iron from the intestine (15, 34). Once in the blood, the iron is bound by the plasma iron transport protein, apotransferrin. Transferrin does not bind Fe(II); hence, a ferroxidase activity on the basolateral membrane surface appears to be essential for the transfer of iron from the gut mucosa to blood (for a review, see Ref. 38).

A candidate protein for the intestinal ferroxidase activity arose from two discoveries. First, a mouse mutant was described in 1962 that exhibited an X-linked, recessive hypochromic, microcytic anemia. The gene responsible for this disorder was called sex-linked anemia or sla (12). Subsequent studies showed that the anemia could be corrected by intra-peritoneal delivery of Fe(II) but not by ingested iron (5) and that the sla mutation involved a defect in the transport of iron from the enterocyte to the blood plasma (11, 33). The second discovery arose from genome sequencing projects in which a ceruloplasmin (Cp) homolog was identified on the mouse (47) and human (45) X chromosomes. The predicted polypeptide shared 50% sequence identity with Cp and was called hephaestin (Hp). Evidence supporting a role for Hp as the cause of the murine hypochromic, microcytic anemia came from the identification of a 582 nucleotide deletion in the Hp mRNA in the sla mouse (47).

Like Cp, Hp is predicted to have six domains forming a pseudo-threefold axis (45). However, unlike Cp (a soluble plasma protein), Hp has an additional 86 amino acids at the COOH-terminus that include a predicted transmembrane domain and a short cytosolic tail. With the exception of the axial type 1 ligand in domain 2, all residues involved in the types 1, 2, and 3 copper binding sites as well as cysteinyl residues involved in disulfide bond formation in Cp are conserved in Hp (45).

The predicted amino acid sequences of human, mouse, and rat Hp were used to identify common peptides in the cytosolic tail of Hp. Various forms of these peptides were synthesized and used to produce antibodies (24, 49). These anti-peptide antibodies were then used to identify the location of Hp in gut tissue from rat and mouse (16, 30) and human cancer cell lines (24, 30) and to confirm that mouse enterocyte membranes contain a protein with ferroxidase activity that comigrates with Hp during polyacrylamide gel electrophoresis (8). Initial im-

Dietary iron absorption occurs in the proximal small intestine by specialized epithelial cells called duodenal enterocytes. The mechanisms for inorganic iron uptake allow for both Fe(II) and Fe(III) to be transported into the enterocyte. McKie et al. (35) have demonstrated that an intestinal ferrireductase (DCytB) reduces Fe(III) to Fe(II), thereby allowing the Fe(II) to be transported into the cell by the apical transmembrane protein divalent metal transporter-1 (DMT-1) (14, 19, 20). If more iron is absorbed into the enterocyte than required, the excess iron is stored in the iron storage protein ferritin until needed or until the enterocyte dies, at which time the stored iron will be lost in the feces.

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Fig. 2. Hp immunoreactivity (IR) in human duodenal cells and tissues remains to be resolved. Furthermore, although the antibodies used in these studies recognize an epitope situated on the opposite side of the membrane from the cytosolic tail. The localization of the human Hp ectodomain within distinct from the functional portion of the protein, which is generally agreed that Hp is oriented extracellularly, several studies have localized Hp within the intracellular environment (30, 44). This introduces the possibility for unknown and novel functions for Hp in humans.

To study the function of Hp, we expressed recombinant human Hp by cloning Hp cDNA from human duodenal tissue and replaced the putative membrane-binding region at the COOH-terminus with a 1D4 affinity purification tag (18). The resulting recombinant soluble human Hp (rHp) was characterized by various physical-chemical techniques and enzymology and was found to be a multicopper ferroxidase of \( M_r \sim 129,600 \) (18).

In the present study, we have generated a rabbit polyclonal antibody against purified rHp. We show localization of the complete functional domain of human Hp by immunocytochemistry in human tissue samples of the stomach, gastrointestinal (GI) tract, and pancreas.

MATERIALS AND METHODS

Polyclonal antibody production. Ethics approval for antibody production in rabbits was obtained from the University of British Columbia (UBC) Research Ethics Board and Animal Care Committee. Two female adult New Zealand White rabbits were used for antibody generation. Prior to immunization, blood was collected from each rabbit for later use as a preimmune serum. rHp was expressed and purified as described previously (18). The priming immunization consisted of an emulsion of equal parts complete Freund’s adjuvant and purified human Hp [0.5 mg/ml in phosphate-buffered saline (PBS)]. Rabbits were injected subcutaneously with a Freund’s adjuvant/rHp emulsion with subsequent immunizations every 2 wk. At the end of the study, rabbits were exsanguinated and serum was prepared. Antibody titers were tested and the sera were stored in aliquots at 4°C, −20°C, and −70°C.

