Studies on the Role of Hephaestin and Transferrin in Iron Transport

by

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Iron homeostasis is essential for maintaining the physiological requirement for iron while preventing iron overload. Multicopper ferroxidases regulate the oxidation of Fe(II) to Fe(III), circumventing the generation of harmful hydroxyl-free radicals. Ceruloplasmin is the major multicopper ferroxidase in blood; however, hephaestin, a membrane-bound ceruloplasmin homolog, has been implicated in the export of iron from duodenal enterocytes into blood. These ferroxidases supply transferrin, the iron-carrier protein in plasma, with Fe(III). Transferrin circulates through blood and delivers iron to cells via the transferrin receptor pathway. Due to the insoluble and reactive nature of free Fe(III), the oxidation of Fe(II) upon exiting the duodenal enterocyte may require an interaction between the ferroxidase and transferrin. In Chapter 3, the putative interaction of transferrin with ceruloplasmin and a soluble form of recombinant hephaestin was investigated. Utilizing native polyacrylamide gel electrophoresis, covalent cross-linking and surface plasmon resonance, a stable interaction between the two proteins was not detected. The lack of interaction between hephaestin and transferrin prompted the investigation into the localization of hephaestin in the human small intestine. Hephaestin has been reported to have both intracellular and extracellular locations in murine tissue. In the Appendix, the location of hephaestin in human tissue was investigated using a novel polyclonal antibody. Hephaestin was localized to the basolateral membrane and an intracellular location of the enterocyte, as well as a novel location in the myenteric plexus of the duodenum. The delivery of iron to cells via the transferrin receptor pathway is well established; however, little is known about the interaction of transferrin with the transferrin receptor at the molecular level. In Chapters 4 and 5, surface plasmon resonance was employed to further
characterize the binding event between transferrin and the transferrin receptor. It was found that mutations affecting iron release in transferrin did not impact receptor binding. However, when N-lobe residues predicted to form contacts with the transferrin receptor were targeted, significant changes in the transferrin receptor binding kinetics and affinity were observed.
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<tr>
<td>Apo-Tf</td>
<td>apotransferrin</td>
</tr>
<tr>
<td>α-rHp</td>
<td>anti-hephaestin antiserum</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney</td>
</tr>
<tr>
<td>BS₃</td>
<td>bis(sulfosuccinimidyl) suberate</td>
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<td>CAPS</td>
<td>N-cyclohexyl-3-aminopropanesulfonic acid</td>
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<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
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<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>Cp</td>
<td>ceruloplasmin</td>
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<td>Cryo-EM</td>
<td>cryoelectron microscope</td>
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<td>diaminobenzidine</td>
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<td>DMT1</td>
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<td>ECL</td>
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<td>Hp</td>
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<td>IFA</td>
<td>incomplete Freund’s adjuvant</td>
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<td>$K_D$</td>
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<tr>
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<td>NHS</td>
<td>N-hydroxyl succinimide</td>
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<td>Polyacrylamide gel electrophoresis</td>
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<td>Paraformaldehyde</td>
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<td>Recombinant hephaestin</td>
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<tr>
<td>rTf</td>
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<tr>
<td>RU</td>
<td>Response unit</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SLA</td>
<td>Sex-linked anemia</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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<td>sTfR</td>
<td>Soluble transferrin receptor</td>
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<tr>
<td>sulfo-KMUS</td>
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<td>Transferrin receptor</td>
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<td>VGH</td>
<td>Vancouver General Hospital</td>
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...this thesis is for Louise.
Chapter 1: Introduction

1.1 Introduction

The average well-nourished human body contains about 4 g of iron of which over two thirds is bound in hemoglobin in red blood cells and their precursor cells (Latunde-Dada et al., 2006a). Before reticulocytes become mature erythrocytes, they must take up iron for hemoglobin production. At least 20 mg of iron is required daily to support the synthesis of the iron-containing heme prosthetic groups of hemoglobin (Koury and Ponka, 2004). This need for elemental iron requires a highly efficient and regulated system for iron absorption and transport.

1.2 Chemistry and biochemistry of iron

Fe(II) and Fe(III) are the physiologically relevant oxidation states of iron. Single electron transfer reactions between these two states make iron beneficial in biological systems. Electron transfer facilitated by iron is reversible within the range of biological oxidants and reductants (Aisen, 2001). Based on the surrounding environment, the reduction potential of the Fe(II)/Fe(III) redox couple can vary significantly (Harris, 2002). Moreover, the reduction potential is subject to alteration via ligand binding to iron, allowing the redox couple to meet the requirements of a wide range of cellular reactions. This variability in redox potential exemplifies the biological importance of iron. The Fe(II)/Fe(III) redox couple is very reactive and participates in a variety of biological processes involving single electron transfer. However, this reactivity comes at a cost as single electron transfer reactions may lead to the formation of damaging oxygen and hydroxyl radicals via the Haber-Weiss-Fenton series of reactions:
Fe$^{2+}$ + O$_2$ $\rightarrow$ Fe$^{3+}$ + O$_2^-$

2O$_2^-$ + 2H$^+$ $\rightarrow$ H$_2$O$_2$ + O$_2$

Fe$^{2+}$ + H$_2$O$_2$ $\rightarrow$ -OH + OH$^-$ + Fe$^{3+}$

The presence of oxygen and hydroxyl free radicals in biological systems can result in oxidative damage to nucleic acids, lipids and proteins. Organisms have evolved biochemical systems for maintaining a balance between the beneficial and potentially cytotoxic properties of iron (Aisen et al., 2001).

1.3 Iron proteins in blood

1.3.1 Transferrin

Although most of the iron in the human body is bound within circulating red blood cells, it is transferrin (Tf) that is the primary iron transport protein in blood. Tf is a single polypeptide composed of two homologous globular lobes, the N-lobe and the C-lobe (Figure 1.1 A). Each lobe is divided into two subdomains (N1 and N2) and (C1 and C2). Joining each subdomain is a metal-binding cleft that is capable of binding a single iron atom. Thus each Tf can bind up to two iron atoms. Iron-loaded Tf is called diferric- or holo-Tf if both lobes contain iron and monoferric if only one lobe has bound iron. When Tf is in an iron depleted state, it is called apo-Tf.
**Figure 1.1.** Structure of human Tf and the Tf receptor. **A.** Ribbon diagram of Tf showing the N- and C-lobe subdomains of the protein as well as the hinge region where iron binding occurs (adapted from Walley et al., 2006). **B.** Ribbon diagram of the Tf receptor showing the separate domains of the homodimer as well as the hypothetical plasma membrane anchor (adapted from Lawrence et al., 1999).
Tf binds Fe(III), but not Fe(II). This binding is both very specific and tight with a $K_D$ of $\sim 10^{-24} \text{M}$ at physiological pH (Richardson and Ponka, 1997). The sequestering of iron is fundamental to the prevention of cell toxicity due to the insoluble nature of free Fe(III) under physiological conditions and the potential for free iron to catalyze superoxide radicals via the Fenton reactions (Aisen et al., 2001). Furthermore, once bound to Tf, Fe(III) is unavailable for the growth of pathogens (Bullen et al., 2000). Human serum Tf circulates through blood at very high concentrations (35-50 $\mu$M). However, $\sim 70\%$ of this is in an iron depleted state and available to act as a buffer against excess free iron (Johnson and Enns, 2004). Tf delivers iron to cells via interaction with the Tf receptor (TfR). The importance of Tf and its receptor in iron homeostasis is exemplified using knockout mice (Rothenberg and Voland, 1996; Levy et al., 1999), as gene deletions in Tf and TfR cause death shortly after birth and in utero, respectively. Interestingly, in patients with hypotransferrinemia, a disease characterized by low transferrin levels, iron absorption is not impeded; in fact, iron delivery to the liver is actually increased (Hayashi et al., 1993; Craven et al., 1987).

1.3.2 Other transferrins

The role of Tf homologs in iron delivery is poorly understood. Lactoferrin (Lf) and melanotransferrin (MTf) share 37-39% sequence identity with serum Tf. Unlike soluble Tf, the majority of MTf is tethered to the cell membrane by a glycosyl phosphatidylinositol (GPI) anchor (Food et al., 1994). However, a small amount of soluble MTf exists in serum, saliva, cerebrospinal fluid and urine (Richardson., 2000). Another difference between Tf and MTf occurs with iron binding, as MTf contains only one iron-binding site (Baker et al., 1992). The most significant difference between these Tf homologs and Tf is possibly that
neither membrane bound MTf (Richardson, 2000) nor soluble MTf (Food et al., 2002) bind TfR and deliver iron to cells. Knockout mice, with a gene deletion for MTf, show no morphological, histological, behavioral, hematological or iron status variation compared to wild-type mice (Dunn et al., 2006). Although several hypotheses have been proposed for MTf function (Dunn et al., 2006), it is perhaps fair to suggest that MTf does not play a significant role in iron transport and delivery (Sekyere and Richardson, 2000).

Lf, a soluble homologue of Tf, possesses both iron-binding sites and is able to bind Fe(III) with a higher affinity than Tf (MacGillivray and Mason, 2002). Human Lf is expressed in glandular epithelial cells and found in breast milk and other mucosal secretions (Ward et al., 2005). There exists some debate regarding the role of Lf in iron metabolism. Like the MTf knockout, deletion of the Lf gene results in no change in phenotype (Ward et al., 2003). Although Lf may have a function in neonate iron absorption, it is generally believed to have a larger role in immunity where it acts as an antimicrobial agent (Ward et al., 2005).

1.3.3 Transferrin receptor

The Tf receptor (TfR) is a ubiquitous protein, anchored to cell membranes by a short cytoplasmic and transmembrane N-terminal region. The TfR forms a homodimer in the phospholipid membrane, joined by two intermolecular disulfide linkages (Figure 1.1 B). The majority of the receptor forms a large globular ectodomain projecting into the extracellular environment (Fuchs et al., 1998). The X-ray crystal structure of the homodimeric TfR ectodomain reveals that each monomer is composed of three distinct domains: a protease domain, an apical domain and a helical domain (Lawrence et al., 1999). The protease
domain (residues 121-188 and 384-606) resembles amino and carboxypeptidases (~ 28% sequence identity), however it lacks protease activity due to missing key residues in the putative active site. The apical domain (residues 189-383) is structurally the furthest distance from the membrane. The helical domain (residues 607-760) contains a four-helix bundle, which is the site of the dimer interface between the two TfR monomeric polypeptides. No structural data exists for the glycosylated stalk region (residues 90-120), the transmembrane domain (residues 62-89), or the N-terminal cytoplasmic domain (residues 1-61).

A soluble form of the TfR circulates in human serum (Kohgo et al., 1986). Soluble TfR (sTfR) is an 85 kDa monomer that results from a cleavage of the full-length TfR after Arg100 (Shih et al., 1990). The mechanism responsible for the generation of sTfR remains to be resolved, but the serum concentration of sTfR does increase during elevated erythropoiesis (Cook et al., 1994).

1.3.4 Ceruloplasmin

Ceruloplasmin (Cp) belongs to the multicopper oxidase family and plays a vital role in mammalian iron homeostasis as the major multicopper ferroxidase in blood (Hellman and Gitlin 2002). Cp requires six copper atoms for functionality and is the primary copper-containing protein in blood, accounting for over 95% of the copper in human plasma. Cp has several physiological functions besides ferroxidase activity, including glutathione peroxidase, ascorbate oxidase and antioxidant activity, as well as a role in copper transport and as a regulator of cellular iron concentrations (Healy and Tipton, 2007). Cp functions in iron homeostasis by controlling the rate of cellular iron efflux (Harris et al., 1998). In the absence of Cp, cellular iron egress is hindered and iron is sequestered within cells (Harris et al.,
1998). This regulatory function of Cp is associated with its role in iron-loading apo-Tf with Fe(III), a process that requires the catalytic oxidation of Fe(II) that is released from cells (Richardson and Ponka, 1997). Cp is most noted for its biological function within the central nervous system (CNS). Deficiencies in Cp result in iron accumulation within the basal ganglia that can lead to progressive neurodegenerative disease (Harris et al., 1995).

1.3.5 Hephaestin

Hephaestin (Hp) is a membrane bound multicopper oxidase that is highly expressed in the basolateral membrane of the duodenal enterocyte (Vulpe et al., 1999). Mice with sex-linked anemia (Sla) have a 582 base pair deletion in the *heph* gene, which results in a 194 amino acid truncation of the Hp protein (Vulpe et al., 1999; Chen et al., 2003). Mice with this condition commonly present with moderate to severe hypochromic, microcytic anemia due to impaired basolateral iron transport (Bannerman et al., 1966; Vulpe et al., 1999). Hp is only the second ferroxidase discovered in vertebrates and the polypeptide encoded by the Hp gene shares 50% sequence identity with the other vertebrate ferroxidase, Cp (Syed et al., 2002). A third mammalian multicopper ferroxidase, Zyklopen, has recently been identified in multiple tissues, but the details of this study have not yet been published in a peer-reviewed paper (Bioiron abstract, April 2007, McArdle and Vulpe). Evidence for Hp as the sole ferroxidase in intestinal iron absorption is provided by patients with aceruloplasminemia, as dietary iron absorption is not impaired in subjects with this condition (Hellman and Gitlin, 2002).

Hp consists of a single 1158 amino acid polypeptide with a predicted mass of ~130,000 Da. The crystal structure of Hp remains to be solved; however, a hypothetical
model of the structure of human Hp was generated using homology modeling with Cp (Figure 1.2 A; Syed et al., 2002). Hp is predicted to have similar activity as Cp, as the residues involved in copper binding and disulfide bond formation are conserved. It has therefore been predicted that Hp can bind six copper atoms. Hp is thought to have an extra 86 amino acids at the C-terminus that include a transmembrane domain and a short cytosolic tail. The model of the Hp structure also reveals a surface exposed region with high negative charge. This region, thought to be an iron-binding site, is potentially involved in the transfer of iron to apo-Tf (Figure 1.2 B).

Early *in situ* hybridization work localized Hp predominantly to the small intestine; however, Hp was also detected in lung, spleen, kidney, testis, brain, muscle, heart and stomach (Vulpe et al., 1999). An anti-peptide antibody raised against a short sequence in the cytoplasmic region of Hp (Frazer et al., 2001), has been employed in multiple immunohistochemical (IHC) and immunocytochemical (ICC) experiments. Initially, Hp was localized to the intracellular environment of rat enterocyte (Frazer et al., 2001). It has since been shown that Hp is also present in an apical supranuclear position and in the recycling endosome compartment of mice enterocytes (Kuo et al., 2004; Chen et al., 2004). It is possible this intracellular localization is simply the processing of the immature protein prior to membrane anchoring. However, more recently Hp has been localized to membranes in the retinal pigment epithelial of mice (Hahn et al., 2004b) and various regions of the rat brain and heart (Qian et al., 2007a; Qian et al., 2007b; Wang et al., 2007).
Figure 1.2. The hypothetical structure of human Hp. **A.** Ribbon diagram of Hp highlighting the putative iron-binding site and the membrane anchor. **B.** Space filling diagram of Hp showing the surface exposed charges: negative (red) and positive (blue). This is proposed to function as an electrostatic pathway for Fe(III) upon release from Hp (adapted from Syed et al., 2001).
Hp must be present on the enterocyte basolateral membrane if Hp is to facilitate iron export from the gut. This is consistent with the small intestine having the highest expression of Hp (Vulpe et al., 1999). However, the presence of Hp in the recycling endosome and apical supranuclear compartments is more difficult to explain. Still more difficult to explain is the presence of Hp in regions of the heart and CNS. It is likely that Hp found in these unique locations may have a novel function that is presently unknown.

