INTESTINE

Mislocalisation of hephaestin, a multicopper ferroxidase involved in basolateral intestinal iron transport, in the sex linked anaemia mouse

Y M Kuo, T Su, H Chen, Z Attieh, B A Syed, A T McKie, G J Anderson, J Gitschier, C D Vulpe

See end of article for authors' affiliations

Correspondence to: Assistant Professor C D Vulpe, Department of Nutrition and Toxicology, University of California, Berkeley, 119 Morgan Hall, Berkeley, CA 94720, USA; vulpe@uclink4.berkeley.edu

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Background: Hephaestin is a multicopper ferroxidase required for basolateral transport of iron from enterocytes. Sex linked anaemia (*sla*) mice have a defect in the release of iron from intestinal enterocytes into the circulation due to an interstitial deletion in the hephaestin gene (*heph*).
Results: We have demonstrated that hephaestin is primarily localised to a supranuclear compartment in both intestinal enterocytes and in cultured cells. In normal intestinal enterocytes, hephaestin was also present on the basolateral surface. In *sla* mice, hephaestin was present only in the supranuclear compartment. In contrast, the iron permease Ireg1 localised to the basolateral membrane in both control and *sla* mice.
Conclusion: We suggest that mislocalisation of hephaestin likely contributes to the functional defect in *sla* intestinal epithelium.

Intestinal absorption of iron is a critical step for regulating whole body iron levels because mammals possess a limited capacity to excrete excess iron.[1] Although iron is crucial for the function of many proteins, the ability of iron to catalyse the formation of reactive oxygen species can contribute to pathological damage.[2] Inherited disturbances of both cellular and whole body iron homeostasis illustrate the critical need to maintain a balance between deficiency and excess of this metal.[3] Within mammalian cells, elaborate regulatory mechanisms exist to regulate iron entry, intracellular sequestration, and mobilisation.[4–6] Recent identification of key components of intestinal iron transport has revealed new insights into molecular mechanisms regulating whole body iron homeostasis.[6]

One such component is hephaestin (Hp), a membrane bound homologue of ceruloplasmin (Cp) that is required for iron egress from the enterocyte. Previously, we discovered this novel protein as defective in mice with sex linked anaemia (*sla*).[7] Apical uptake of iron in enterocytes of *sla* mice is normal but basolateral export is decreased, leading to iron accumulation in enterocytes.[8 9] *sla* mice contain an inframe deletion of 582 bases in the *Heph* gene resulting in a truncated protein.[10] Hp has an oxidase activity like Cp[11] which may facilitate iron export from the intestine to plasma[7 12] where it is found bound as Fe(III) to transferrin. Hp may work in concert with a basolaterally located Fe (II) permease, Ireg1 (also known as ferroportin or MTP1),[13–15] in the export of iron from the enterocytes.[16] For example, oxidation to Fe (III) by Hp may directly assist in the release of iron from Ireg1 or indirectly by maintaining an Fe(II) gradient. The activities of both Cp[17–19] and the *S cerevisiae* multicopper ferroxidase Fet3p[20 21] are crucial for iron transport. Interestingly, a functional Fet3p is required for proper plasma membrane localisation of the yeast high affinity iron importer Ftr1p.[22]

Abbreviations: Hp, hephaestin; Cp, ceruloplasmin; *sla*, sex linked anaemia; Tfr, transferrin receptor; DAB, 3,3′-diaminobenzidine