Immunocytochemistry. Ethics approval for specimen collection was obtained from the Clinical Research Ethics Committee administered by both UBC and the Vancouver Coastal Health Authority. Human tissue samples were fixed in Bouin’s solution for 2 h, dehydrated, and embedded in wax. Full thickness tissue sections (5 \( \mu m \)) were cut and mounted on glass slides. Sections were dewaxed in xylene, cleared in petroleum ether, incubated with 1% sodium dodecylsulfate (SDS) in PBS (pH 7.4) for 5 min (as an antigen retrieval technique), then washed three times for 5 min each in PBS. The relevant sections were incubated in a solution of glycine and \( \text{NH}_4 \text{Cl} \) (50 mM each) for 15 min to block endogenous fluorescein or in 3% \( \text{H}_2\text{O}_2 \) for 5 min to block endogenous peroxidase. Nonspecific binding was blocked by incubation with 10% normal horse serum in PBS for a minimum of 1 h. Tissue sections were incubated overnight at 4°C with the following antibodies and dilutions: Hp (rabbit; 1:1,000), insulin (mouse monoclonal, Meridian Life Science, Saco, ME; 1:2,000), glucagon (mouse monoclonal; Sigma, MO; 1:6,000), somatostatin (mouse monoclonal;
Antibodies were visualized by enhanced chemiluminescence (GE peroxidase-conjugated anti-rabbit IgG (Sigma, 1:50,000) were incubated with Alexa Fluor 594-conjugated anti-mouse IgG (Molecular Probes, Invitrogen, Burlington, ON, Canada; 1:2,000). Sections were incubated with the second and third layers for 1 h at room temperature. Some sections incubated with Hp and Fpn1 antibodies were localized by the avidin-biotin complex (ABC) method (Vector Laboratories). Negative controls consisted of preimmune serum (same dilution as the primary antibody) and omission of the primary antibody. Stained sections were examined with a Leica microscope equipped with epifluorescence. Images were acquired by use of a Retiga EXi camera (Qimaging, Surrey, BC, Canada) and Improvision Openlab software (Version 4.0.2, Improvision, Waltham, MA).

PCR. A sample of human islet cDNA was generously provided by Dr. Jim Johnson of the Diabetes Research Group, Life Sciences Institute, UBC. Primer pair sequences are as follows: Hp forward primer 5'-GCTGAGATGTTGCGCTGGGAACC-3', reverse primer 5'-CCCAG-GATTCCAAGAGTGCCTACTCT-3'; Fpn1 forward primer 5'-ATGAC-CAGGCGGAGGAT-3', reverse primer 5'-CCCATCTCATCTCGGAA-GGTA-3'; insulin forward primer 5'-AGCCCTTTGTGAACCA-ACACC-3', reverse primer 5'-GTCGGTAGAGGGAGCAGATG-3'. Hp and Fpn1 primer pairs span an intron to differentiate whether a PCR product is amplified from genomic DNA or cDNA. Product sizes of 374 base pairs (bp) for Hp and 898 bp for Fpn1 reflect priming from an intron. The product is amplified from genomic DNA or cDNA. Product sizes of 245 bp. The expected single band of 150 kDa was found for rHp, whereas Cp showed a dominant band with some minor proteolysis as is often observed with plasma.

RESULTS

Polyclonal antibody raised against a soluble, recombinant form of human Hp. To date, our laboratory is the only group to have expressed a recombinant, soluble form of human Hp (18). By using pure rHp as the antigen, a polyclonal antibody (α-rHp) was generated in rabbits. This is the first polyclonal antibody that specifically recognizes the complete functional ectodomain of Hp. Antibody specificity and titer were verified by Western blot analysis (Fig. 1). Because the rHp was used as an antigen contained the 1D4 affinity tag, there was a possibility that the polyclonal antibodies might be dominated by antibodies recognizing this highly antigenic 1D4 fusion tag. To address this issue, the antibody was tested for reactivity against the rHp ectodomain before and after removal of the 1D4 tag by factor Xa. Western blot analysis showed that the α-rHp recognized the rHp before and after cleavage of the 1D4 tag (Fig. 1A, lanes 1 and 2). In contrast, an antibody to the 1D4 tag recognized the rHp prior to factor Xa cleavage but not after the 1D4 tag had been removed (Fig. 1A, lanes 3 and 4). We concluded that the α-rHp antibody predominantly recognized the rHp and not the 1D4 affinity tag.