1.4 Dietary iron uptake

1.4.1 Physiology of the gut

The human gastrointestinal tract consists of the mouth, esophagus, stomach, small intestine and large intestine. The intestine serves as the body’s primary digestive organ and is the predominant site of nutrient absorption into the bloodstream. Several layers of surface area expansion optimize nutrient absorption in the small intestine (Bloom and Fawcett, 1975). First and perhaps most obvious, the sizable length of the small intestines provides an extensive surface for absorption. The small intestine is 2-3 cm in diameter and extends about 4 m in length. The intestinal lumen is lined with finger-like folds or projections known as villi, which provide a large surface area for absorption (~200 m²). Brush border cells line the villi and create additional folds in the epithelial cell membrane. The core of these villi projections is lined with connective tissue, known as the lamina propria. The intestine is connected to the vasculature within the lamina propria. The small intestine connects to the systemic blood supply through mesenteric arteries and veins. Nutrients in the blood travel through the hepatic portal vein to the liver, where they can be utilized by the body. The small intestine has three major sections: the duodenum, jejunum and ileum. The division of
these sections is somewhat arbitrary, based mostly on structural and functional features (Bloom and Fawcett, 1975).

### 1.4.2 Small intestine

The duodenum is composed of the first 30 cm of the small intestine. It contains a thick wall, with deeply folded mucous membrane and duodenal digestive glands. The duodenum contains a common duct linking it to the pancreas and gall bladder, which connect it to pancreatic and bile secretions that help in the digestive process (Bloom and Fawcett, 1975).

The jejunum is almost a meter in length. Like the duodenum, the jejunum has a deeply folded mucous membrane, but it also has a thicker wall and is more vascularized than the duodenum. The villi in the jejunum are much longer than those in the duodenum or ileum. While nutrient absorption occurs in all three segments of the small intestine, the primary location of absorption is the jejunum (Bloom and Fawcett, 1975).

The ileum is the longest section of the small intestine at over two meters in length. It has smaller and fewer villi than the jejunum and duodenum. The walls of the ileum have more lymphatic follicles than elsewhere in the intestine (Bloom and Fawcett, 1975).

Dietary iron uptake occurs primarily in the duodenum and the proximal region of the jejunum (Mackenzie and Garrick, 2005). However, iron absorption may occur along the entire length of the small and large intestine (Brookes et al., 2004). Specialized intestinal epithelial cells called enterocytes form a barrier between the intestinal lumen and the vasculature (Frazer and Anderson, 2005). These enterocytes of the intestinal mucosa and the
epithelial cells lining of the colon, termed colonocytes, are also responsible for the absorption of nutrients (Brookes et al., 2005).

The life cycle of the enterocytes start off as undifferentiated cells located at the base of the villi folds, known as the crypts. As the undifferentiated cells mature, they migrate apically along the villi membrane until ultimately they slough off, in a process commonly termed cell shedding. Maturation of the cells occurs over a 2-3 day period, upon which time cells fully differentiate into mature enterocytes (Bullen et al., 2006).

1.4.3 Iron as nutrient

Iron is the fourth most prevalent element in the Earth’s crust (Harris, 2002); however, despite this abundance, iron deficiency is the most common and widespread nutritional disorder in the world (World Health Organization Statistics, 2003). In food sources, iron is found in two basic forms: inorganic iron (as Fe(II) or Fe(III)) and heme (as a protoporphyrin IX complex). These iron sources are not equally available as inorganic iron accounts for approximately 90% of total iron content in the average diet worldwide (Anderson and Frazer, 2005; Sharp and Skrai, 2007). In the developed world where meat consumption is relatively high, over half of all absorbed iron comes from heme, whereas the rest of the world obtains iron mostly as Fe(III) from plant sources (Fleming and Bacon, 2005). Pharmaceutical iron supplements of Fe(II) provide a viable alternative source of iron in some diets (Umbreit et al., 2005).

Remarkably, even with diets rich in iron, estimates reveal that less than 10% of ingested iron is absorbed (Linder et al., 2003). Part of this relatively low absorption is due to the complex chemistry of Fe(III) in aqueous solutions that precludes its solubility at neutral
pH. Fe(II) and Fe(III) are both soluble in acidic environments (pH < 2); however, Fe(III) forms the insoluble \([\text{Fe(H}_2\text{O)}_3\text{(OH)}_3]\) complex as the pH rises. Furthermore, in the presence of physiological oxygen concentrations, Fe(II) will auto-oxidize to Fe(III), contributing to the insolubility of iron at neutral pH (Aisen et al., 2001). This exemplifies the requirement for ligands to increase the solubility of iron at physiological oxygen tensions and pH. Biological ligands such as citrate, histidine and acidic amino acids commonly coordinate dietary iron (Conrad and Umbreit, 2000).

1.4.4 Iron absorption pathways

Both Fe(III) and Fe(II) are transported into the enterocytes via different mechanisms, i.e., three pathways are believed to exist for the intestinal uptake of iron: 1) the divalent metal transporter-1 (DMT1) pathway; 2) the heme pathway, and 3) the integrin-mobilferrin pathway. The first two pathways are shown in Figure 1.3.

The best characterized of these pathways for the transport of iron into enterocytes is the DMT1 pathway. An initial step in iron absorption from plant sources is the reduction of Fe(III) to Fe(II), this occurs naturally in the low-pH environment of the gut with the aid of ascorbic acid. Fe(III) reduction is also facilitated by ferrireductases localized to the apical membrane of the enterocyte, such as DCytB (McKie et al., 2001). This allows the Fe(II) to be transported across the apical membrane into the cell via the transmembrane protein DMT1 (Fleming et al., 1997). Reduced expression of DMT1 results in severe anemia that is untreatable by oral or intravenous iron supplement (Fleming et al., 1997).
Figure 1.3. Intestinal iron absorption pathways. Heme iron, the dietary form of iron from meat sources, enters the enterocyte via HCP1. Inside the enterocyte, the heme porphyrin is degraded and Fe(II) joins the intracellular free iron pool. The dietary form of iron from plant sources, Fe(III), is reduced to Fe(II) by the ferrireductase DcytB prior to entering the duodenal enterocyte. Fe(II) is transported across the apical membrane by DMT1. Inside the enterocyte, Fe(II) can either be stored ferritin (Fr) or transported across basolateral membrane by Fpn1. Outside the enterocyte, the ferroxidase Hp oxidizes Fe(II) to Fe(III). Fe(III) is taken up by transferrin and transported from the gut mucosa to blood. This figure is courtesy of Ann Wong, University of British Columbia.
Until very recently, the mechanism for heme iron absorption was poorly understood (Latunde-Dada et al., 2006b). The identification of the mouse intestinal heme carrier protein 1 (HCP1) has greatly increased our understanding in the absorption process (Shayeghi et al., 2005). HCP1 is a transmembrane protein that transports heme across the apical membrane as an intact molecule. Heme oxygenase catabolizes the iron porphyrin ring inside the enterocyte (Latunde-Dada et al., 2006b). Soluble Fe(II) is then free to join the pool of iron transported across the plasma membrane via the DMT1 pathway.

Perhaps the least characterized of these iron pathways is the integrin-mobilferrin pathway (for this reason it has been omitted from Figure 1.3) (Conrad et al., 1994). Mobilferrin is a homologue of calreticulin, a common chaperone protein within the endoplasmic reticulum, and β3 integrin is a known adhesion protein (Conrad and Umbreit, 2002). Despite a decade of study, this pathway remains poorly understood.
The integrin-mobilferrin pathway only transports iron in an inorganic Fe(III) state (Conrad et al., 2000). During iron deficiency, mobilferrin is secreted into the lumen of the small intestine along with the mucosal surface protein, mucin, where they chelate free Fe(III) (Conrad et al., 1993). The soluble Fe(III) complex is transported to the plasma membrane and Fe(III) is taken up into the enterocyte by β3 integrin (Simovich et al., 2003). Inside the cytosol, a complex of β3 integrin, mobilferrin, flavin monooxygenase and DMT1 is formed (Conrad and Umbreit, 2002). This complex, known as paraferritin, has sufficient ferrireductase activity to reduce the newly transported Fe(III) to Fe(II) (Umbreit et al., 1996). The Fe(II) is allowed to join the low molecular weight iron pool and be exported from the cell.
Once across the apical membrane of the enterocyte, Fe(II) is presumably transported by a currently unidentified iron chaperone and delivered to the basolateral membrane for export. Absorbed dietary iron that exceeds the body’s requirement for the metal is stored in ferritin, the iron storage protein, for later use. Iron must be exported into the bloodstream to maintain systemic iron homeostasis. Essential to this process is the integral membrane permease ferroportin 1 (Fpn1) which exports Fe(II) across the basolateral membrane of the enterocyte (Fleming, 2005). A recent study on glioma cells and astrocytes has shown that Fpn1 is stabilized at the cell surface by multi-copper oxidases (De Domenico et al., 2007a). Loss of ferroxidase activity resulted in loss of Fpn1 mediated iron transport as well as increased FPN internalization and ubiquitination leading to degradation (De Domenico et al., 2007a). Outside the cell, Fe(II) is oxidized to Fe(III) by the ferroxidase Hp (Vulpe et al., 1999). Ferroxidases are proposed to function in iron release from iron storage cells through the generation of a steep concentration gradient of Fe(II) (De Domenico et al., 2007a). This allows for highly efficient efflux of iron, which is then bound by Tf as Fe(III). To prevent damage resulting from unbound Fe(III), it is proposed Tf and Hp may function together to export iron out of the duodenal enterocyte (Griffiths et al., 2005). However, the details of the Hp catalyzed oxidation of Fe(II) to Fe(III) in vivo and subsequent transfer of Fe(III) to apo-Tf are presently unknown.

1.4.5 Regulation of iron absorption

Strict regulation of iron homeostasis is essential as revealed by numerous human disorders including cancer, anemia, Parkinson’s disease and Alzheimer’s disease that are associated with improper iron homeostasis (Brookes et al., 2006; Zecca et al., 2004). The
regulation of iron absorption is a complex process that centers on the antimicrobial peptide hepcidin (Ganz, 2003). Hepcidin is synthesized by the liver as an 84 amino acid prepropeptide; however, post-translational cleavages yield the active 25 amino acid hormonal peptide (Valore and Ganz, 2007). Hepcidin was identified as a negative regulator of iron transport and has been coined the “master iron regulatory hormone” (Nicolas et al., 2001; Nicolas et al., 2002; Pigeon et al., 2001; Fleming, 2005). Hepcidin down regulates iron absorption by binding to Fpn1 and inducing its internalization and degradation (Figure 1.3) (Nemeth et al., 2004). The internalized Fpn1 is trafficked through multivesicular bodies to lysosomes, in a process mediated by the endosome sorting complex required for transport proteins (ESCRT) (De Domenico et al., 2007b). Moreover, increased intracellular iron levels lower the expression of DMT1, also decreasing iron uptake (Yamaji et al., 2004).

Hepcidin expression is modulated at the transcription level by multiple factors, including inflammation, anemia, hypoxia, and iron stores (De Domenico et al., 2008). Defective erythropoiesis and hypoxia induce separate signaling pathways that repress the transcription of hepcidin. Conversely, hepcidin transcription is up regulated with inflammation and increased iron stores.

In the favored model for the regulation of intestinal iron absorption, Tf and TfR function at the nexus of the entire process, acting as indicators for systemic iron levels (Frazer and Anderson, 2005). In this model, levels of Tf-bound iron in circulation correlate directly with changes in body iron usage. When the systemic demand for iron is high the levels of circulating diferric-Tf are reduced, whereas when the systemic iron demand is low serum concentrations of diferrie-Tf increase. The TfR plays a role in iron homeostasis through competitive interactions between diferrie Tf and the hereditary hemochromatosis...
protein, HFE. The precise role of HFE in the regulation of iron absorption remains to be elucidated. The absence of HFE is associated with iron overloading while over-expression of HFE reduces iron absorption (Sebastiani and Walker, 2007). The HFE polypeptide is comprised of a small extracellular domain, a TM domain and a short cytosolic tail. HFE forms a complex with β2 microglobulin, which can then interact with the helical domain of TfR (Lebron et al., 1998; Feder et al., 1998).

At high iron concentrations TfR preferentially binds to diferric Tf; the unbound HFE ultimately leads to the increased expression of the iron regulatory protein hepcidin (Fleming and Britton, 2006). This may involve an interaction with the TfR homolog, TfR2, which results in a signaling pathway that activates hepcidin transcription (De Domenico et al., 2008). The exact function of TfR2 is unclear, however it thought to be connected to hepcidin expression as mutations in TfR2 are associated with decreased hepcidin expression and iron overload disease. TfR2 is capable of binding and internalizing diferric Tf, in fact the cell surface stability of TfR2 is increased in the presence of diferric Tf (Goswami and Andrews, 2006). However, it likely that TfR2 serves as an iron sensor rather than a cellular iron uptake mechanism (Andrews and Schmidt, 2007). Thus, HFE and TfR2 are proposed to sense systemic iron requirements based on the level of Tf saturation with iron.

1.4.6 Diseases of iron imbalance

Mutations of transporter proteins involved in dietary iron uptake can result in anemia. Loss-of-function mutations in DMT1 are autosomal recessive and cause hypochromic microcytic anemia and hepatic iron accumulation. DMT1 mutations can be caused by point mutations, in-frame deletion of a single amino acid or the complete deletion of an exon due
Hereditary hemochromatosis is a primary iron overload disease with subsequent tissue damage from iron-induced oxidative stress. Hereditary hemochromatosis is ascribed to mutations in four genes encoding proteins involved in iron homeostasis. Hemochromatosis type I is caused by a C282Y mutation in HFE. C282Y disrupts a disulfide bond required for the HFE-β2 microglobulin interaction, which results in unstable surface expression of the protein (Feder et al., 1996). Most patients with HFE C282Y do not develop clinical disease, suggesting incomplete penetrance of this mutant (Sebastiani and Walker, 2007). Mutations in hemojuvelin or hepcidin are known as hemochromatosis type II or juvenile hemochromatosis. The majority of type II cases are caused by mutations in hemojuvelin. This is a severe form of hereditary hemochromatosis and is characterized by an early onset age. Hemochromatosis type III is associated with mutations in TfR2 (Robson et al., 2004). Unlike hemochromatosis types I, II and III, which result in abnormally low levels of hepcidin, hemochromatosis type IV results from mutations in Fpn1 (De Domenico et al., 2006). All Fpn1 mutations reported to date involve either a single base change or a deletion.

Aceruloplasminemia is an autosomal recessive disorder caused by a loss-of-function mutation in the gene for Cp (Gitlin, 1998). Some mutations in Cp cause premature termination of Cp translation; the resulting truncated product is not copper loaded (Vassiliev et al., 2005). Patients with aceruloplasminemia have a complete absence of serum Cp and are anemic due to the low serum iron concentration and high serum ferritin level (Madsen and Gitlin, 2007). Despite the low serum iron level, iron accumulates in the brain and
visceral organs such as the liver and pancreas. Excess iron deposition in the brain leads to loss of neurons in the basal ganglia (Madsen and Gitlin, 2007).