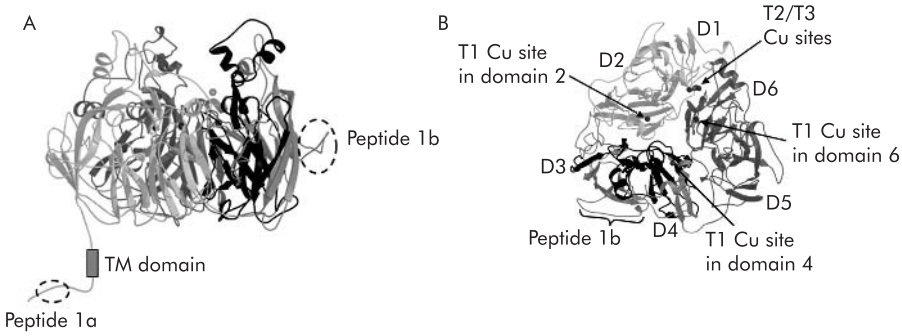

Figure 1 Location of peptide epitopes on hephaestin (Hp) and deleted region in sex linked anaemia (*sla*) mice. A ribbon diagram of a molecular model of mouse Hp is presented. (A) Side view of the mouse Hp molecule almost perpendicular to the pseudo-3-fold axis. Location of the two peptide epitopes (Hp peptide 1a and Hp peptide 1b) are indicated. The region deleted in *sla* is shown in black. (B) Top view along the pseudo-3-fold axis showing the predicted domain structure of Hp with copper binding sites indicated, the Hp1b peptide epitope, and the region deleted in *sla* in dark black. The figures were generated using a modified version of Molscript[24] and subsequently rendered in Raster3D version 2.0.[25 26]

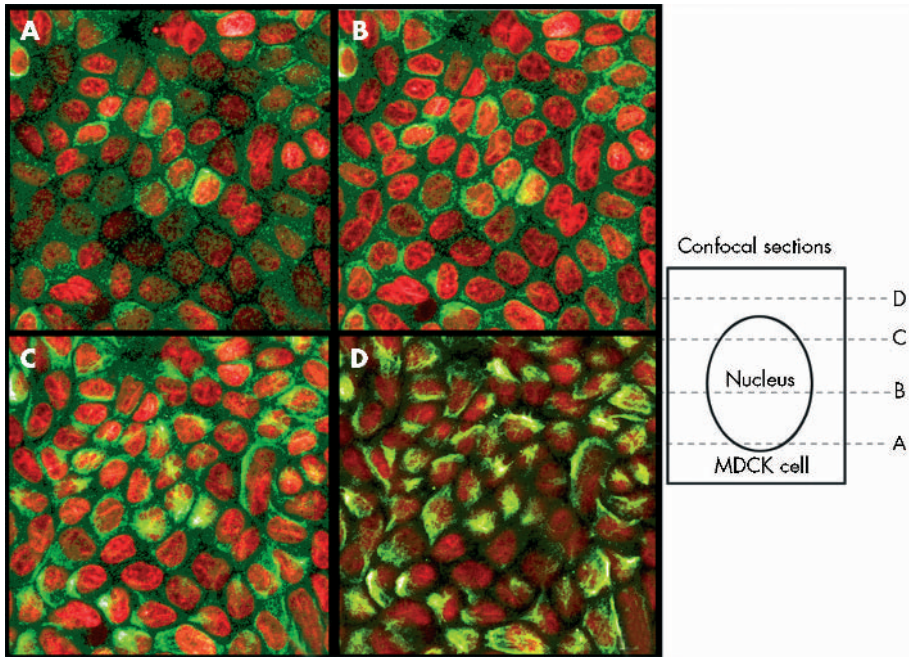

Figure 2 Apical supranuclear location of hephaestin (Hp) in MDCK cells. Confocal immunofluorescence microscopy was carried out using an affinity purified antiserum to the C terminus of Hp in cultured MDCK cells. Hp staining is represented in green and nuclei are in red. Sequential confocal reconstructions from the bottom (A) to the top (D) of the cell showed predominantly apical perinuclear staining of Hp.

Here we show that Hp is located primarily in apical supranuclear and basolateral membranes in mature intestinal enterocytes. The truncated version of Hp in the *sla* mouse is detectable only in a supranuclear intracellular compartment. Targeting of Ireg1 is not affected in the *sla* mouse and the predicted basolateral location was seen. We suggest that improper localisation of Hp contributes to the defect in basolateral iron transport in *sla* mice.