Because of the ~50% sequence identity between Hp and Cp, we also wanted to exclude the possibility that the α-rHp antibody cross-reacted with Cp. Figure 1B shows 2-μg aliquots of purified rHp and Cp subjected to SDS-PAGE and stained with Coomassie blue. The expected single band of ~M, 150 kDa was found for rHp, whereas Cp showed a dominant band with some minor proteolysis as is often observed with plasma.
When diluted aliquots of rHp and Cp were subjected to SDS-PAGE and Western blot analysis, the α-rHp antibody did not detect Cp in the range of 100–500 ng (Fig. 1B, lanes 2 and 4); however, the α-rHp antibodies were able to detect rHp at concentrations of 100 and 500 ng (lanes 1 and 3). We concluded that cross-reaction between the α-rHp antibody and human Cp is negligible.

The α-rHp antibody was also tested for specificity using whole cell extracts from human duodenal mucosa. A protein the same size as Hp (approximate molecular mass of 150 kDa) and some smaller proteins assumed to be degradation products were detected by the α-rHp antibody (Fig. 1C). As shown in Fig. 1C, the α-rHp antibodies were of high affinity and could be diluted to 1:25,000 while detecting 100 ng of rHp by Western blot analysis.

Hp and Fpn1 are expressed throughout the GI tract. As shown in Fig. 2, Hp was present in human primary enterocytes in the duodenum and was localized at or very close to the basolateral membrane (Fig. 2A). Controls using α-rHp antibody preabsorbed with excess rHp (1 μM) or preimmune serum confirmed the specificity of the α-rHp antibody and the absence of preexisting specific antibodies in the rabbit serum (Fig. 2B). Hp immunoreactivity (Hp-IR) was also found in the mature enterocytes of the jejunum, ileum (Fig. 3, A and B), and colon (not shown). Very little if any Hp staining was observed in the crypt regions of the mucosa. We also investigated Hp expression in the antral portion of the stomach, a tissue not previously found to express Hp. Hp was present on the basolateral membranes of antral mucosal cells (Fig. 3C).

Apart from its expression in mature enterocytes (Fig. 4A), Fpn-IR was observed in Brunner’s glands (submucosal glands that secrete alkaline fluid into the duodenum) (Fig. 4B) and crypts of the jejunum and ileum (Fig. 4C). In some cases, the intracellular location of Fpn1 differed from that of Hp with Fpn-IR being found in a subapical location in some duodenal enterocytes and mucosal cells of the antrum (Fig. 4D).

Hp and Fpn1 are expressed in enteric nerves. Interestingly, Hp-IR and Fpn-IR were also found in some of the cells bodies of the submucous plexus and myenteric plexus (SMP and MYP, respectively); see Fig. 5. These plexi represent two nerve networks that run the entire length of the gut, one just under the mucosa (SMP) and the other between the outer longitudinal and inner circular muscle layers (MYP). Hp-IR in the SMP and MYP was observed not only in the duodenum, but also in the jejunum and ileum (Fig. 5). Unlike the villi, where the Hp-IR is restricted to the basolateral membrane region of the cell, Hp-IR and Fpn-IR in the nerve cell bodies are found in an intracellular location (Fig. 5).

Colocalization of Hp and insulin in pancreatic β-cells. An unexpected finding was that Hp is expressed by pancreatic islets (Fig. 6). Colocalization of insulin and Hp showed that Hp is confined to the β-cells (Fig. 6, A and B). An individual islet is shown at a higher magnification in Fig. 6, C and D further confirming the colocalization of Hp and insulin. Double labeling of pancreas sections indicated that somatostatin-containing δ-cells and glucagon-containing α-cells did not express Hp (not shown). The staining is intracellular and the punctate pattern suggests a location in the secretory granules of β-cells. Fpn-IR was also observed in the pancreas (Fig. 7). Very faint staining was observed in the pancreatic islets (Fig. 7A), but much stronger staining was observed in what appear to be macrophage/dendritic cells within the endocrine and exocrine pancreas (Fig. 7B). In addition, Fpn1 is expressed by pancreatic duct cells in the plasma membrane. These immunocytochemistry results were verified by RT-PCR, demonstrating mRNA expression for both Hp and Fpn1 in human islets (Fig. 8).