1.5 Iron uptake by other cells

1.5.1 Transferrin-transferrin receptor pathway

In erythroid precursor cells, iron uptake is a well-characterized pH-dependent process of receptor-mediated endocytosis (Enns, 2002). This process relies on the high serum concentration of Tf and the ubiquitous nature of TfR on plasma membranes, as depicted in Figure 1.4. The soluble extracellular domain of TfR extends into the plasma and is available for Tf binding. Diferric Tf enters cells by preferentially binding to the TfR with high affinity ($K_D \sim 0.1\text{-}10 \text{ nM}$) (Dautry-Varsat et al., 1983; Lebron et al., 1998; Giannetti et al., 2005). Monoferric Tf binds an order of magnitude weaker relative to diferric Tf, and an additional order of magnitude weaker for apo-Tf (Mason et al., 1998; Evans et al., 1994). The Tf-TfR complex is internalized by endocytosis into a clathrin coated pit.

An ATPase pump acidifies the endosome to pH $\sim 5.6$ (Dautry-Varsat et al., 1983). At this pH, Fe(III) is released from Tf, while apo-Tf remains bound to the receptor. The removal of Fe(III) from Tf is a complex and incompletely understood process involving salt, pH and a potential unknown chelator inside the endosome (Mckie, 2005). Upon release it is thought that Fe(III) is reduced to Fe(II) by a ferrireductase, such as Steap3 (Ohgami et al., 2005). The Fe(II) is then transported across the endosomal membrane by DMT1 and released into the intracellular environment (Fleming et al., 1999). Reticulocytes utilize $\sim 85\%$ of Tf-bound iron for incorporation into hemoglobin. The remaining iron is found in other iron-containing proteins such as the cytochromes and myoglobin.
Figure 1.4. Depiction of the uptake of cellular iron via the TfR pathway. Diferric Tf (blue and green structure); TfR (yellow structure); iron (red dot). Diferric Tf binds the TfR at physiological pH. The Diferric Tf-TfR complex is internalized via endocytosis of a clathrin coated pit. The resulting endosome is acidified to pH ~5.6 by an ATPase proton pump. At pH 5.6, diferric Tf releases iron but remains bound to the TfR. Fe(III) is reduced to Fe(II) by the ferrireductase Steap3 and is transported into the cytoplasm through DMT1. The endosome is recycled to the cell surface, where at physiological pH apo Tf is released from the TfR. This figure is courtesy of Dr. Anne Mason and Dr. Stephen Everse, University of Vermont.
Inside the endosome, apo-Tf remains bound to the TfR until the Tf-TfR complex is recycled to the outside of the cell, where at physiological pH, release of apo-Tf takes place. The entire process of cellular iron uptake occurs in less than 3 min (Enns, 2002).

### 1.5.2 Iron release from transferrin

Iron release from the individual lobes of Tf has been extensively studied (He and Mason, 2002). As outlined above, several factors play critical roles to ensure iron-release occurs in a physiologically relevant timeframe. These include pH, anion concentration, presence of a chelator, and the TfR. The iron delivery pathway occurs in cycles of 2-3 min duration; however, the process can slow to over 3 hours in the absence of such factors (He and Mason, 2002).

The molecular mechanism of iron release is still undergoing elucidation. Iron is bound to four residues in each lobe of serum Tf: His, Asp, and two Tyr residues (Anderson, et al., 1989). A synergistic anion, usually carbonate, is also required for complete binding (Schlabach and Bates, 1975). Upon iron release, the Tf clefts are proposed to rotate and open around the hinge from a closed conformation (iron bound) to an open conformation (iron free). Interestingly, it is the contribution of residues not directly involved in iron binding, coined the “second shell” residues, which provide much of the insight into the mechanism of iron-release.

In the N-lobe, iron release is believed to be “triggered” by two Lys residues, known as the dilysine trigger (Dewan et al., 1993). Each Lys is located in a separate subdomain of the N-lobe (206Lys in N1 subdomain; 296Lys in N2 subdomain). At pH 7.4, the ε-amino groups of the two Lys residues, which are located 2.3 Å apart, form a hydrogen bond. This
stabilizes iron bound Tf in a closed cleft conformation. Upon acidification of the endosome, both Lys residues become fully protonated, leading to charge repulsion and cleft opening (MacGillivray et al., 1998).

Although not as well characterized as N-lobe iron release, the C-lobe is believed to function in a similar pH-dependant conformational change as the N-lobe dilysine trigger (Wally et al., 2006). The C-lobe lacks a dilysine trigger, but does contain three residues (534Lys, 632Arg and 634Asp), coined the pH sensitive triad, which likely plays a similar role in iron release (Halbrooks et al., 2003). It has been suggested that only the C-lobe is involved in cellular iron delivery, with monoferric Tf being recycled to the extracellular environment (Cheng et al., 2004).

Iron can also be removed from Tf through the action of bacterial Fe(III)-chelating molecules called siderophores. Siderophores are small peptide-based molecules synthesized and secreted by bacteria (Wandersman and Delepelaire, 2004). Upon binding Fe(III), siderophores return to the bacterial cell where they are recognized by surface proteins and internalized. Iron may be released from siderophores by reduction of Fe(III) to Fe(II) or by breaking down the siderophores and releasing the iron (Wandersman and Delepelaire, 2004).

1.5.3 Structure of the transferrin receptor-transferrin complex

An electron density map of the diferric Tf-TfR complex was produced using cryo-electron microscopy (cryo-EM) (Cheng et al., 2004). A 7.5 Å resolution predicted atomic model was generated by docking the known crystal structures of diferric Tf and the TfR ectodomain into the electron density map of the complex (Figure 1.5 A; Cheng et al., 2004).
Figure 1.5. Structure of the Tf-TfR complex. A. Ribbon diagram of the Tf-TfR complex as determined from the cryo-EM atomic model. TfR protease-like domain (red); TfR helical domain (yellow); TfR apical domain (orange); Tf C-lobe (dark green); Tf N-lobe (light green) B. Interaction between the Tf C-lobe and TfR. C. Interaction between the Tf N-lobe and TfR. (Adapted from Cheng et al., 2004).
This model offers a preliminary view of the molecular interactions between Tf and the receptor. Based on the model, both the N1- and N2-subdomains of the Tf N-lobe make contact with the receptor, whereas only the C1-subdomain of the C-lobe is involved in the interaction. This is an interesting finding as the C-lobe was thought to contribute 76% of the binding energy in the Tf-TfR interaction (Zak et al., 1994).

The cryo-EM model reveals an expansive, predominantly negatively charged patch (residues 349-372) of the C1-subdomain is involved in the interaction with an equally large and predominantly positive charged patch (residues 619-651) of the TfR helical domain. This suggests the involvement of numerous salt bridges in the interaction, covering a large surface area. In the model, the C2-subdomain does not make contact with the receptor and is therefore thought to swing freely.

The N-lobe subdomains are predicted to contribute four residues each in the interaction. Only two residues in the TfR helical domain (Asn662 and Glu664) make contact with the N1-subdomain (residues Tyr71-Leu72-Ala73-Pro74) (Figure 1.5 B). Residues Pro142-Arg143-Lys144-Pro145 of the N2-subdomain make contacts with the protease domain of the TfR (residues Leu122-Tyr123-Trp124-Asp125) (Figure 1.5 C). The large number of nonpolar residue contacts between the N-lobe and TfR is consistent with a complex interaction involving a combination of hydrophobic and ionic bonds.

1.6 Transferrin interaction with the transferrin receptor

1.6.1 Cell binding studies

The binding interaction between Tf and TfR is traditionally measured using cell-binding assays (Aisen et al., 1978; Dautry-Varsat et al., 1983). These assays require
radiolabeled Tf and are performed using various cell types that express TfR on their plasma membrane surfaces. Such assays make it very difficult to determine detailed kinetics of the binding events as they are not typically performed in real time. Furthermore, due to a lack of sensitivity, it is nearly impossible to resolve the separate binding constants of each Tf interactions, i.e. when the TfR homodimer sequentially binds two Tfs.

1.6.2 Biosensor studies

A comprehensive library of mutations in TfR was created by the Bjorkman group (Giannetti et al., 2003; West et al., 2001). Analysis of these mutants provided needed insight into the critical residues involved in Tf binding. These studies were the first to employ surface plasmon resonance (SPR) to investigate the interaction between Tf and its receptor. The sensitivity of this technique has resulted in several novel ideas about the interaction. For example, it was observed that Tf does not bind equally to each monomer of the TfR dimer (West et al., 2001). In other words, there are two equilibrium dissociation constants ($K_D$) for each binding event that occurs between Tf and the TfR dimer. The kinetics and binding constants of the TfR mutants have been analyzed using SPR and specific residues in the receptor involved in the interaction have been predicted. Unfortunately, these studies were not complemented by similar measurements using Tf mutants, to address the involvement of Tf residues in the interaction.
1.7 Surface plasmon resonance

1.7.1 Overview

Biacore instruments employ SPR to monitor the real time formation and breakdown of complexes between an immobilized ligand and mobile analyte. Using this optical biosensor technique, detailed information is provided about rate constants and the binding mechanisms associated with macromolecular interactions. An SPR experiment allows analysis of several different types of interactions, but the most common is perhaps protein-protein interactions. Briefly, this involves the examination of the interaction between a protein immobilized on a surface (referred to as immobilized ligand) and a protein applied to the surface in solution (referred to as analyte). Biacore (Piscataway, NJ) provides several commercial biosensor chips to accommodate numerous surface preparations and experimental designs. The CM5 chip allows for ligand immobilization using standard amine coupling chemistry. The CM5 chip has carboxymethyl dextran chains (a carboxymethyl glucose polymer) that have been covalently attached to the gold surface of the CM5 chip through gold-thiolate bonds. The terminal carboxymethyl groups of these long dextran chains allows for the amine coupling of virtually any polypeptide ligand. The ligand is immobilized to the carboxyl groups of the dextran via a 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)–N-hydroxyl succinimide (NHS) coupling reaction. Ethanolamine is used to block all unreacted carboxyl groups. An interaction between the analyte protein and the immobilized ligand results in a surface mass change, which is detected and signaled in real time. There are several advantages in using SPR to study interactions including lack of labeling, low sample consumption and high sensitivity.
1.7.2 Optical configuration and detection principles

Functionally, SPR requires only a source of polarized light and a glass prism coated with metal, most commonly gold (Hall, 2001) (Figure 1.6). The system relies on a principle known as total internal reflection. Polarized light is directed towards a glass prism and at a critical angle the incident light is completely reflected. In other words, at the angle of total internal reflection the light photons can not permeate the prism and are reflected. SPR utilizes the “Kretchmann Configuration”, whereby the prism is coated with a thin (50 nm) gold film (Schuck, 1997). In this configuration, the total internal reflection of light excites surface plasmons in the metal surface. A surface plasmon is an oscillating wave of charge density that travels along the surface of the metal. Only at specific angles of incident light, termed the resonance angle (θ), is the energy from the photons transferred to surface plasmons. The resonance of surface plasmons produces an electromagnetic field of evanescent waves that decay exponentially with increasing distance perpendicular to the sensor surface. The resonance causes an energy loss in the reflected light, which is visible as a sharp minimum in the angle-dependent reflectance, an experimentally recorded quantity. The resonance angle is dependent on the refractive index of the sample traveling through the evanescent field above the sensor surface (n_{surf}). Adsorption and desorption of analyte at the sensor surface causes a change in the local refractive index and produces a shift in resonance angle that can be precisely measured. The resonance angle shift is directly proportional to the mass bound to the sensor surface (Schuck, 1997).
Figure 1.6. Detection principle of SPR. Schematic diagram illustrating a SPR biosensor. TIR: total internal reflection; $n_{surf}$: refractive index of the bulk solution in the vicinity of the sensor surface (adapted from Schuck, 1997).
The Biacore 3000 instrument has four flow cells in parallel that allow experiments and controls to be performed with the same analyte sample simultaneously. The surface of the sensor chip serves as the base of each flow cell (dimensions: \( l = 2.4 \text{ mm}, w = 0.5 \text{ mm}, h = 0.05 \text{ mm} \)). During SPR, a photo-detector accurately monitors all angles of reflected light. The SPR-angle that causes surface resonance is used to quantitatively measure the binding of analyte to the surface. The SPR-angle response is converted to Response Unit (1 RU = 0.0001°), which is equivalent to 1 pg/mm² of bound protein (Stenberg et al., 1991). The raw data acquired from an SPR experiment is presented in the form of a sensorgram plot (RUs vs. time).

1.8 Objectives and overview

Although an abundance of information has been gathered characterizing the processes of iron homeostasis, many questions remain unanswered. This thesis addresses issues of both iron absorption and transport. Hp is only the second vertebrate ferroxidase to be discovered and although it has been proposed to play a role in iron absorption, the specific details of its function are still unclear. Virtually no studies have characterized the transport of Fe(III) from Hp to the circulation. Due to the insoluble nature of free Fe(III) under physiological conditions, in Chapter 3 I posit that the release of unbound Fe(III) is prevented by a direct protein-protein interaction between Tf and Hp during intestinal iron export. No interaction between Tf and Hp or Tf and Cp has been observed.

In Chapters 4 and 5, the focus changes from iron absorption to iron transport and uptake. The Tf-TfR iron uptake pathway has been the focus of considerable iron transport research for years. However, little is known regarding the interaction between Tf and TfR at
the molecular level. In this section of the thesis, SPR was employed to characterize the binding event between Tf and TfR. The significance of the findings and future directions are also discussed.

Questions regarding the potential interaction between Hp and Tf prompted the investigation into the localization of the Hp in the human small intestine. Is Hp actually located extracellular to the basolateral membrane of the enterocyte and readily available for interaction with blood proteins? In Appendix 1, the location of Hp in human tissue is clarified using a novel polyclonal antibody against Hp.

1.9 Hypotheses

Four hypotheses were tested in this dissertation:

1. As a means of preventing the release of free Fe(III) during intestinal iron transport, Hp interacts with Tf to form a stable complex.

2. The distribution of human Hp is not restricted to the basolateral membrane of the duodenal enterocyte.

3. Mutations to residues affecting iron-release in recombinant Tf do not alter TfR binding.

4. N-lobe residues involved in the TfR interaction, as judged by the cryo-EM model, are necessary for receptor binding.
Chapter 2: Materials and methods

2.1 Materials

All recombinant Tf (rTf) proteins were kindly provided by Dr. Anne Mason (Department of Biochemistry, University of Vermont). Soluble His-tagged TfR, expressed in BHK cells as described (Byrne et al., 2006), was also provided by Dr. Anne Mason (University of Vermont). Human apo-Tf and holo-Tf were purchased from Sigma-Aldrich (Oakville, ON). Human Cp was purchased from VitalProducts Inc. (Boynton Beach, FL). Anti-bovine rhodopsin monoclonal antibody (anti-1D4) was kindly provided by Dr. Robert Molday (Department of Biochemistry and Molecular Biology, University of British Columbia). Large unilamellar vesicles composed of equimolar amounts of N,N-dioleyl-N,N-dimethylammonium chloride : dioleoylphosphatidylethanolamine (DODAC:DOPE) were kindly provided by Dr. Pieter Cullis (Department of Biochemistry and Molecular Biology, University of British Columbia).

All sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed using established protocols (Sambrook and Russell, 2001). Western Blots and IHC were carried out using commercial antibodies. Primary antibodies: goat anti-human Tf (whole antiserum) (Sigma-Aldrich, Oakville, ON); secondary antibodies: HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich, Oakville, ON), Texas Red-conjugated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, USA), HRP-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, USA), biotinylated horse anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA).
Protein concentrations were determined by absorbance using the following mass extinction coefficients (E280 0.1%, 1cm) and molecular weights; holo-Tf: 1.41, 80,000; apo-Tf: 1.14, 80,000; TfR: 1.20, 159,500; rHp: 1.66, 129,600. All SPR reagents, including CM5 sensor chips, HBS and HBS-P running buffer were purchased from Biacore (Biacore Inc., Piscataway, NJ). Plastic vials, rubber caps and 96-well plates were supplied by Biacore. Cross-linking reagents bis(sulfosuccinimidyl) suberate (BS³) and N-[κ-maleimidoundecanoyloxy] sulfosuccinimide ester (sulfo-KMUS) were purchased from the Pierce Chemical Company (Rockford, IL). Ferrous ammonium sulfate hexahydrate (ReagentPlus grade), Ferrozine, complete Freund’s adjuvant and incomplete Freund’s adjuvant were obtained from Sigma-Aldrich (Oakville, ON). Tissue culture multi-well plates were supplied by BD Falcon (Oakville, ON), Corning expanded surface roller bottles and 100 mm diameter Petri dishes were from Fisher Scientific (Ottawa, ON). All other chemicals were from Sigma Chemical Co. and were of the highest grade available.

2.2 Protein expression

Soluble human recombinant Hp (rHp) was expressed and purified as previously described by Griffiths et al., 2005. Briefly, the rHp construct was engineered to express a soluble protein by replacing the C-terminal transmembrane domain of rHp with a cleavable 1D4 epitope tag. The construct was cloned into a pNUT expression vector and expressed in baby hamster kidney (BHK) cells. The BHK cells were transfected with the rHp construct and grown at 37°C in a 5% CO₂ environment in a culture media of Dulbecco’s modified Eagle’s medium-Ham F12 (DMEM-F12), 2% (w/v) Ultraser G (BioSepra, Marlorough, MA) and 10 μM CuSO₄. Cells were passaged into expanded surface roller bottles, and media was
collected every three days. rHp was purified using an anti-ID4 immunoaffinity CNBr-activated Sepharose 4B column (anti-ID4 Sepharose 4B).

2.3 Ferroxidase activity

Oxidation of Fe(II) by rHp was determined using a ferrozine-based ferroxidase assay as described previously (Reilly and Aust, 1997). Briefly, 1.0 μM rHp was incubated with 100 molar equivalents of Fe(II):imidazole (1:5) in HBS (HEPES-buffered saline, 0.01M HEPES pH 7.4 containing 0.15M NaCl) in the presence or absence of 1.0 μM apo-Tf. At given time points, 100 μl aliquots of each reaction were mixed with 200 μl of 15 mM ferrozine directly in a 96-well plate. Ferrozine quenches the ferroxidase reaction by binding residual Fe(II). The ferrous:ferrozine complex was measured spectrophotometrically (ε_{562} = 27900 M⁻¹ cm⁻¹). All solutions were purged with nitrogen gas before the addition of protein. Autoxidation was determined in the absence of rHp and Tf. All assays were performed in triplicate.

2.4 Gel electrophoresis and western blotting

Native PAGE was performed using 5% polyacrylamide gels in the absence of detergent, reducing agent and sample heating. Protein samples were incubated at room temperature for 30 min in HBS prior to being electrophoresed at 5 mA. All other gel analyses utilized 7.5% or 10% polyacrylamide gels containing 1% SDS and 125 mM dithiothreitol (Sambrook and Russell, 2001). Gels were stained with EZBlue™ Coomassie Brilliant Blue G-250 (Sigma-Aldrich, Oakville, ON).
For western analysis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane in N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer for 90 min at 400 mA. The membrane was blocked with 1% (w/v) skim milk powder, incubated with the primary and secondary antibodies as indicated on figure legends and developed using Enhanced Chemiluminescence (ECL Plus Western Blotting Detection System, GE Healthcare, Buckinghamshire, UK).

2.5 Covalent chemical cross-linking

Optimal chemical cross-linking conditions were determined empirically using the homo-bifunctional chemical cross-linking reagent BS$_3$. Equimolar concentrations of each protein (5 μM) were incubated in HBS at room temperature for 15 min prior to being treated with 5 mM BS$_3$ for 2 hours at room temperature. Untreated samples were prepared in the absence of BS$_3$.

2.6 Polyclonal antibody production

Ethical approval for this part of the study was obtained from the UBC research ethics board and animal care committee (Animal Care Certificate A06-0014). Animals were cared for at the UBC Animal Care Centre. Two female adult New Zealand White rabbits were used for antibody generation. A preimmunization bleed (10 mL), containing the preimmune serum, was collected from each rabbit to ensure that antibodies to Hp were not present in the animals. The priming immunization consisted of an emulsion of equal parts complete Freund’s adjuvant and purified rHp (0.5 mg/mL in PBS, phosphate-buffered saline, 0.01 M PO$_4$ pH 7.4 containing 0.15 M NaCl). Rabbits were injected subcutaneously with complete
Freund’s adjuvant/rHp emulsion (1 mL each) by the attending veterinarian. Subsequent immunizations were given every 2 weeks as intramuscular injections using incomplete Freund’s adjuvant. Antibody titer was tested after each booster injection. Rabbits were exsanguinated at the end of the study. Serum was prepared by allowing blood to clot for 4 hours at room temperature followed by an overnight incubation at 4°C. The blood sample was centrifuged at 2700 x g for 10 min at 4°C and the supernatant collected. Serum was stored in aliquots at 4°C, -20°C and -70°C.

2.7 Immunohistochemistry

Ethical approval to conduct this research was obtained from UBC and Vancouver Coastal Health Authority. Prepared slides of paraffin-embedded tissue were kindly provided by Dr. Alison Buchan (Department of Cellular and Physiological Sciences, University of British Columbia). Full thickness human tissue samples of duodenum, jejunum, ileum, colon and stomach were used in this study. IHC staining was performed using the rabbit-generated antiserum to human Hp (α-rHp) at a dilution of 1:1000. Control experiments included preabsorption of the antibody with 1 μM purified rHp for 60 min at room temperature (blocked α-rHp) and probing the tissue with the preimmune serum. The paraffin-embedded tissues were dewaxed in xylene (2×2 min) and dipped in petroleum ether. Tissues were rehydrated in graded alcohols and quenched of endogenous peroxidase activity by placing tissue sections in 0.3% (v/v) H₂O₂ in distilled H₂O for 15 min at room temperature. All slides were blocked for non-specific staining with 10% (v/v) normal horse serum and 0.3% (v/v) Triton X-100 in PBS for 60 min at room temperature. Antigen retrieval was carried out using 1% (v/v) SDS in PBS for 5 min at room temperature. The sections were incubated
with α-rHp diluted in 5% (v/v) normal horse serum and 0.3% (v/v) Triton X-100 in PBS for 12-72 hours at 4°C. Sections were incubated with 1:1000 dilution of biotinylated anti-rabbit secondary antibody in 5% (v/v) normal horse serum and 0.3% (v/v) Triton X-100 in PBS for 60 min at room temperature. Proteins were visualized using the Vectastain Elite ABC kit and diaminobenzidine (DAB) substrate kit for peroxidase (Vector Laboratories Inc., Burlingame, CA, USA). Coverslips were applied to sections in PBS-glycerine (1:9). All tissue sections were viewed under a Zeiss Axioplan 2 microscope (Zeiss, Don Mills, Ontario.).

2.8 Surface plasmon resonance

2.8.1 Ligand immobilization

SPR experiments employed a Biacore 3000 (Uppsala, Sweden). All ligands were immobilized on a CM5 sensor chip (Biacore AB) in random orientation using amine coupling chemistry. The immobilization procedure consists of three basic steps: 1) activation, 2) coupling, and 3) deactivation. The carboxyl groups of the dextran surface were activated with NHS and EDC (1:1). A ligand was applied to the activated surface until sufficient coupling was achieved (see below). Finally, a blocking solution (ethanolamine) was applied to the coupled surface to quench the remaining activated sites. All Biacore buffers were filtered through a 0.22 μm filter and degassed prior to use. Stock solutions were briefly centrifuged prior to remove insoluble material.

2.8.1.1 Random orientation

rHp and Cp (150 μg/mL in 10 mM acetate, pH 5.0) were immobilized to 7000-8500 RU. sTfR (20 μg/ml in 5 mM maleate, pH 6.0) was immobilized to 500-1900 RUs. Prior to
protein binding, flow cells were equilibrated with running buffer until the baseline stabilized to less than 0.05 RU/min.

2.8.1.2 Directed orientation

sTfR was also immobilized using an oriented coupling procedure. Briefly, anti-His-tag antibody (10 μg/ml in 10 mM acetate, pH 5.0; anti-PentaHis, Qiagen, Mississauga) was randomly coupled to a CM5 chip to ~ 3000 RU. 6x-His-tagged sTfR was applied over the antibody-bound flow cell at a flow rate of 30 μL/min to a density of 200-400 RU. A large amount of the bound sTfR immediately dissociated from the anti-His antibody, but the baseline drift eventually stabilizes less than 2 RU/min.

2.8.2 Protein binding experiments

All protein binding experiments were performed at 24°C. Protein binding was examined over a concentration series typically spanning a 10-fold range above and below the $K_D$ of the interaction. All SPR experiments were carried out in duplicate.

2.8.2.1 Transferrin-hephaestin interaction

The Tf-rHp interaction was carried out in HBS with a flow rate of 10 μL/min. Data collection for association phase was 5 min with a dissociation phase of 6 min. A regeneration phase of 2 min with 500 mM NaCl followed each cycle.
2.8.2.2 1D4 antibody-hephaestin interaction

The 1D4 antibody-rHp interaction was carried out in HBS with a flow rate of 10 μL/min. Data collection for association and dissociation phases was 6 min each. A regeneration phase of 30 min with 1.2 μg/μl 1D4 peptide followed each cycle in the antibody titration series to return the response signal to baseline.

2.8.2.3 Transferrin-ceruloplasmin interaction

The Tf-Cp interaction was carried out in HBS with a flow rate of 10 μL/min. Data collection for association and dissociation phases was 2 min each. A regeneration phase of 2 min with 500 mM NaCl followed each cycle.

2.8.2.4 Transferrin-transferrin receptor interaction in random orientation

The Tf-TfR interaction was carried out in HBS and 0.005% (v/v) surfactant P20 (HBS-P) with a flow rate of 20 μL/min as previously described (Lebron et al., 1998). Data collection for association phase was 4 to 6 min with a dissociation phase of 6 min. A regeneration phase of 500 mM MgCl₂ (30 min) followed each cycle to return the response signal to baseline.

2.8.2.5 Transferrin-transferrin receptor interaction in directed orientation

The directed orientation Tf-TfR interaction was carried out in HBS-P with a flow rate of 50 μL/min as previously described (Giannetti et al., 2005). Data collection for association and dissociation phases was 4 min each. A regeneration phase of 500 mM MgCl₂ (30 sec) followed each cycle to return the response signal to baseline.
2.8.3 Data analysis

Multiple software programs were used for data analysis. BIAevaluation v4.1 (Biacore) and the Scrubber software package (BioLogic Software, Campbell, Australia; www.biologic.com.au) were used to preprocess raw sensorgram data and make reference corrections and to display sensorgram data (RU vs. time). All data was reference corrected for non-specific binding and bulk refractive index changes by subtracting the response from the reference flow cell. A second reference correction was made by subtracting the response from the average of buffer-only injections to eliminate potential systematic instrument artifacts.

2.8.3.1 Near steady state analysis

The equilibrium dissociation constant \((K_D)\) values were determined using two methods. In the first, binding reactions were allowed to approach equilibrium by using longer injection times at lower flow rates with ligand coupled to the chip at higher densities. The near steady state response units \((R_{eq})\) were measured at several ligand concentrations. Graphpad Prism 4.1 (GraphPad Software, San Diego, CA) was used to fit binding data globally to either a one-site binding hyperbola according to the relationship:
\[
R_{eq} = \frac{R_{max} C}{K_D + C},
\]

or two-site binding hyperbola according to the relationship:
\[
R_{eq} = \frac{R_{max1} C}{K_{D1} + C} + \frac{R_{max2} C}{K_{D2} + C},
\]

where the subscripts 1 and 2 represent the first and second binding sites, respectively. \(R_{max}\), \(R_{max1}\) and \(R_{max2}\) are the response signals at saturation, \(K_D\), \(K_{D1}\) and \(K_{D2}\) are the equilibrium dissociation constants, and \(C\) corresponds to the injected analyte concentration. The dissociation constant is an approximation since the
binding may to be more complex than the fitted one- and two-site binding models. These constants are of course dependent on to the experimental conditions used for SPR analysis.

2.8.3.2 Kinetic analysis

Kinetic constants were determined from binding experiments using shorter injection times at faster flow rates with ligand coupled to the chip at lower densities. Sensorgram data was analyzed and kinetic constants were calculated from global fittings of the association and dissociation phases using the program CLAMP© (Myszka, 1998). CLAMP© is a data analysis program designed to interpret the kinetics of binding reactions by fitting sensorgram data to models simulated from hypothetical mechanisms (Myszka, 1998). The $K_D$ values were determined from the ratio of the dissociation and association rate constants, $k_{off}$ (s$^{-1}$) and $k_{on}$ (M$^{-1}$*s$^{-1}$). Sensorgrams curves were fit to the bivalent ligand model:

\[
\begin{align*}
Tf + TfR & \leftrightarrow Tf:TfR; \quad K_{D1} = \frac{k_{off,1}}{k_{on,1}} \\
Tf + Tf:TfR & \leftrightarrow Tf:TfR:Tf; \quad K_{D2} = \frac{k_{off,2}}{k_{on,2}}
\end{align*}
\]
Chapter 3: Studies on the interaction of transferrin with the multicopper ferroxidases, hephaestin and ceruloplasmin

The majority of the results from this chapter were recently published (Hudson et al., 2008). I completed all the experiments. Tanya Griffiths supplied the initial purified rHp used in this study and Michael Krisinger provided advice and guidance with the SPR studies.

3.1 Rationale

Iron homeostasis is essential for maintaining the physiological requirement for iron while preventing iron overload. Due to the insoluble nature of free Fe(III) under physiological conditions and the potential for both Fe(II) and Fe(III) to generate toxic hydroxyl free radicals, the regulation of iron is fundamental to the prevention of cell toxicity (Aisen et al., 2001). The multicopper ferroxidases regulate the oxidation of Fe(II) to Fe(III), circumventing the generation of these harmful by-products. It has been suggested that as a means of preventing the release of unbound Fe(III), a direct protein-protein interaction may occur between Tf and Hp during intestinal iron export (Syed et al., 2002; Griffiths et al., 2005). Support for this hypothesis arises from a recent study reporting the formation of a stable complex between a multicopper ferroxidase and Tf in algae (Paz et al., 2007). Furthermore, the identification of an interaction between Cp and Lf (a Tf analogue) under physiological conditions provides additional support (Sokolov et al., 2006; Pulina et al., 2002). However, there have been no reports to date that confirm an interaction between serum Tf and any human multicopper ferroxidases.

Previous work in this laboratory revealed the loading of Tf with Fe(II) is enhanced by a soluble form of Hp (rHp) (Griffiths et al., 2005). This is consistent with the specificity of Tf for Fe(III) as well as a potential interaction between the two proteins. In the present
study, the putative interaction between Tf and both rHp and Cp was investigated. Native PAGE and covalent chemical cross-linking were utilized to visualize any potential protein complexes. SPR was employed to study more transient interactions between the proteins.

3.2 Ferroxidase activity

To investigate a potential interaction with Tf, a soluble form of the Hp ectodomain was expressed with a C-terminal 1D4 epitope tag. BHK cell cultures were grown in a roller bottle culture system. As BHK cells are adherent, the conditioned medium was collected from the roller bottles and purified directly. Typically, the BHK cells expressing rHp were maintained for at least 2-3 weeks before cell detachment and death became significant. The switch from newborn calf serum to the serum replacement, Ultroser G, was employed to decrease the levels of other proteins, especially albumin, and to facilitate purification; it has also been observed that Ultroser G increases the production of recombinant protein expression possibly by stressing the cells (personal communication, Dr. Anne Mason and Dr. Ross MacGillivray). As medium was collected every three days, purification of rHp was performed every three days for the entire time period the BHK cells were alive and producing protein.