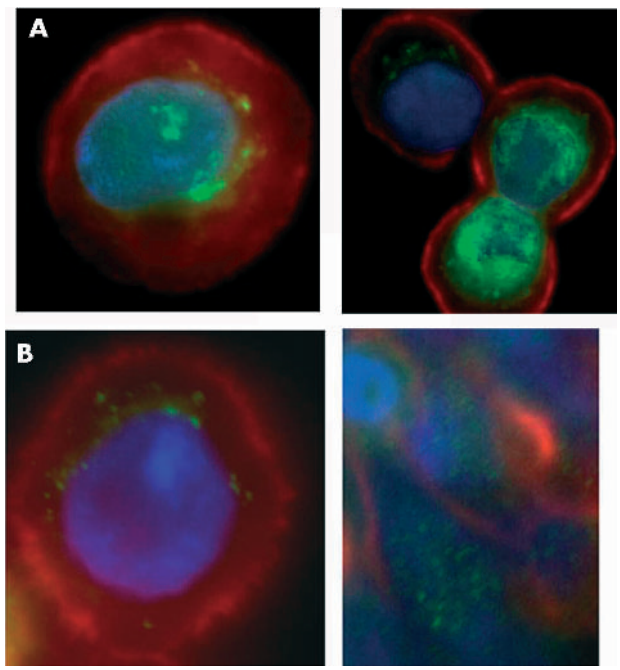

Figure 3 Apical supranuclear location of hephaestin (Hp) in HT29 cells. (A) Immunofluorescence microscopy using an affinity purified antiserum to the C terminus of Hp (Hp1A) in cultured HT29 cells. (B) Immunofluorescence microscopy using an affinity purified antiserum to the more N terminal part of Hp (Hp2a) in cultured HT29 cells. A single cell is shown in panels to the left and several cells are shown in the right hand panels. Hp staining is shown in green, actin in red, and nuclei in blue.

METHODS

Antisera to hephaestin and Ireg1

We used two affinity purified anti-Hp peptide antisera which we previously demonstrated to be specific for Hp from multiple species with no cross hybridisation to Cp.[10] Hp, in contrast with Cp, has a C terminal transmembrane domain and presumed cytosolic domain (fig 1). One antiserum, Hp1a, was raised against a peptide corresponding to the C terminal 15 amino acids (QHRQRKLRRNRRSIL), predicted to be on the cytoplasmic surface of the membrane. The second antiserum, Hp1b, was raised against a peptide corresponding to amino acids 435–52 (AFQDETFQERVHQEEETH) of mouse Hp (Genbank accession: AAD16035). This sequence resides within Hp domain 2,[23] predicted by homology modelling to be located on a solvent accessible portion of the Hp protein.[23] The *sla* mouse contains deletion of 192 amino acids (shown in black in fig 1) which does not include the regions to which the antisera were raised. Affinity purified rabbit anti-Ireg1 (CGKQLTSPKDTEPKPLEGTH) antiserum was made using the same protocol as above. We have previously demonstrated the specificity of this antiserum for Ireg1.[10]

Cell immunostaining

MDCK cells were grown on Transwell filters (Costar-Corning Life Sciences, Acton, Massachusetts, USA) and Cos7 and HT29 cells were grown on LabTekII chamber slides (Nalge Nunc International, Rochester, New York, USA) in Dulbecco's modified Eagle's medium. Non-differentiated HT29 cells were grown in McCoy's medium until 70% confluency. Cell media was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin cocktail. Cells were fixed for 20 minutes in 4% paraformaldehyde. Cells were made permeable with 0.25% saponin (Sigma, St Louis, Missouri, USA), 0.7% bovine serum albumin (Sigma), and 0.7% normal