DISCUSSION

Previous investigation into the localization of Hp has always involved the use of antibodies generated against a
short peptide sequence in the cytoplasmic region of Hp (16, 24, 30, 49). In the present study, we generated a polyclonal α-rHp antibody using purified rHp as the antigen. The expression of Hp in primary mature enterocytes in the human gut and its location in the basolateral membranes of the enterocytes is in agreement with a previous study involving an immortalized human colorectal adenocarcinoma cell line (24). Hp has previously been localized to the duodenum and colon in mouse tissue (47), to normal and cancerous human colon (6), and to rat duodenum, jejunum, ileum, and colon (49); however, identification of Hp in human jejunum and ileum has not been reported. In the rat, Hp-IR has been reported along the entire small and large intestine but only in a supranuclear location (16). In mouse primary enterocytes, Hp-IR was first found in a supranuclear location but more sensitive immunocytochemical techniques revealed Hp-IR in the lateral portion of the membrane (30). Kuo et al. (30) also reported Hp in a supranuclear location in two nonhuman kidney cell lines (MDCK and Cos7) and a human colonic adenocarcinoma cell line (HT29). We did not observe Hp in a supranuclear location. These discrepancies could be related to species differences and/or differences between normal and transformed cells or to differences in the antibodies used.

Fig. 5. Hp-IR and Fpn-IR in cell bodies of the submucous plexus and myenteric plexus. Arrows indicate Hp and Fpn1 positively stained nerve cell bodies. A: Hp in the myenteric plexus of the ileum. B: Hp in the submucous plexus in the ileum. C: Fpn1 containing nerve cell bodies in the myenteric plexus of the duodenum. D: Fpn1-positive nerve cell bodies in the submucous plexus in the ileum. For all images, magnification is ×400.

Fig. 6. Hp immunoreactivity in human pancreatic islets. Hp is colocalized with insulin in the same tissue sections. Colocalization of Hp (A), and insulin (B). For both images, magnification is ×200. Higher magnification (×400) images of an individual islet are shown in C (Hp) and D (insulin).
Contrary to studies in the rat where Hp was not expressed in the stomach (16), strong staining was found in the upper glands of the antral portion of the stomach. The staining pattern is the same as that found in the basolateral membrane of duodenal enterocytes. This finding was unexpected since very few substances are absorbed by the mucosa of the stomach. It is not known whether the corpus epithelial cells also express Hp. It has recently been suggested that iron absorption may occur not predominantly in the duodenal enterocytes as previously thought, but along the entire small and large intestine (6). However, the stomach has never been considered a tissue involved in iron absorption, suggesting an alternative role for Hp at these sites; as a result, the stomach has received less scrutiny when localizing proteins involved in iron metabolism. However, the non-iron-responsive element isoform of the DMT1 gene is expressed in the antral portion of the rat stomach (16), and both the iron-responsive and non-iron-responsive genes are expressed in the mouse stomach (26). Since these studies only examined mRNA expression, the cellular distribution of DMT1 (apical membrane vs. intracellular) could not be determined. The stomach, therefore, may be another site of iron absorption, or perhaps the epithelial cells of the antral mucosa participate in a bacteriostatic function by reducing the amount of iron in the stomach. Indeed, a major role of acid in the stomach is to act as a bacteriostat. Iron in the acid environment of the stomach lumen would be in the form of Fe(III), necessitating a reductase in order for DMT1 to transport iron into the cell. Fpn in the antrum was found in an intracellular location. This raises the possibility that it may be involved in movement of intracellular iron into or out of various intracellular compartments. Conversely, it could represent internalized and ubiquitinated Fpn1 that is targeted for destruction. Electron microscopy could pinpoint the exact intracellular location of Fpn1.

The finding of Hp expression in enteric nerves has not been reported previously, although the Hp homolog Cp is expressed in astrocytes and neurons of the central nervous system (CNS) (27, 37). In addition, Hp and Fpn1 have now been identified together in different cell types including neurons in numerous parts of the brain (48). The role of glycoprophosphatidylinositol-anchored Cp in the CNS is not well understood but it is believed to function in both neuronal iron efflux or influx (41). It is tempting to propose a similar function for Hp; however, neither Hp nor Fpn1 is localized to the plasma membrane in enteric neurons. This is in agreement with other studies involving rat brain neurons of the substantia nigra, which have a similar intracellular staining pattern (48). Because the single gene for Hp contains a signal sequence that directs the growing polypeptide to pass into the endoplasmic reticulum (45), the Hp staining pattern observed in the present study probably reflects membrane-bound Hp rather than soluble Hp in the cytoplasm (to date, there is no evidence for a soluble form of Hp).