Ferroxidase activity was confirmed using an assay in which rHp oxidized Fe(II) to Fe(III) (Figure 3.1). It has previously been shown that apoferritin, but not apo-Tf, increases the ferroxidase activity of Cp due to a direct interaction (Reilly and Aust, 1997; Reilly et al., 1998).
Figure 3.1. Ferroxidase activity of rHp in the presence and absence of Tf using a ferrozine-based ferroxidase assay. Hp (■), Hp and Tf (□), Tf (●), autoxidation in the absence of added proteins (○). Data represent the mean ± standard deviation.
Neither apo-Tf (Figure 3.1) nor ferritin (data not shown) enhanced the ferroxidase activity of rHp, from which I conclude a similar interaction is absent. I posit that Tf does not act as a sink for released Fe(III). Under the conditions employed, both autoxidation from atmospheric oxygen tension and Tf was negligible.

3.3 Gel electrophoresis

Native PAGE was used in the initial investigation to detect protein complexes (Figure 3.2). A complex of proteins should migrate separately from the individual proteins. This was observed with the sTfR + holo-Tf complex (lane 6), which migrated between both of the individually electrophoresed proteins (lanes 2 and 3). No distinct molecular weight species were detected in the mixed sample of rHp and apo-Tf (lane 8), or Cp and apo-Tf (lane 7) compared to the individual proteins (lanes 1, 4 and 5), which is not consistent with the presence of a stable protein-protein complexes.

The possibility that a complex could be formed in solution but dissociate during the electrophoresis was also examined. The homo-bifunctional cross-linking reagent BS$_3$ was employed to covalently join transient complexes in solution. Initial evaluation revealed that BS$_3$ was able to capture homodimers of both sTfR (sTfR)$_2$ and rHp (rHp)$_2$ (Figure 3.3; lanes 4 and 10). This result is expected for the sTfR as it is known to exist as a disulfide-linked dimer. However, it is not known if rHp oligomerizes in nature, making the presence of the (rHp)$_2$ dimer difficult to evaluate. A high molecular weight band was observed with the sTfR + holo-Tf cross-linked complex (lane 1), that was absent in the individually cross-linked and untreated samples (lanes 2-5).
Figure 3.2. Putative complex formation between rHp and Tf analyzed by 5% native PAGE. Proteins (10 μg) were prepared in the absence of detergent, reducing agent and heating. Lane 1, apo-Tf; lane 2, holo-Tf; lane 3, sTfR; lane 4, rHp; lane 5, Cp; lane 6, sTfR and holo-Tf; lane 7, Cp and apo-Tf; lane 8, rHp and apo-Tf.
Figure 3.3. Analysis of putative protein-protein interactions using chemical cross-linking. After cross-linking with BS\textsuperscript{3} protein species were analyzed on 7.5% SDS PAGE. Lane 1, sTfR + holo-Tf + BS\textsuperscript{3}; lane 2, holo-Tf + BS\textsuperscript{3}; lane 3, untreated holo-Tf; lane 4, sTfR + BS\textsuperscript{3}; Lane 5, untreated sTfR; lane 6, rHp + apo-Tf + BS\textsuperscript{3}; lane 7, Cp + apo-Tf + BS\textsuperscript{3}; lane 8, apo-Tf + BS\textsuperscript{3}; lane 9, untreated apo-Tf; lane 10, rHp + BS\textsuperscript{3}; lane 11, untreated rHp; lane 12, Cp + BS\textsuperscript{3}; lane 12, Cp untreated.
The absence of a higher molecular weight species in the BS3-treated samples of rHp and apo-Tf (lane 6) and Cp and apo-Tf (lane 7) compared to the controls (lanes 8-13) is inconsistent with the proposal that the ferroxidases form a complex with Tf \textit{in vitro}. Similar cross-linking experiments using the long-chain heterobifunctional cross-linker sulfo-KMUS (Pierce) also failed to reveal an interaction (data not shown).

3.4 Transient interactions

SPR analysis was used to determine whether rHp or Cp was involved in a transient interaction with Tf in real time. No responses were observed in the rHp-bound flow cell with Tf concentrations up to 30 μM (using apo- and holo-Tf) (Figure 3.4 A). Moreover, no evidence of an interaction was found between Cp and Tf under similar conditions (Figure 3.4 C). Immobilization of rHp in directed orientation using a 1D4 antibody capture method also failed to reveal an interaction (data not shown). These observations are inconsistent with the hypothesis that these multicopper ferroxidases participate in a direct protein-protein interaction with Tf.

The presence of rHp on the biosensor chip was verified by injecting anti-1D4 antibody under identical conditions as the injection of Tf ($K_D = 134 \pm 30$ nM; Figure 3.4 A). Comparatively, holo-Tf bound to immobilized sTfR at concentrations as low as 0.25 nM. Near steady state analysis of holo-Tf binding to sTfR resulted in two dissociation constants when fitted to a bivalent ligand (2:1) model. Each sTfR was able to bind two holo-Tf molecules in a stepwise manner with $K_{D1} = 10.7 \pm 1.2$ nM and $K_{D2} = 158 \pm 39$ nM (Figure 3.4 B).
Figure 3.4. SPR analysis of Tf binding to immobilized rHp, sTfR and Cp.  

A. Binding profiles of apo- and holo-Tf (3.0 and 30 μM; *indicated by arrow*) and anti-1D4 antibody (5.0, 10, 25, 50, 100, 200 ng/μL) to immobilized rHp  

B. Binding profiles of holo-Tf (0, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 200, 500 nM) to immobilized sTfR  

C. Binding profiles of apo- and holo-Tf (0, 3.0 and 30 μM) to immobilized Cp.
These values are in reasonable agreement with previous studies using other preparations of recombinant, soluble TfR \((K_{D1} \sim 3 \text{ nM and } K_{D2} \sim 29 \text{ nM}; \text{(Lebron et al., 1998)})\) and membrane bound TfR \((K_D \sim 7 \text{ nM}; \text{(Dautry-Varsat et al., 1983)})\).

### 3.5 Discussion

All assays were designed to facilitate the comparison of a Tf interaction with each rHp, Cp and sTfR. sTfR was used as a positive control since TfR is the known physiological receptor for Tf. Furthermore, both rHp and sTfR are membrane bound proteins that are expressed as soluble recombinant proteins using analogous expression systems.

Multicopper ferroxidases such as Hp and Cp provide the catalysis for the oxidation of Fe(II) to Fe(III) and are essential for the mobilization of intracellular iron into the extracellular environment (Vulpe et al., 1999). As a means of preventing potential damage caused by unbound Fe(III), it has been proposed that Tf and Hp may function together in the export of iron out of the duodenal enterocyte (Syed et al., 2002; Griffiths et al., 2005). In the present experiments, no evidence was found for the formation of stable complexes of the ferroxidases Hp and Cp with Tf in solution. Moreover the possibility of transient interactions, which could have been obscured in the gel analysis, is excluded based on the SPR results.

Cp and Hp are paralogous proteins that have nonidentical functions in iron metabolism (Chen et al., 2004). Although Hp functions as a ferroxidase (Griffiths et al., 2005; Frazer et al., 2001), its specific role in iron metabolism remains unclear. The localization of Hp to both the intracellular and extracellular environments presents another element of uncertainty concerning its function, as it is not clear how an intracellular
ferroxidase would function in iron transport. A recent study, however, has implicated an intracellular Cp as well as Hp as having roles in iron absorption (Cherukuri et al., 2005). As well, both DMT1 and apo-Tf have also recently been localized to the supranuclear compartment in an intestinal cell line (Ma et al., 2002), supporting the possibility of a role for an intracellular ferroxidase in vesicular transport or transcytosis. The processing of absorbed iron by Hp or Cp inside the cell could also explain the absence of a direct interaction with Tf observed in this study.

Whether Tf actually plays a significant role in iron absorption must be considered. It has been suggested that during iron absorption from the enterocyte, the levels of free iron released exceed the binding capacity of Tf (Morgan, 1980). Furthermore, in separate studies using hypotransferrinemic mice as a model, it was proposed that Tf may not be required for intestinal iron absorption (Craven et al., 1987; Raja et al., 1999). Another explanation for the lack of interaction between the ferroxidase and Tf is based on their physical locations; upon oxidation, Fe(III) would be released into the mucosa, while Tf is typically found circulating throughout the vasculature. In this situation an unknown iron carrying intermediate, such as citrate, could transport iron through the interstitial fluid to portal blood; thereby bypassing the need for a direct ferroxidase-Tf interaction. On the other hand, with Tf serum concentrations as high as ~ 50 μM (Richardson and Ponka, 1997), Tf may act as an efficient scavenger for released Fe(III) without the requirement for intermediates or direct protein-protein interactions.

Finally, it is possible that Tf is only capable of recognizing membrane-bound Hp if Fpn1 is present, rather than the soluble ferroxidase alone (used in the current studies). Indirect evidence for an interaction between Hp and Fpn1 arose from studies of the yeast Hp
homologue Fet3p and the iron transporter Ftr1p (Bonaccorsi di Patti et al., 2005; Kwok et al., 2006). Moreover, Fpn1 and Hp co-localize on the basolateral membrane of human intestinal absorptive cells (Han and Kim, 2007). Furthermore, Fpn1 has been shown to export iron both in the presence of Tf, but the absence of ferroxidase (Donovan et al., 2000), and the presence of ferroxidase, but the absence of Tf (McKie et al., 2000). Taken together, these data are consistent with a complex network of interactions between these proteins that remains to be resolved. Further work is required to elucidate the role of each of these proteins in the process of duodenal iron absorption. Additional studies are also required to clarify the localization and function of Hp (see Appendix).
Chapter 4: Studies on the interaction of transferrin iron-release mutants with the soluble transferrin receptor

This work is part of a larger study in collaboration with Dr. Dan Kamei at UCLA. The majority of the results from this chapter are in preparation for publication:


4.1 Rationale

One of the mechanisms by which cells communicate with the extracellular environment is through the directed trafficking of proteins through intracellular pathways (Varga et al., 2000). The cellular iron uptake mechanism is perhaps one of the most well characterized examples of this process (Dunn et al., 2007; Ponka and Lok, 1999; Richardson and Ponka, 1997). Once the diferric-Tf-TfR complex is internalized via receptor-mediated endocytosis, the newly formed endosome can deliver iron to the cytoplasm. This process is complete when the apo-Tf-TfR complex is shuttled back to the outside of the cell. With a turnover of just minutes, this physiological recycling pathway is both specific and efficient (Enns, 2002).

This efficiency has led to the manipulation of the iron uptake pathway as a potential targeted drug delivery system to cancer cells. Multiple studies investigating the cytotoxicity of anticancer agents conjugated to Tf have been reported, using polyethylene glycol, methotrexate, artemisinin and diphtheria toxin (Jiang et al., 2007; Lim et al., 2004; Lai et al., 2005; Johnson et al., 1988). TfR is commonly over expressed on the surface of cancer cells.
as high iron levels are required for rapid cell turnover (Cazzola et al., 1990). The specific targeting of drugs to cancer cells aims to decrease the general toxicity associated with non-specific treatments such as radiation and chemotherapy.

Exploiting a pre-existing physiological system for use as a therapeutic delivery agent against cancer cells is an obvious challenge to scientists. The major problem with developing a targeted drug carrier system using the iron delivery pathway is the rapid recycling of Tf through the endocytic pathway (Lim and Shen, 2004). Accordingly, it has been demonstrated that cytotoxic conjugates are more effective with increased intracellular trafficking (Yazdi et al., 1994; Yazdi and Murphy, 1994; Wenning et al., 1998). Several strategies for engineering Tf modify its normal trafficking pattern such that cellular association is increased (Rao et al., 2005). The most obvious strategy involves altering the binding of the Tf-TfR interaction outside and inside the cell. Unfortunately, this is also the most difficult approach as this binding interaction remains poorly characterized (Lebron et al., 1998; Gianetti et al., 2005). Another approach is to alter the release rate of iron from Tf. Reducing the rate of iron release from diferric-Tf will increase the length of cellular association (Lao et al., 2007).

Mutations in both lobes of rTf result in slowed or inhibited iron release from Tf. A point mutation in the dilysine trigger (K206E) results in the inhibition of iron release from the N-lobe of Tf (Halbrooks et al., 2003). The dilysine trigger is absent from the C-lobe of Tf and believed to be replaced by pH sensitive triad, composed of K534, R632 and D634. Point mutations to alanine (K534A, R632A and D634A) greatly impede iron release from the C-lobe (Halbrooks et al., 2003). Iron release is completely inhibited at pH 7.4 and extremely slow at pH 5.6. Combining the above mutations in both lobes is believed to prevent the
release of iron in a physiologically relevant time frame. Furthermore, it has been shown that replacing the physiological synergistic anion carbonate for oxalate reduces the rate of iron release over 10-fold (Halbrooks et al., 2004).

This study was part of a larger effort in collaboration with Dr. Dan Kamei at UCLA. The research goal for the overall project was to create a more efficient drug carrier by modifying the iron release rate of drug-conjugated rTfs (Lao et al., 2007). In the present study the rate of iron release from Tf was investigated as a factor influencing cellular association. The experimental design is unique as the model investigates the ligand-metal interaction instead of the ligand-receptor interaction as a factor influencing cellular association. As such, it was essential to the model to ensure that changes in the ligand-receptor interaction between the tested rTfs were not affecting the degree of cellular association. SPR was employed to ensure that the selected rTf mutants did not exhibit altered TfR-binding properties compared with wild-type Tf.

4.2 Recombinant transferrin mutants

In this study four rTf proteins with reduced rates of iron release were tested for TfR binding affinity. The strategies for reducing the rates of iron release included replacing the synergistic anion carbonate with oxalate, altering a single lysine in the dilysine trigger of the N-lobe, as well as mutating two residues from the triad of the C-lobe. Each strategy yields Tf with a reduced rate of iron release (Halbrooks et al., 2004; Halbrooks et al., 2003). All rTfs contained an N-terminal poly-His tag to aid purification. Diferric rTf with carbonate as synergistic anion was used as a control for this study. Tested proteins are listed below:

1: diferric rTf with carbonate as synergistic anion
2: diferric rTf with oxalate as synergistic anion
3: diferric rTf K206E with carbonate
4: diferric rTf K206E/R632A with carbonate
5: diferric rTf K206E/K534A with carbonate

4.3 Immobilization

The $K_D$ values for these binding experiments were derived using near-steady state conditions. To this end, binding reactions were allowed to closely approach equilibrium by using slightly longer injection times combined with slower flow rates. Ligands were also immobilized to the chip surface at high densities allowing for a near-equilibrium state to be approached in an appropriate time frame (Figure 4.1 A). The immobilized ligand was monitored for baseline stability ($< 0.05$ RU/min) for over 60 min before initial binding experiment (Figure 4.1 B).