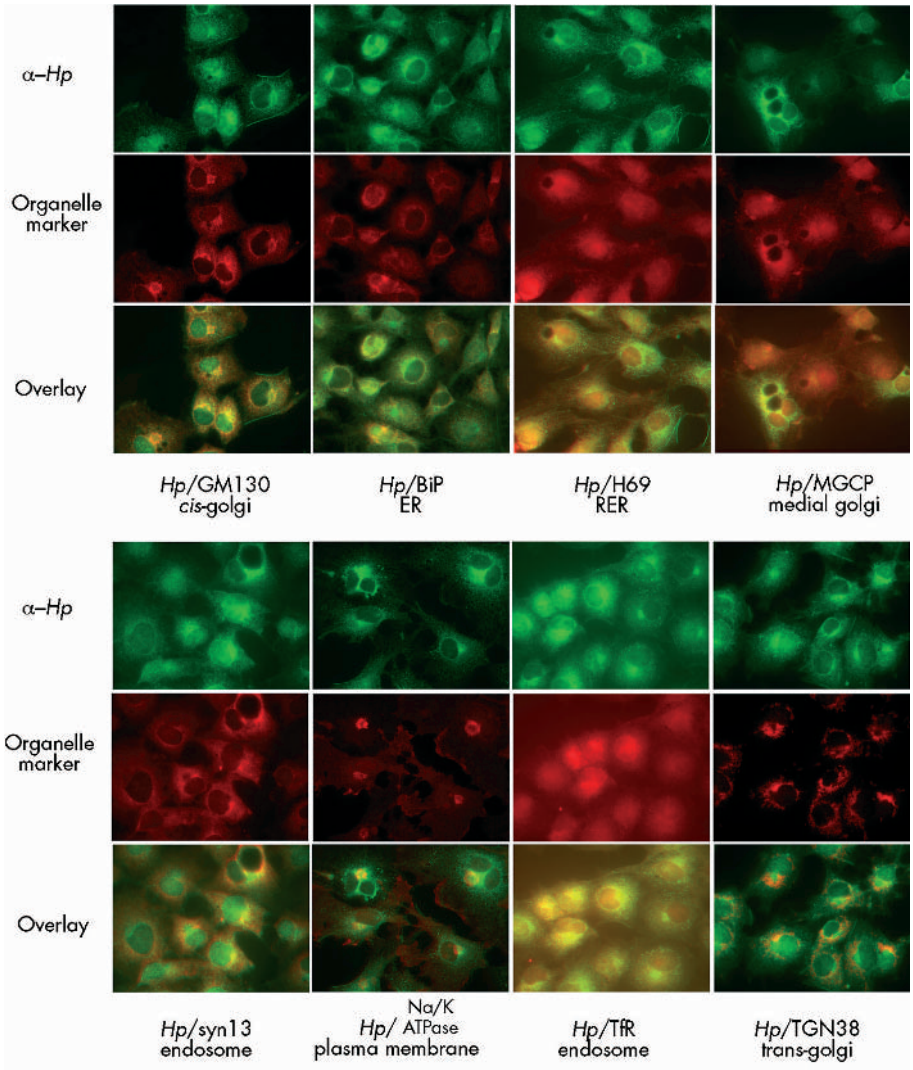

Figure 4 Colocalisation of hephaestin (Hp) with subcellular markers. Colocalisation of affinity purified antibodies to the C terminus of Hp (Hp1A) and antibodies to various subcellular markers in cultured CoS7 cells. Hp staining is represented in green and the markers (TfR, TGN38, GM130, BiP, H69, Na,K-ATPase, MGCP, and syntaxin 13) are shown in red. Yellow in the overlays indicates colocalisation. ER, endoplasmic reticulum; RER, rough endoplasmic reticulum; Tfr, transferrin receptor.

goat serum (Cappel-MP Biomedicals Inc., Irvine, California, USA). Primary and secondary antibodies were diluted in 1% normal goat serum, 0.7% bovine serum albumin, and 0.1% saponin. The rabbit antimouse Hp antibody 1a (1:200) was visualised using a goat AlexaFluor488 antirabbit IgG antibody (molecular probes and nuclei visualised with propidium iodide; Molecular Probes, Eugene, Oregon, USA) or 4′-6-diamidino-2-phenylindole (Molecular Probes) staining. Co-immunolocalisation studies involved the following antisera: rat antimouse-CD71 (transferrin receptor (Tfr)) for endosomes (Serotec, Raleigh, North Carolina, USA); mouse anti-TGN38 for trans- Golgi network (Transduction Laboratories, BD Biosciences, San Jose, California, USA); mouse anti-GM130 for cis-Golgi (Transduction Laboratories); mouse anti-BiP/GRP78 for endoplasmic reticulum (Transduction Laboratories); rat anti-H69 for rough endoplasmic reticulum (Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA); mouse anti-Na^+,K^+-ATPase for plasma membrane (Developmental Studies Hybridoma Bank); rat anti-ABL70 for medial Golgi cisternae (Developmental Studies Hybridoma Bank); and mouse antisyntaxin 13 for endosomes (Stressgen, San Diego, California, USA). Alexa 594 labelled antirat or antimouse IgG (Molecular Probes) were used as secondary antisera. F-actin of HT29 cells was stained with 1:4000 rhodamine phalloidin (Molecular Probes, R-415) for one hour at room temperature. Cells were examined at 100× magnification on a Nikon E800 microscope and images captured using a Spot II digital camera. Confocal images were visualised and captured as a Z series using a Bio-Rad confocal microscope.