An increased need for iron regulation in neurons due to the high energy requirement of the CNS could explain the requirement for multiple membrane-bound and intracellular ferroxidases. Thus it is not surprising that a ferroxidase is expressed by neurons in the peripheral nervous system. In addition, neurons contain large cell bodies that are susceptible to free radical damage as a result of their elevated metabolic rates and poor antioxidant defense (42). Hp was first reported to have an alternate function in addition to iron absorption when it was localized to the Muller glia and the retinal pigment epithelium and shown to play a critical role in CNS iron homeostasis (23). Thus mice deficient in both Hp and Cp are prone to age-dependent retinal neurodegeneration (23). It is not clear why Cp and Hp would both be required to fulfill the same apparent enzymatic function. One hypothesis to account for the functional redundancy is that it may be due to regulatory differences in expression between the ferroxidases. It has been shown that both Cp (36) and Fpn1 (22) are upregulated in response to serum iron deficiency through a negative-response feedback mechanism, thereby restoring systemic iron levels to normal. In contrast, Hp expression is not regulated in response...
to variations in iron status in duodenum (2, 3) or brain (40) but instead is maintained at a steady expression level. The constitutive expression of Hp in these locations may serve as protection against the potential damaging effects caused by iron overload.

It is becoming apparent that there is a correlation between glucose and iron metabolism (13). Hp and Fpn1 expression in pancreatic islets is perhaps not so surprising in light of the recent discovery that human and rat β-cells also express hepcidin (29). Hepcidin is located in the insulin secretory granules and therefore would be cosecreted with insulin in response to glucose (29), indicating a role for the β-cell in both iron and glucose metabolism. Iron-overload disorders such as hemochromatosis and aceruloplasminemia are frequently accompanied by insulin deficiency, thought to be secondary to the iron overload (9). In many cases, individuals with these disorders develop diabetes mellitus as the β-cells of the islets accumulate excessive iron. This may be explained by the fact that β-cells express high levels of DMT1 (4, 28) and very large amounts of (apo)feritin (even greater than the liver, which is considered to be a major iron storage tissue (32)). Messenger RNA levels for the heavy (H) ferritin in the β-cell are responsive to increasing glucose concentrations (32). As β-cell zinc levels are 2,000 times that of iron, the high levels of ferritin may serve as a buffer, protecting the β-cell from zinc toxicity. However, in pathological conditions of iron overload, the iron binding capacity of the β-cell ferritin is probably insufficient to avoid the excess iron being toxic to the cell (32). Iron can induce free radical generation, and β-cells are particularly sensitive to oxidative damage since they have very low levels of free radical scavengers (17). The heavy (H) chain of ferritin specifically enhances the ferroxidase activity of Cp (21); this is the same ferritin chain that is preferentially expressed by β-cells. In the β-cell, Hp may catalyze the oxidation and incorporation of iron into ferritin, thereby minimizing the concentrations of toxic ferrous ions. The iron requirement of the β-cell may be higher than other cells owing to its high rate of glucose metabolism, which, unlike in other cells, increases in direct proportion to increasing plasma glucose levels. In fact, it is the metabolism of glucose and the consequent increase in ATP levels that provides the signal for glucose-induced insulin secretion (25).

In mammals both glucose and iron circulating levels are maintained within a very narrow range (~4–8 mM for glucose and ~10–30 μM for iron). The pancreas receives the first pass of blood directly from the gut, so iron- and glucose-sensing β-cells are in a prime location to function as sensors of nutrient absorption. This may provide a mechanism for very precise regulation of iron levels in the body, since excess iron cannot be excreted. The question still remains as to whether Hp is functioning as a ferroxidase in these locations. Interestingly, Fpn1 is expressed in the membrane of pancreatic ductal epithelial cells. These cells are responsible for secreting water and bicarbonate ions that buffer the acidity of gastric secretions in the duodenum and provide an optimal pH for digestive enzymes to function (43). Secretion occurs via an anion exchanger: SLC26, a member of the SLC superfamily of membrane transport proteins that includes Fpn1 (SLC40a1) (10). The physiological function of Fpn1 in the pancreatic duct cells remains to be elucidated.

Most of the previous work on the localization of Hp has been performed on animal tissues and immortal cell lines and human cell lines. This study examined the expression of Hp in normal human stomach, GI tract, and pancreas. It is evident that further studies are required to explain possible novel functions of Hp. In the last few years, Hp has been localized to the retinal pigment epithelium of the mouse CNS (23) as well as various regions of rat brain and heart (39, 40, 48). It is becoming clear that the membrane-bound ferroxidase Hp plays a role in iron metabolism well outside the realm of intestinal iron absorption.

REFERENCES