4.4 Binding analysis

SPR was used to confirm that the rTf mutants all exhibited similar receptor binding affinity. Binding isotherms were generated from equilibrium analysis of sensorgrams using the Scrubber software package. All data was globally fitted to a 2:1 binding model. Similar binding response patterns were observed for all rTfs (Figure 4.2). Equilibrium binding analysis was used to verify the binding constants of each mutant to the sTfR.
Figure 4.1. Surface preparation for the Tf-TfR near-steady state binding experiments. A. Immobilization procedure for sTfR on a CM5 chip to a level of ~ 1000 RUs. Steps: a, pre-concentration step; b, EDC/NHS; c, TfR injection; d, ethanolamine. B. Baseline stability test. After immobilization of His-tagged sTfR, the system was monitored for over 60 min to ensure minimal fluctuation (< 0.05 RU/min).
Figure 4.2. SPR analysis of rTf binding to immobilized sTfR. The left panels show the binding profiles of rTf (0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 200, 500 nM) to immobilized sTfR. The right panels show the plots of equilibrium binding response versus the concentration of injected protein derived from biosensor experiments. A, rTf; B, rTf-oxalate; C, rTf K206E; D, rTf K206E/R632A; E, rTf K206E/K534A.
As seen in Figure 4.3, the $K_D$ values calculated from the equilibrium binding response isotherms were 1.54 +/- 0.09 nM for rTf, 2.88 +/- 0.26 nM for rTf-oxalate, 1.44 +/- 0.10 nM for rTf K206E, 0.93 +/- 0.11 nM for rTf K206E/R632A, and 1.12 +/- 0.19 nM for rTf K206E/K534A. It should be noted that diferric rTf with oxalate as synergistic anion bound approximately 2-fold weaker than other mutants. However, from this data it was concluded that all mutants bind the sTfR within the acceptable published range for wild-type Tf ($K_D = 0.5-10$ nM) (Lebron et al., 1998; Dautry-Varsat et al., 1983). The Scrubber analysis of the raw data shows that differences in iron affinity do not cause differences in the receptor binding affinity.

### 4.5 Discussion

SPR was employed to investigate the interaction between recombinant iron-release rTf mutants and the sTfR. The research goal for the overall project was to create a more efficient drug carrier by modifying the rate of iron release in rTfs (Lao et al., 2007). The immediate goal of my research was to confirm that the binding constants of the rTf-sTfR interaction were not affected by the iron-binding mutations. This allowed the Kamei group to test the theoretical model that iron release rates affect cellular association, independent of the affinity of the rTf-sTfR interaction.

The binding interactions between various rTf and sTfR constructs are known (Lebron et al., 1998; West et al., 2000; Gianetti et al., 2005). In the current study, diferric rTf with carbonate as synergistic anion was used as a positive control for the iron release mutants.
**Figure 4.3.** Summary of the $K_{D_1}$ values from the steady-state Tf-TfR interaction. Values were derived from the Scrubber software program. Standard error bars are shown. 1, rTf; 2, rTf-oxalate; 3, rTf K206E; 4, rTf K206E/R632A; 5, rTf K206E/K534A.
All binding constants were within the acceptable range for the Tf-TfR interaction, with oxalate-bound rTf binding 2-fold weaker than the other rTfs. The potential role of oxalate in reducing the binding affinity of the Tf-TfR interaction is not understood.

A consideration of experimental discrepancies is revealed by how the overall experiment was setup. Random orientation immobilization was selected for its ease and convenience. However, an artifact of the random orientation amine coupling strategy is the potential occlusion of one of the sTfR dimer binding sites during immobilization. In this situation, only one binding site of the dimer is free to bind Tf and therefore the primary dissociation constant ($K_{D1}$) is unaffected by immobilization. However, an unknown amount of the second binding site can be largely blocked, therefore the secondary dissociation constant ($K_{D2}$) is misrepresented. The bivalent-ligand model is still used to fit the data, but $K_{D2}$ is simply there to assist a better fit. As such, random orientation immobilization allows for analysis using only $K_{D1}$ in bivalent-ligand model. It should be noted however, that this immobilization strategy has been successfully employed in the past (Lebron et al., 1998; Giannetti et al., 2005). As such, there exists a precedent for evaluating only one $K_D$ in a bivalent ligand system, as in current study.
Chapter 5: **Studies on the interaction of monoferric N-lobe transferrin mutants with the soluble transferrin receptor**

This work is part of a larger study in collaboration with Dr. Anne Mason at the University of Vermont. The majority of the results from this chapter are in preparation for publication: Byrne, S.L.*, Hudson, D.M.*, James, N.G., Smith, V.C., MacGillivray, R.T., and Mason, A.B. (2008). Mutational analysis of the interaction of the N-lobe of transferrin with the transferrin receptor. (in preparation, * joint first authors). I designed and performed all the binding experiments using SPR.

**5.1 Rationale**

There is considerable research characterizing the Tf-TfR interaction. It is well established that diferric Tf binds to the TfR with high affinity, monoferric Tfs bind with moderate affinity, and apo-Tf binds with low affinity (Dautry-Varsat et al., 1983; Mason et al., 1998). As well, the Tf lobes have different binding sites on the TfR (Cheng et al., 2004). The N-lobe alone does not bind to TfR, whereas the C-lobe alone binds with low affinity (Zak et al., 1994). Lastly, it has been determined that high affinity binding requires both lobes of Tf (Zak et al., 1994). Efforts to uncover the specific residues of TfR involved in the interaction have also been examined with mutagenesis and SPR (Giannetti et al., 2003). Perhaps the most significant work is the cryo-EM model of the Tf-TfR complex which provided the first visualization of the regions that contribute to the high affinity binding of the Tf-TfR interaction (Cheng et al., 2004). Yet despite the wealth of research amassed over the last few decades, very little is known about the molecular involvement of the specific Tf residues involved in TfR binding.
The cryo-EM model reveals that the predominant contacts in the complex are found between the TfR helical domain and the Tf C1-subdomain (Cheng et al., 2004). This finding is supported from work done in the Aisen lab, where it was reported that 76% of binding energy from the Tf-TfR complex comes from the C-lobe of Tf (Zak et al., 1994). Together these studies point to the C-lobe as the primary region of Tf involved in the TfR interaction. However, recent studies by the Mason lab indicate that each lobe contributes equally to receptor binding (Mason et al., 2005). They report that each lobe equally contributes 82% of total binding energy in a non-additive manner (Mason et al., 2005), consistent with a greater role for the N-lobe in receptor binding than previously thought. The identities of the N-lobe residues in receptor binding are less certain than residues from the C-lobe in the cryo-EM structure (Cheng et al., 2004). However, residues 71-74 (Tyr-Leu-Ala-Pro) of the N1-subdomain and residues 142-145 (Pro-Arg-Lys-Pro) of the N2-subdomain may be in close enough proximity to the TfR to form potential contacts (Cheng et al., 2004).

The purpose of the present study was to determine the role played by the four amino acids of the Tf N2-subdomain in TfR binding. To this end, each residue was individually mutated to an alanine and binding analysis was performed using SPR. This work was completed in collaboration with Dr. Anne Mason at the University of Vermont. All construct mutations were made by Valerie Smith.

5.2 Recombinant transferrin mutants

Four rTf proteins with mutations to the N2-subdomain were tested for TfR binding affinity. The mutations were based on the cryo-EM model (Cheng et al., 2004). It is well established that the Tf lobes are incapable of binding the TfR in an iron depleted (apo) state.
at physiological pH. Therefore, iron-binding capacity was selectively removed from the lobes of several rTf constructs by replacing the specific iron-binding tyrosine ligands with phenylalanine residues. This study utilized full length rTf mutants with either one or two apo-lobes, which were consequently unable to bind the sTfR at physiological pH. This strategy was utilized to create monoferric N-lobe rTf constructs with the mutations in residues 142-145. Thus, all changes in observed binding affinity could be attributed to the N-lobe mutations. The experimental setup was validated using previously studied rTf constructs, including wild-type diferric rTf, monoferric C-lobe rTf (rTf with capacity to bind iron in C-lobe only), monoferric N-lobe rTf (rTf with capacity to bind iron in N-lobe only), and Apo rTf (rTf with no iron-binding capacity). All rTfs were expressed with an N-terminal poly-His tag to aid in purification. The poly-His tag was cleaved from all rTfs prior to SPR analysis. The tested proteins included:

**Controls**

Diferric rTf

Apo rTf: (Y95F/Y188F/ Y426F/Y517F)

Mono-C rTf: Monoferric C-lobe (Y95F/Y188F)

Mono-N rTf: Monoferric N-lobe (Y426F/Y517F)

**Test samples**

Mono-N-P142A: Monoferric-N rTf (Y426F/Y517F) with P142A mutation

Mono-N-R143A: Monoferric-N rTf (Y426F/Y517F) with R143A mutation

Mono-N-K144A: Monoferric-N rTf (Y426F/Y517F) with K144A mutation

Mono-N-P145A: Monoferric-N rTf (Y426F/Y517F) with P145A mutation
5.3 Immobilization

The kinetic parameters of binding were determined for each rTf construct. Kinetic analysis of binding events using SPR requires a specific experimental setup. Binding reactions were analyzed over shorter injection times with higher flow rates and ligands were immobilized to the chip surface at lower densities. These criteria were designed to minimize any mass transport effects that might affect the kinetics of the binding reactions. The TfR was immobilized using an oriented coupling procedure whereby an anti-His antibody was first coupled to the chip in random orientation to a level of ~2000 RU (Figure 5.1 A). Following this, the His-tagged TfR was applied over the same flow cell to a level between 200-400 RU (Figure 5.1 B). A steady rate of TfR dissociation was observed (~2 RU/min; Figure 5.1 B), which never fully stabilized. However, I found the majority of the TfR remained bound during the course of the experiment. This was compensated for by including blank buffer injections sporadically throughout the dilution series to act as double reference subtractions for the raw data.

5.4 Binding analysis

Binding experiments were performed at a flow rate of 50 µL/min with an association and dissociation phase of 4 min. The binding response sensorgrams were prepared using the Scrubber software (Figure 5.2). Kinetic constants were calculated from sensorgram data using simultaneous fitting of the association and dissociation phases derived from a bivalent ligand model with global fitting to all curves in the working set using CLAMP software (Figure 5.3).
Figure 5.1. Surface preparation for Tf-TfR kinetic binding experiments. A. Immobilization procedure on CM5 chip with an immobilized anti-His antibody level of 3000 RUs. Steps: a, EDC/NHS; b, anti-His antibody injection; c, ethanolamine. B. Baseline stability test. Steps: a, His-tagged sTfR injection; b. steady dissociation was observed for ~ 10 min.
Figure 5.2. SPR analysis of rTf binding to antibody-coupled sTfR. Sensorgram binding profiles of analyte rTf (0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 200, 500 nM) to ligand sTfR. A, diferric rTf; B, Mono-C rTf; C, Mono-N rTf; D, Apo rTf; E, Mono-N-P142A; F, Mono-N-R143A; G Mono-N-K144A; H, Mono-N-P145A.
Figure 5.3. SPR fitting analysis of rTf binding to antibody-coupled sTfR. Experimentally observed responses are shown as black lines with best fit binding curves (red lines) derived from a bivalent ligand model superimposed. Residuals between fits and data are shown above each panel. Selected concentrations from each sensorgram were chosen for analysis A, diferric rTf; B, Mono-C rTf; C, Mono-N rTf; D, Apo rTf; E, Mono-N-P142A; F, Mono-N-R143A; G Mono-N-K144A; H, Mono-N-P145A.
Not every binding curve was used to fit raw data to simulated curves in the CLAMP software. This is because of an artifact in the Biacore system, whereby at high analyte concentrations, the system starts to fail and report irregular values. The exact reason for this is unknown; however, it is common for experimental failure to occur in SPR when concentrations vastly exceeding the $K_D$ are tested (personal communication, Dr. Anthony Giannetti). It should be noted that the quality of the fits as judged by the residuals between the estimation model and data was often found to be outside an acceptable range (see 3 largest responses in Figure 5.3 B). Ideally the sum of the residuals squared is equal to the short term noise of the instrument. As the simulated fit and raw data diverge, the residual squared value increases.

Equilibrium dissociation constants were calculated from association and dissociation rate constants ($K_D = k_{off}/k_{on}$). A summary of the kinetic constants and residual squared values (Res. Ssq) is shown in Table 5.1. The controls aligned reasonably well with previously determined $K_D$ using a similar SPR experimental setup (diferric Tf, 0.68 nM; monoferric C-lobe, 26.41 nM; monoferric N-lobe, 4.31 nM; apo-Tf, 1891 nM; Mason et al., unpublished results). The N-lobe mutations in residues 142 to 144 did not result in any significant change in binding affinity. However, from the current SPR data it appears that mutation P145A resulted in a 9-fold weaker binding affinity compared to the monoferric N-lobe control (Figure 5.4).
Table 5.1. Summary of binding and kinetic constants as interpreted by Scrubber and Clamp software. The residual squared (Res. Ssq) values determine goodness of fit.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$k_{\text{on,1}}/k_{\text{on,2}}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{\text{off,1}}/k_{\text{off,2}}$ (s$^{-1}$)</th>
<th>$K_{D1}$ (nM)</th>
<th>$K_{D2}$ (nM)</th>
<th>Res. Ssq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono-C-rTf</td>
<td>1.10e+06/1.73e+05</td>
<td>9.20e-03/8.39e-03</td>
<td>8.37</td>
<td>48.4</td>
<td>2.88</td>
</tr>
<tr>
<td>Mono-N-rTf</td>
<td>8.11e+05/1.47e+06</td>
<td>0.0103/0.117</td>
<td>12.7</td>
<td>79.6</td>
<td>1.98</td>
</tr>
<tr>
<td>Mono-N-rTf (P142A)</td>
<td>8.67e+05/1.39e+06</td>
<td>8.47e-03/0.105</td>
<td>9.76</td>
<td>75.4</td>
<td>4.45</td>
</tr>
<tr>
<td>Mono-N-rTf (R143A)</td>
<td>1.21e+06/1.50e+06</td>
<td>0.0146/0.120</td>
<td>12.1</td>
<td>79.6</td>
<td>4.28</td>
</tr>
<tr>
<td>Mono-N-rTf (K144A)</td>
<td>7.02e+05/1.77e+06</td>
<td>0.0124/0.189</td>
<td>17.7</td>
<td>108</td>
<td>2.87</td>
</tr>
<tr>
<td>Mono-N-rTf (P145A)</td>
<td>6.59e+05/1.180</td>
<td>0.0735/2.01e-03</td>
<td>112</td>
<td>1700</td>
<td>2.88</td>
</tr>
<tr>
<td>Apo-rTf</td>
<td>1.84e+05/9.270</td>
<td>0.245/9.81e-04</td>
<td>1330</td>
<td>106</td>
<td>1.26</td>
</tr>
<tr>
<td>Diferric-rTf</td>
<td>1.81e+06/2.46e+05</td>
<td>6.55e-04/3.45e-03</td>
<td>0.362</td>
<td>14.0</td>
<td>1.49</td>
</tr>
</tbody>
</table>
**Figure 5.4.** Summary of the $K_{D1}$ values from the kinetic analysis of the Tf-TfR interaction. For clarity the $K_{D1}$ of apo-Tf value was omitted. Standard error bars are shown. 1, Mono-C rTf; 2, Mono-N rTf; 3, Mono-N-P142A; 4, Mono-N-R143A; 5 Mono-N-K144A; 6, Mono-N-P145A; 7, diferric rTf.
5.5 Discussion

The goal of the study was to examine the interaction between recombinant N-lobe Tf mutants and the soluble TfR. The controls used to validate the experimental setup were all within the range of previously published values. Among the tested monoferric rTf mutants, only mutation P145A reduced rTf binding affinity for sTfR. Unfortunately, the residuals between the simulated fits and raw data were often found to be unacceptable (Figure 5.3). While the diferric rTf binding curves fit within the acceptable range for the CLAMP program, all monoferric rTf constructs fit quite poorly to the simulated curves.