Immunostaining for Hp and Ireg1 in mouse duodenal sections

The *sla* mice were originally obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and maintained on a C57BL/6J background at the Queensland Institute of Medical Research and University of California, Berkeley (California, USA) for nine years prior to this study. Mouse duodenum from C57BL/6J or *sla* mice were isolated and fixed for 12–14 hours in Bouins fixative (Sigma), organs were washed in 70% ethanol, dehydrated, embedded in paraffin, and sectioned (8μM) as previously described.[27] Sections were immunostained using standard procedures with the affinity purified antiserum to the C terminus of Hp (1a) or Ireg1at

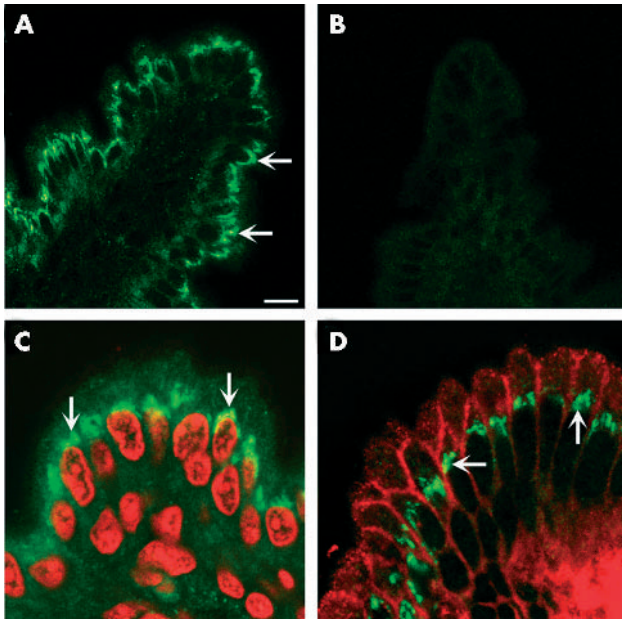

Figure 5 Predominant supranuclear location of hephaestin (Hp) in duodenal enterocytes. Immunofluorescent staining of duodenal sections from C57BL/6J mice on control diets using an antiserum to the C terminus of Hp demonstrated predominantly supranuclear staining. (A) Hp alone (green). (B) Preincubation with Hp peptide. (C) Propidium iodide staining of nucleus (in red) and Hp staining (in green). (D) Immunolocalisation of Hp (green) and Na^+,K^+-ATPase (red).

1:200 dilution and enhanced with ABC Elite Vector Stain Substrate Kit (Vector Laboratories, Burlingame, California, USA) using the manufacture's protocol. Staining was visualised with 3,3′-diaminobenzidine (DAB substrate kit; Vector Laboratories) and counterstained with Gill's haematoxylin No 2 (Polysciences Inc., Warrington, Pennsylvania, USA) including the nickel solution for Hp staining. For peptide blocking studies, anti-Hp1a 1:200 was preadsorbed

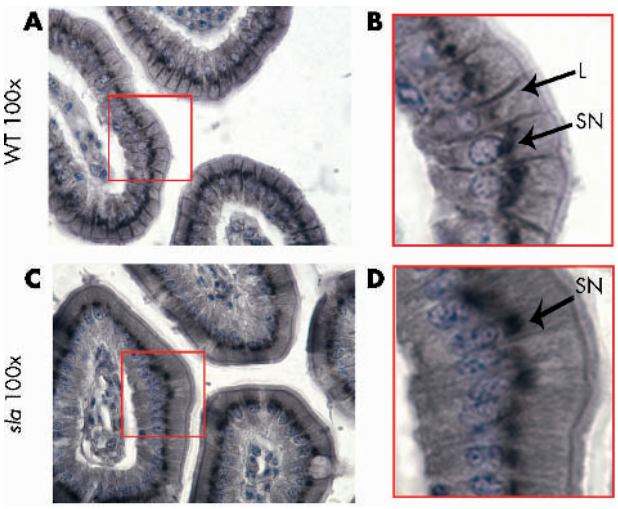

Figure 6 Absence of basolateral hephaestin (Hp) on duodenal enterocytes from sex linked anaemia (*sla*) mice. 3,3′-Diaminobenzidine immunohistochemistry of duodenal sections using an antiserum to the C terminus of Hp. (A) 100× of C57BL/6J (WT) duodenal sections; (B) 3× magnification of boxed area in (A). Arrows indicate lateral (L) and supranuclear (SN) staining. (C) 100× of *sla* duodenal sections; (D) 3× magnification of boxed area in (C). Arrows indicate supranuclear (SN) staining. There was no appreciable lateral staining.