The poor fitting of the binding data may be due to complicated binding patterns associated with monoferric rTfs. It is obvious from the EM structure (Cheng et al., 2004) and the extensive mutagenesis analysis of the TfR (Giannetti et al., 2003; Giannetti et al., 2005) that there are multiple sites of contact between Tf and TfR. Accordingly, it is possible that such a complex binding interaction will not fit well to a simple binding model. Complex binding patterns might be the result of weak apo-C-lobe interactions contributing to the diferric-N-lobe interactions with the sTfR.

Another explanation for this fitting discrepancy is the aggregation of monoferric rTfs. A common problem with analyzing monoferric rTfs using SPR is that the system is sensitive enough to detect potential analyte aggregation (personal communication, Dr. Anthony Giannetti). Analyte aggregation is observed if sensorgrams show rapid reversibility but do not return to baseline (personal communication, Dr. Anthony Giannetti); a phenomenon observed in the monoferric sample runs of Figure 5.2. This effect might result from increased binding avidity of the aggregates. Even when the aggregates constitute a small fraction of the total sample, the effects become more dominant at higher concentrations. This
was observed with the raw data of the monoferric rTf binding curves using Scrubber (Figure 5.2).

Solutions to this problem include running high concentrations of the rTf samples through a size exclusion column to remove any existing aggregates. The collected samples should not be concentrated following the chromatography, as this may induce more aggregate formation. Although practical, this procedure required more sample than I had available and was therefore omitted. Some alternative procedures include 0.2 µm syringe filtering the samples (this would also result in sample loss and would only be effective with rather large aggregates). Finally, high-speed centrifugation could be used to remove aggregates with reduced sample loss; again however this procedure is dependent on fairly large aggregates.

The presence of monoferric Tf aggregates complicates analysis of an already complex binding event, as was the case for many of the experiments carried out in this study. Interpretation of simulated fits evaluating the complex kinetic data obtained from SPR is often difficult for any given protein-protein interaction. This is often described as major disadvantage of using SPR, which is currently still in its infancy. However, with further study these complexities should be overcome and new insights gained. Unfortunately, when data fails to fit well to a known model, the mechanistic details behind the discrepancy are often difficult to decipher. Hopefully, further experiments using a simpler experimental model of the interacting proteins (diferric Tf and sTfR) will provide us with the answers to this complicated interaction.
5.6 Limitations

This work requires repetition with additional rTf constructs, due to the potential complications with analyzing the binding patterns of monoferric rTfs. It was decided to make the N2-subdomain mutations (residues 142-145) in diferric rTf. This will simplify the comparative analysis of the mutant binding pattern with the well-established wild-type rTf binding pattern. This work is not included in my thesis due to the lengthy times required for mutagenesis of the new constructs, transfection of the BHK cells, and expression and purification of the new rTf mutants, but it is currently ongoing.
Chapter 6: Summary and general discussion

6.1 Lack of interaction between ferroxidase and iron-carrier

The localization of Hp to the basolateral membrane of duodenal enterocytes (Kuo et al., 2004), is consistent with a role for Hp as the ferroxidase that catalyzes the oxidation of Fe(II) on the serosal side of the enteric epithelium for loading of Fe(III) onto apo-Tf. It has previously been shown in this lab that the ferroxidase activity of Hp promotes the formation of diferric Tf upon incubation of rHp with apo-Tf and Fe(II) (Griffiths et al., 2005). Under identical conditions, incubation of apo-Tf and Fe(II) without Hp resulted in formation of little or no diferric Tf (Griffiths et al., 2005). This finding led to the investigation of a potential interaction between the Hp and Tf. Cp was tested in parallel to complement the study. I was not able to detect an interaction between either Hp or Cp and Tf.

A concern from the beginning of this study was the quality of recombinant Hp. In our lab, ICP MS analysis revealed that rHp contained an average of 3.13 copper atoms/protein molecule rather than the predicted six copper atoms (Griffiths et al., 2005). The ferroxidase activity of the Hp prepared in this manner was rigorously tested and the recombinant protein was shown to be functional (Griffiths et al., 2005). The ferroxidase activity was re-evaluated in this study and again the rHp was determined to be active. As this activity presumably requires the presence of a trinuclear copper site and a minimum of one T1 copper, at least some of the Hp present in samples prepared as described in this thesis must possess a minimum of four copper atoms distributed in this manner. This heterogeneity of sample and potential incomplete activity could alter the folding and structure of the ferroxidase. If so, this might account for the observed lack of interaction. It should be noted
that in this study, no interaction was observed between Tf and plasma derived Cp. Given the potential structural similarity between Hp and Cp, this suggests that the lack of interaction was probably not solely due to folding artifacts in the recombinant ferroxidase.

Two separate studies warrant mention in the discussion of the current research. The first is the extensive characterization of the interaction between human Lf and Cp over the last decade (Zakharova et al., 2000, Sokolov et al., 2005a; Sokolov et al., 2005b; Sokolov et al., 2006; Pulina et al., 2002; Sabatucci et al., 2007). The Lf-Cp complex has been reported \textit{in vitro} and \textit{in vivo}, and was found to increases the ferroxidase activity of Cp (Zakharova et al., 2000; Sokolov et al., 2005a; Sokolov et al., 2006). The Lf-Cp interaction has been proposed to have a predominantly electrostatic binding force, specifically between a positive cluster at the N-terminus of Lf and a negatively charged region at the C-terminus of Cp (Sabatucci et al., 2007). The specific residues of Cp involved in this interaction remain to be elucidated, but given the 50% sequence identity between Cp and Hp, they are likely to be present in both ferroxidases.

Interestingly, a similar study was published immediately prior to the submission of the current work for publication. Paz et al. (2007) reported a novel interaction between a Tf-like iron carrier and a multicopper ferroxidase in the algae \textit{Dunaliella salina}. Many of the same techniques were used in both studies to investigate the presence of an interaction, including native PAGE and chemical cross-linkers. The relevance of these findings to the current work is not certain as plants and humans have very different iron absorption pathways (Paz et al., 2007). Interestingly, aside from \textit{Dunaliella}, the presence of Tf has never been described in plants or related organisms (Paz et al., 2007).
It is clear that functional and properly localized Hp is necessary for iron absorption. This is consistent with the observation that in sla mice the Hp mutant is misdirected to an intracellular location resulting in the development of hypochromic, microcytic anemia (Kuo et al., 2004). Interestingly, the mutant Hp that exists in enterocytes of sla mice still exhibits ferroxidase activity, though at a reduced level (Kuo et al., 2004). This provided the first evidence for alternative potential locations and functions for the ferroxidase protein. This fact in combination with the current lack of interaction study prompted us to re-evaluate the localization of Hp (see Appendix).

6.2 Localization of hephaestin in duodenum

Hp is highly expressed in the duodenum, supporting its role as the intestinal ferroxidase (Vulpe et al., 1999). The need for Hp in duodenal iron absorption is exemplified by uninterrupted dietary iron absorption in patients with aceruloplasminemia (Hellman and Gitlin, 2002). The expression of Hp in other tissues, like the brain, heart and retinal pigment epithelial is more difficult to explain. The current discovery of Hp in the myenteric plexus of duodenum adds to the growing list of novel locations for the membrane-bound ferroxidase (please refer to Appendix). Perhaps the most difficult finding to substantiate is the seemingly redundant requirement for two homologous ferroxidases in many of these locations, such as brain and neuronal tissue. However, the requirement for both ferroxidases in a singular location is not unprecedented. The work by Hahn et al. (2004b) is the first and only study to demonstrate that perhaps Cp and Hp work in concert to facilitate iron export from the retinal pigment epithelium in the CNS. Unlike humans with aceruloplasminemia, Cp knockout mice do not develop severe iron overloading in the CNS. In the mouse model, the absence of both
Cp and Hp are required to detect the same levels of CNS iron overloading as is observed in patients with aceruloplasminemia (Hahn et al., 2004b).

To better understand this requirement, a general overview of brain and neuronal iron metabolism is required. Many parallels exist between systemic and neuronal iron uptake. Iron is essential to the normal functioning of neurons (Rouault and Cooperman, 2006). Neurons require iron for the same purpose as other cells, such as energy production and enzymatic function, but also for specific roles like lipid biosynthesis for the GABA (γ-aminobutyric acid)-ergic system (Thomas and Jankovic, 2004). TfR and DMT1 are highly expressed in neurons, consistent with a role for diferric-Tf in iron absorption similar to that of other cells (Moos, 1996). Like its systemic counterpart, the details of intracellular iron trafficking in neurons remains to be elucidated. Furthermore, very little is known about iron export from the CNS or PNS. It is not clear whether this export process requires the oxidation of Fe(II) by a ferroxidase, such as Cp or Hp (Moos et al., 2007). It is known that Fpn1 and GPI-anchored Cp are expressed in brain neurons, but their specific functions remain unclear (Patel and David, 1997; Wu et al., 2004). Interestingly, the levels of both Tf and Cp in brain are quite low compared with serum levels (Rouault, 2001); this observation is implicit for the requirement for a second ferroxidase, such as Hp. The importance of ferroxidase activity in neurons is highlighted by histological studies on brain tissue from patients with aceruloplasminemia, which reveals excess iron in neurons and glia cells along with neuronal cell death (Morita et al., 1995; Madsen and Gitlin, 2007).

The processes of iron storage are where systemic and neuronal iron metabolic pathways diverge. In most cells, excess iron is taken up by the iron storage protein ferritin. Ferritin is present in the axons of neurons in the brain (Zhang et al., 2005) and retina (Hahn
et al., 2004b). However, an alternative function is anticipated for ferritin in these sites. Due to the relatively low expression levels of ferritin in neurons ferritin might only play a minor role in neuronal iron storage (Moos and Morgan, 2004). In this situation, iron taken in via the TfR pathway would have to be immediately processed for the metabolic needs of the neuron, with excess iron being exported from the cell rather than being stored in ferritin (Moos and Morgan, 2004). It has also been proposed that neuronal ferritin may potentially aid in the intracellular trafficking of iron rather than storage (Rouault and Cooperman, 2006).

It is becoming clear that Hp plays an important role in brain and neuronal tissue. It is possible that with the reduced iron storage capacity in neurons, efficient iron export machinery is required to prevent iron toxicity. This could account for the presence of Hp in tissues outside of the enterocytes. Perhaps the requirement for both Cp and Hp in certain tissues can be viewed as a collaboration rather than a redundancy. As the exact subcellular location of either ferroxidase within many of these tissues including retinal pigment epithelium and myenteric plexus is still unknown, it is possible that the two proteins could have similar functions in slightly different intracellular compartments (Hahn et al., 2004b).

### 6.3 Transferrin interaction with the transferrin receptor

Chapter 3 of my thesis explored the involvement of Tf in duodenal iron absorption through potential protein interactions with the human ferroxidases. In Chapters 4 and 5, I further characterized the well known interaction of Tf with the TfR. These interactions are pivotal steps in the processes of iron absorption and uptake and demonstrate the importance of Tf in iron metabolism.
Interestingly, despite the abundance of research characterizing the binding event between Tf and TfR, very little is known about the interaction at the molecular level. The transport of iron by Tf across plasma membranes via the TfR interaction depends on the effective contribution of a number of amino acid contacts. As such, a sensitive and quantitative assay describing the affinity and the kinetics governing association and dissociation is an essential tool for understanding the function and regulation of these proteins. SPR was employed to assess the protein-protein interaction of Tf and TfR.

In Chapter 4, the effect of Tf iron-binding mutations on the binding interaction between Tf and TfR was investigated. It should be noted that due to the immobilization technique, only binding affinity ($K_{D,i}$) could be obtained for this part of the study. Nevertheless, the SPR results give a reasonable estimate of the near steady state dissociation constant. It was clear from the results that with the possible exception of the oxalate containing protein, none of the Tf iron-binding mutations result in a significant change in TfR binding. The oxalate-containing rTf binds the TfR 2-fold weaker than wild-type Tf. It is not clear how the replacement of oxalate for carbonate as the synergistic anion could have this effect. A 1.2 Å resolution structure of the N-lobe of human serum Tf with oxalate as the synergistic anion revealed no significant changes in protein folding or domain orientation (Halbrooks et al., 2004). This suggests that the differences observed in receptor binding between Tf using carbonate and oxalate as a synergistic anion are not due to structural changes in the binding site of Tf. One explanation for the finding might simply be due to the use of SPR as a highly sensitive technique for studying this interaction. Previous studies may have missed this observation because other techniques used to study the interaction have lacked the sensitivity needed to detect the minor difference. The weaker binding affinity of
oxalate-containing rTf was verified using the oriented coupling procedure and a 2-fold weaker binding affinity was still observed (data not shown). This demonstrates that the observation is not an artifact of the experimental set up. Despite the minor discrepancy between affinities, it is still clear that all the rTf proteins tested in Chapter 4 bound within the acceptable range for the Tf-TfR interaction.

In Chapter 5, the goal was to eliminate or reduce the binding strength of the Tf-TfR interaction. It is generally thought that the Tf N-lobe interacts with the TfR with a lower affinity than the C-lobe (Zak et al., 1994). By eliminating the binding potential of the C-lobe, contributions from the N-lobe were more easily assessed. Residues 142-145 were targeted for mutation based on the cryo-EM structure of the Tf-TfF complex. As discussed in Chapter 5, potential aggregation of the mono-ferric rTf samples limited the complete analysis of the raw data. Despite a lack of quantitative data, it is plausible to speculate qualitatively into the results of the binding analysis. For example, it is apparent that no single mutation in the tested region was solely responsible for the interaction with the receptor. However, from my data I propose that Pro145 has the most significant effect on receptor binding. From the cryo-EM model, the Tf N2-subdomain might interact with several residues from the TfR protease-like domain (Leu122, Tyr123, Trp124, and Asp125) (Cheng et al., 2004). With the TfR contributing binding energy from three nonpolar residues and an acidic residue, the Tf-TfR binding event might be composed of hydrophobic and ionic interactions. Extensive mutagenesis of TfR reveals only one mutation in protease domain (Tyr123) that results in a significantly weakened Tf interaction (Giannetti et al., 2003).

The contribution from the N1 subdomain must also be considered. The cryo-EM model predicts an interaction between residues 71-74 of Tf and two helices of the TfR helical
domain (Cheng et al., 2004). This potential site of interaction is probably having a distal effect on the overall binding kinetics of the interaction. However, changes in binding kinetics due to mutations in the N2 subdomain should still be apparent regardless of contributions from the N1-subdomain.

One final consideration in this analysis is the use of sTfR. It is obvious from the cryo-EM model that the TfR stalk potentially forms contacts with the N-lobe (Cheng et al., 2004). This may have an altering effect in vivo from my observations with sTfR. It is difficult to reconcile the potential binding energy lost with the use of sTfR in these binding experiments. Perhaps in vivo cell binding studies could corroborate the data obtained from the current in vitro assays.

6.4 Significance of the work

It is becoming more and more apparent that iron homeostasis is essential to human health. This is demonstrated by disorders resulting from iron overload and iron deficiency, including Alzheimer’s disease, Parkinson’s disease, age-related macular degeneration, and anemia. Recently Brookes et al., (2006) proposed that a reduction in Hp activity directly correlates with an increase in colorectal cancer. It is now becoming increasingly clear that copper and iron metabolism are intimately linked as revealed by the anemia suffered by individuals with aceruloplasminemia (Harris et al., 1995), and the sla mouse (Vulpe et al., 1999). Moreover, the absence of both Hp and Cp in mouse leads to severe iron overloading in the retinal pigment epithelium (Hahn et al., 2004b).