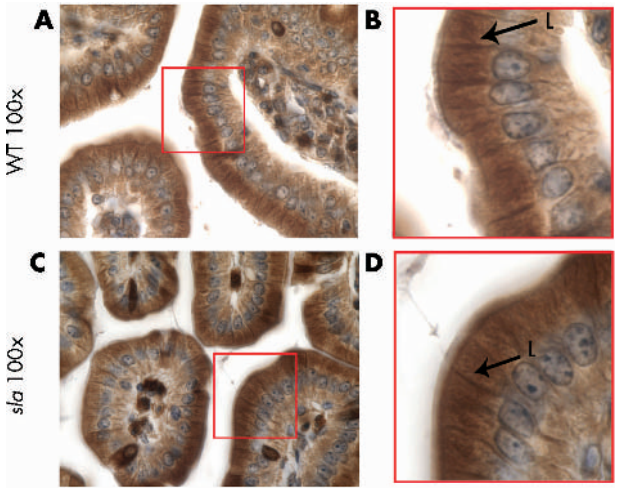

Figure 7 Basolateral Ireg1 in duodenal enterocytes from *sla* mice. 3,3′-Diaminobenzidine immunohistochemistry of duodenal sections using an antiserum to Ireg1. (A) 100× of C57BL/6J (WT) duodenal sections; (B) 3× magnification of boxed area in (A). Arrows indicate lateral (L) staining. (C) 100× of *sla* duodenal sections; (D) 3× magnification of boxed area in (C).

with 10^{-5}M peptide (the same peptide to which the antiserum was raised) for 48 hours at 4°C and then used as above. Co-immunolocalisation with mouse anti-Na^+,K^+-ATPase was as described above for cell culture. Sections were examined using a Nikon E800 microscope and images captured using a Spot II digital camera.

RESULTS

Predominantly supranuclear localisation of hephaestin in cultured cells

Immunofluorescence studies with affinity purified polyclonal antisera to Hp in the cultured canine kidney (MDCK) and intestinal (HT29) cell lines showed a predominantly supranuclear distribution under standard culture conditions (figs 2 and 3). Confocal sections of the MDCK cells (fig 2) showed clear supranuclear staining (in green) with the antiserum to the C terminus of Hp. Similarly, supranuclear staining was seen in HT29 cells grown in standard culture conditions with antisera to both the C terminal and N terminal regions of Hp. We therefore concluded that Hp is primarily localised in a supranuclear location in cultured cells.

Colocalisation studies suggest recycling endosome and/or cis-Golgi location of Hp

To identify the subcellular compartment containing Hp, we carried out colocalisation studies with a series of antisera to organelle specific marker proteins, as detailed in the methods section above. Cos7 cells were used because they express abundant Hp and have a large cytoplasm/nucleus ratio. These studies suggest that Hp colocalises with the recycling endosome compartment identified by Tfr (fig 4). There was also considerable colocalisation with the cis-Golgi compartment identified by the GM130 marker (fig 4).

Supranuclear and basolateral location of hephaestin in enterocytes

We investigated the cellular localisation of Hp in C57BL/6J mice. Immunofluorescence studies showed an apical supranuclear signal in duodenal enterocytes (fig 5A, C) which was blocked by the immunising peptide or was absent in sera adsorbed with the immunising peptide (fig 5B). Given the

role of Hp in the basolateral export of iron, we conducted colocalisation studies using antibodies to both Hp and the basolateral marker Na^+,K^+-ATPase. Surprisingly, these studies showed little overlap between the two proteins (fig 5D). We conclude that the majority of Hp protein is located in an apical supranuclear compartment in C57BL/6J mice.

Although immunofluorescence studies using fresh frozen and PFA fixed tissues provide an excellent means of detecting the predominant location of a protein, secondary less abundant populations of protein may be overlooked. We therefore used paraffin embedded intestinal sections and staining with avidin-biotin complex horseradish peroxidase followed by DAB staining. As seen in fig 6A and B, this approach revealed that Hp was present on the lateral surfaces of the enterocytes in C57BL/6J mice as well as abundant in an apical supranuclear location.

Hephaestin in *sla* mice is primarily intracellular

In marked contrast, Hp expression in *sla* mice was confined to the supranuclear location with no appreciable basolateral staining (fig 6C, D). The absence of Hp in the basolateral membrane may contribute to the decreased transfer of iron into the circulation and contribute to the iron deficiency in *sla* mice.