The current study further characterizes the role played by Hp in intestinal iron export as well as identified a novel location of Hp in the enteric nervous system (see Appendix). As
novel locations for Hp continue to be reported, these studies should open the way for further functional studies that can define the roles and potential interactions of human Hp with other proteins involved in iron homeostasis, such as Fpn1.

The immediate benefits of the development of a targeted drug delivery system are palpable. Eliminating or reducing the side effects of non-specific cancer chemotherapy is a long term goal in oncology. The use of Tf as an effective drug delivery tool has been tested in human in clinical trials (Weaver and Laske, 2003). The current work is directed at improving the cellular delivery and drug efficacy of conjugated-Tf. Once in vitro studies are completed, the next step will be to test the model in vivo and eventually move on to a clinical trial.

Understanding the function of Tf in iron absorption, delivery and uptake on a molecular level is a major aim in studies on iron homeostasis. The characterization of individual residues of Tf involved in receptor binding provides insight into the iron uptake process. Resolving this complex molecular interaction will contribute to our overall understanding of iron homeostasis.

6.5 Future Directions

The present study investigates the general role played by human Hp and Tf in iron transport across plasma membranes. In addressing current unknowns in the literature, further questions arose that require answers. As such, several aspects of my studies remain to be resolved.

In Chapter 3, I demonstrated that Hp and Tf do not interact. This is an observation that I assume to be consistent with the events of intestinal iron absorption. This work could
be modified to include Fpn1. It is possible that Tf is only capable of recognizing membrane-bound Hp if Fpn1 is present. Once a clone of Fpn1 is obtained, this work will be made easier by cloning various extracellular regions of Fpn1 and investigating potential interactions with Hp and Tf.

In Chapters 4 and 5, further work needs to be completed by our collaborators. Yet further Tf constructs that are to be designed for enhanced drug delivery can easily be tested for receptor binding using our SPR experimental assay developed in this study. As alluded to in Chapter 5, much work remains to be resolved in this study. The current study is to be completed using the new constructs (mutations in residues 142-145 in diferric rTf). In addition, residues from different regions of Tf are to be targeted. The model generated by Cheng et al. (2004) revealed areas in the C-lobe as well as the N1 subdomain. Constructs with mutations in residues 71-74 (Tyr-Leu-Ala-Pro) of the N1-subdomain have been generated and are currently being expressed by our collaborators. The residues in the C-lobe positioned close to the helical domain of TfR are His349, Arg352, Leu353, Asp356, Glu357, Ser359, Val360, Glu367, Glu369, Ser370, and Glu372. These mutations will be individually addressed. Combinations of mutations will also be examined once more information is obtained into the potential roles played by the individual residues.

In the Appendix, I revealed the presence of Hp in the myenteric plexus of the enteric nervous system. This finding adds to the growing list of novel locations for Hp, outside the realm of intestinal iron absorption. Much work is needed to determine the function of both Hp and Cp as players in the CNS and PNS. It would also be of interest to test sla mice with the current polyclonal antibody (α-rHp) in concert with the commercially available α-Hp.
Lastly, a long-term goal of our lab is to obtain a crystal structure of Hp. To this end, our laboratory is currently developing an expression system in yeast that should result in greater yields of expressed protein. Crystallization screens for subsequent structure determination of Hp will be done in collaboration with Dr. Natalie Strynadka’s laboratory (Department of Biochemistry and Molecular Biology, University of British Columbia). The blue color of Hp should facilitate crystal identification during the screens. A structural model of Hp will complement the existing structures for human Cp (Zaitseva et al., 1996) and *Saccharomyces cerevisiae* Fet3p (Taylor et al., 2005).
References


Appendix: Localization of hephaestin in the myenteric plexus of the human gastrointestinal tract

The majority of the results from the appendix are in preparation for publication. I completed all IHC experiments. Valerie Smith provided guidance with the IHC experiments and Dr. Alison Buchan supplied the tissue and provided advice in experimental design and analysis.

A.1 Rationale

Hp acts as a transmembrane multicopper ferroxidase and has been implicated in dietary iron absorption (Chen et al., 2004; Griffiths et al., 2005). However, the lack of interaction between Hp and Tf in iron absorption prompted me to re-investigate the localization of the ferroxidase in the human gastrointestinal tract (Hudson et al., 2008). Initial immunofluorescence studies localized Hp to the duodenal enterocytes of rat, mouse and human (Frazer et al., 2001; Kuo et al., 2004; Brookes et al., 2006). Unfortunately, the antibodies used in these studies recognize an epitope distinct from the functional portion of the protein, which is situated opposite the membrane. The localization of the human Hp ectodomain within cells and tissues remains to be resolved. Furthermore, although it is generally agreed that Hp is orientated extracellularly, several studies have localized Hp within the intracellular environment (Simovich et al., 2002; Kuo et al., 2004). This introduces the possibility for unknown and novel functions for the ferroxidase.

In this study, a polyclonal antibody against the soluble human rHp was generated in rabbits. Localization of the complete functional domain of human Hp was carried out using immunochemistry techniques in various paraffin-embedded human gastrointestinal tissues. The tissue preparations included full thickness sections of human duodenum, jejunum, ileum
and colon. The localization of Hp from the current study is consistent with a potentially unidentified function for the multicopper ferroxidase outside the realm of duodenal iron absorption.

A.2 Polyclonal antibody production

To date, our laboratory is the only group to have expressed a recombinant, soluble form of human Hp (Griffiths et al., 2005). Using this recombinant protein, a polyclonal antibody against rHp (α-rHp) was generated in rabbits. Rabbit serum containing the α-rHp (called antiserum) was isolated for these studies. To our knowledge, this is the first polyclonal antibody that specifically recognizes the Hp ectodomain. Antibody specificity and titer were verified using western blot analysis (Figure A.1). The possibility that the polyclonal antibodies might be dominated by antibodies recognizing the highly antigenic 1D4 fusion tag was addressed. The antibody was tested for reactivity against the rHp ectodomain by removing the 1D4 tag via a factor Xa cleavage site upstream of the tag (Figure A.1). The α-rHp was highly specific and had a high affinity as it could be diluted 1:50,000 and could detect 90 ng of rHp via western blot analysis (Figure A.2).

A.3 Tissue distribution and localization

Prepared slides of paraffin-embedded and Bouin’s fixed tissue section were kindly provided by Dr. Alison Buchan. The tissue preparations included full thickness sections of human duodenum, jejunum, ileum, colon and stomach, all of which had been stored for 10 to 25 years.
Figure A.1. Western blot analysis of rabbit antiserum (α-rHp). Antiserum specificity to rHp ectodomain was determined by testing reactivity before and after the 1D4 antigenic tag was removed with FXa (1 μg for 30 min). Lanes 1 and 3, rHp; lanes 2 and 4, rHp treated with FXa. Each lane contained 150 ng of rHp. Antiserum was used at a titer of 1:10,000 to determine antiserum specificity; anti-1D4 was used at a titer of 1:500 to confirm 1D4-tag cleavage.
Figure A.2. Determination of α-rHp antibody titer using western blot analysis. Western blots containing purified rHp (90 ng) were probed with decreasing concentrations of rabbit antiserum (α-rHp). Lane 1, 1:20,000; lane 2, 1:40,000; lane 3, 1:50,000.
Tissue sections were processed for IHC, however the age and quality of the tissue sections prevented the use of fluorescence detection; therefore, the secondary antibody for all IHC was stained with a DAB substrate.

The rabbit antiserum was tested using standard IHC techniques to localize Hp in various human tissues. The results obtained from this preliminary study confirmed the presence of Hp in the human duodenal sections. In accord with Hp gene expression (Vulpe., et 1999) and previous IHC studies in rat and mouse (Frazer et al., 2001; Kuo et al., 2004), Hp was localized to the basolateral region in the mature human villi duodenal enterocytes, with no staining in the crypts (10X magnification) (Figure A.3 A). Unfortunately, due to the age and quality of the tissue samples, increased magnification failed to reveal greater detail, as the resolution was found to decrease with increased magnification. However, staining occurred not only along the basolateral membrane of the human enterocytes, but also within the intracellular milieu of the basolateral region (Figure A.3 B). Controls using blocked α-rHp (antiserum pre-incubated with purified rHp) and preimmune serum confirmed the specificity of the α-rHp and the lack of pre-existing specific antibodies in the rabbit serum, respectively (Figure A.3 C, D).

Staining for Hp was observed in each human tissue section tested, except for stomach (Figure A.4). The stomach sample served as a negative control since Hp is not expressed in stomach (Vulpe et al., 1999). The localization of Hp in jejunum, ileum and colon closely resemble that of the duodenum with staining mostly occurring at the basolateral region of the villi cells. This finding is novel in that Hp protein has never before been detected in all these tissues in human.
Figure A.3. Immunolocalization of Hp in duodenum tissue. Sections of human duodenum were processed for IHC using the α-rHp antibody and detected with ABC peroxidase conjugated secondary antibody. Hp staining is found predominantly in the basolateral region in the mature villus enterocytes (labels, L:lumen; V:villi; C:crypts; LP: lamina propria; arrows indicate positive staining); A.10X magnification; label B. 40X magnification; C. blocked α-rHp (antiserum pre-incubated with purified rHp at 10X magnification); D. preimmune serum, 10X magnification.
Figure A.4. Immunolocalization of Hp in the gastrointestinal tract. Sections of human tissue were processed as in Figure A.3 and viewed at 10X magnification (arrows indicate positive staining). A. Jejunum; B. Ileum; C. Colon; D. Stomach.
Due to the decreased resolution, it was difficult to determine the exact location of α-rHp staining within the colon tissue; however, Hp has previously been localized to the basolateral region of human colonocytes (Brookes et al., 2006). A similar pattern for protein localization and gene expression was previously reported in rat gastrointestinal tissue (Frazer et al., 2001).

A.4 Novel location and potential function

A novel location was also uncovered for the ferroxidase in the enteric nervous system. Hp-specific staining was detected in the myenteric plexus of duodenum tissue (Figure A.5 A). The enteric nervous system consists of three major ganglionated plexuses: the myenteric plexus, the submucous plexus and the mucous plexus. The myenteric plexus is located between the outer longitudinal and the circular muscle layers. At 40X magnification the staining revealed that Hp was not simply localized to the plasma membrane of the nerve bundles but also to the intracellular compartment (Figure A.5 B). The blocked α-rHp and preimmune serum controls confirmed the location of Hp within the nerve bundles (Figure A.5 C, D).

A.5 Discussion

A novel ectodomain-specific antibody was used to investigate the localization of Hp in the human gastrointestinal tract. Hp has previously been localized to the duodenum and colon of human tissue (Vulpe et al., 1999; Brookes et al., 2006); however, identification of protein in human jejunum and ileum has not been reported previously.
Figure A.5. Immunolocalization of Hp in myenteric plexus. Sections were processed as in Figure A.3. Arrows indicate positive staining in myenteric plexus. A. 10X magnification; B. 40X magnification; C. blocked α-rHp (antiserum pre-incubated with purified rHp at 10X magnification); D. preimmune serum 10X magnification.
These findings are not entirely unexpected as protein and gene expression of Hp was previously reported along the entire small and large intestine of rat (Frazer et al., 2001). Furthermore, it has recently been suggested that iron absorption occurs along the entire small and large intestine (Brookes et al., 2006).

I also detected Hp in the myenteric plexus of duodenal tissue. At the time of this finding, it was unclear as to the role of Hp in the enteric nervous system. Interestingly, Cp is expressed in astrocytes and neurons of the CNS (Patel and David, 1997; Jeong and David, 2006). This could set the precedent for the current neuronal location of Hp, which was predicted to have a singular role in intestinal iron absorption. Furthermore, Cp expressed in brain is predominantly GPI-anchored. This introduces another similarity between the normally soluble Cp and the membrane-bound Hp. In contrast to Hp, Cp does not have a transmembrane spanning domain, but is only anchored by the GPI addition (Patel and David, 1997). The exact role of GPI-anchored Cp in the CNS is not well understood, but it is believed to function in either neuronal iron efflux or influx (Qian and Ke, 2001). Hypothetically, Hp could function in a similar fashion.

The enteric nervous system plays important roles in gastrointestinal function including motility, blood flow and exocrine and endocrine secretions (Hansen, 2003a, b). Neurons contain large cell bodies that are susceptible to free radical damage as a result of their elevated metabolic rates and poor antioxidant defense (Rouault, 2001). An increased need for iron regulation in neurons could explain the requirement for multiple membrane-bound ferroxidases. The disorder aceruloplasminemia exemplifies the importance of Cp in the CNS, as it results in iron overload in the brain and visceral organs as well as an iron
decrease in the serum (Miyajima H, 2003; Madsen and Gitlin, 2007). This may also suggest a role for Hp in a similar or complementary process.

Hp was first reported to have a function in addition to iron absorption when Hp was localized to the Muller glia and the retinal pigment epithelium and shown to play a critical role in CNS iron homeostasis (Hahn et al., 2004b). Thus, mice deficient in both Hp and Cp are prone to age-dependent retinal neurodegeneration (Hahn et al., 2004b). As mentioned above, 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 may be due to differences in expression regulation between the ferroxidases. It has been shown that both Cp (Mukhopadhyay et al., 2000) and Fpn1 (Hahn et al., 2004a) are up-regulated 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 (Anderson et al., 2002a; Anderson et al., 2002b) or brain (Qian et al., 2007b), but instead maintains a steady expression level. The constitutive expression of Hp may serve as a “safeguard” against the potential damaging effects caused by iron overload.

It is evident that more research is required to explain possible novel functions of Hp. In the last few years, Hp has been localized to the retinal pigment epithelial of the mouse CNS (Hahn et al., 2004b) as well as various regions of rat heart and brain (Qian et al., 2007a; Qian et al., 2007b; Wang et al., 2007). It is becoming quite clear that the membrane-bound ferroxidase, Hp, plays a role in iron metabolism well outside the realm of intestinal iron absorption.
A.6 Limitations

Due to a shortage of available tissue, further IHC studies were not possible. To complement the results obtained from the stored tissue, ethical approval for the acquisition of human gastro-intestinal tissue was obtained from the UBC clinical research ethics board in November 2007 (UBC CREB number H07-00244). Following UBC approval, ethical approval from the Vancouver Coastal Health Authority was obtained in March 2008 to conduct research at Vancouver General Hospital (VGH). We are currently awaiting fresh duodenal samples to complete this study.

The attending surgeon at VGH will be Dr. Charles Scudamore. After obtaining informed consent, full thickness human tissue samples will be collected from five subjects as a by product of the surgical procedure known as the Whipple operation. In this procedure the duodenum, the gallbladder and portions of the pancreas and bile duct are surgically removed. In some cases, a portion of the stomach may also be removed. This procedure is commonly required for pancreatic cancer or cancer of the small intestine. The extra tissue obtained from these surgeries will allow us to perform the proper controls to validate our current findings. Tissue samples will be fixed with a 4% (v/v) solution of paraformaldehyde. To ensure maximum antigen exposure, the tissues will be sectioned using two approaches: paraffin-embedded wax sections and cryostat-frozen sections. All tissue preparation will be carried out at VGH Department of Pathology under the supervision of Dr. David Owen. I would like to repeat the current IHC analysis with this new tissue and test for the location of several other iron metabolism proteins using a suite of commercially available antibodies. The antibodies to be tested include α-Fp1, α-Cp, α-TfR, α-DMT1 and α-Hp. The α-Hp results could prove most interesting as it and the α-rHp antibody recognize distinct regions on Hp:
the cytoplasmic tail and the ectodomain, respectively. Unfortunately, these new samples will not be available until after submission of the thesis.