Ireg1 in *sla* mice located on the basolateral membrane

To address whether inappropriate localisation of Hp in *sla* leads to changes in Ireg1 targeting, we immunolocalised Ireg1 in intestinal enterocytes of C57Bl6/J type and *sla* mice (fig 7). We found Ireg1 on the basolateral surfaces of enterocytes in both control and mutant mice. We conclude that inappropriate localisation of Hp does not affect the location of the Ireg1 protein.

DISCUSSION

We have proposed that Hp ferroxidase activity may be necessary for effective release of iron following transport through the basolateral membrane by the iron transporter Ireg1.[7] Our finding of Hp on or near the basolateral membrane of the intestinal enterocyte provides support for this hypothesis. However, the predominant apical supranuclear location in multiple cultured cell lines and mature enterocytes suggests intracellular trafficking of Hp, an additional intracellular function, or both. The exact cellular compartment remains uncertain as immunolocalised Hp did not completely colocalise with any one organelle marker. Considerable overlap with the transferrin receptor (Tfr) suggests the recycling endosome but cis-Golgi remains a possibility, or both locations. Hp is predicted to be a multicopper ferroxidase based on sequence similarity with Cp and conservation of structural features.[23] Copper is assembled into Cp in the Golgi[28] and it is reasonable to suggest copper is also incorporated into Hp at this point. Detection of Hp in Golgi may reflect this assembly process. Localisation in recycling endosomes is more difficult to explain and suggests that Hp, like Tfr, may cycle between the basolateral membrane and the recycling endosome, with the bulk of the protein at any one time in the endosome. Alternatively, the supranuclear compartment may not represent the recycling endosome or cis-Golgi but a distinct compartment of unknown function. The functional role of Hp, if any, in this location is not known, particularly as Ireg1 is not found in this location.

Despite an inframe interstitial deletion removing 192 amino acids, *sla* mice still produce a truncated Hp protein.[10] In *sla* mice, Hp is located exclusively (within the limits of our detection) within a supranuclear compartment. Absence of Hp from the basolateral membrane in *sla* mice, and thus its unavailability for interaction with the basolaterally located Ireg1, may contribute to the observed iron transport defects in *sla*. For example, Ireg1 may have diminished efflux capacity in the absence of Hp. The supranuclear localisation of Hp in *sla* could result from constitutive inappropriate localisation of the truncated Hp protein. The mutant protein may not fold appropriately leading to retention in the supranuclear region or the region deleted in *sla* could contain necessary basolateral targeting information. In contrast with the Fet3p/Ftr1p complex in yeast where the proper localisation of Ftr1p (the Ireg1 functional orthologue) on the plasma membrane depends on a functional Fet3p (the hephaestin-like molecule),[22] the basolateral location of Ireg1 in *sla* suggests that proper targeting of Ireg1 is not dependent on Hp.

ACKNOWLEDGEMENTS

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Authors' affiliations

Y M Kuo, J Gitschier, Departments of Medicine and Pediatrics and Howard Hughes Medical Institute, University of California, San Francisco, CA 94143-0794, USA
T Su, H Chen, Z Attieh, C D Vulpe, Department of Nutritional Sciences and Toxicology, University of California, Berkeley, CA 94720-3104, USA
B A Syed, Metalloprotein Research Group, The Randall Centre, New Hunts House, Kings College London, SE1 9RT, UK
A T McKie, Department of Molecular Medicine, King's College, London SE5 9NU, UK
G J Anderson, Queensland Institute of Medical Research and the University of Queensland, Post Office Royal Brisbane Hospital, Brisbane, Queensland 4029, Australia

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GI SNAPSHOT

Answer

From question on page 161

Although anticoagulation for cerebral sinus thrombosis remains controversial, a recent Cochrane review concluded that anticoagulation was safe and associated with a potentially important reduction in the risk of death or dependency. In this case, following discussion with neurologists, she was anticoagulated for a four month period. Her vision slowly improved but did not return to normal. No prothrombotic state other than her inflammatory bowel disease (IBD) and her being on the pill could be identified. The pill was stopped and she was counselled on different methods of contraception.

In IBD, histological and haematological studies suggest that a hypercoaguable state is involved in the pathogenesis of venous thrombosis. The exact mechanism is unknown but is bound to be multifactorial. During acute flare up there are increases in factor VIII, fibrinogen, platelets, and factor V, and a decrease in antithrombin III, all of which may contribute. Thrombosis in IBD is important because it occurs in young patients, often in unusual sites such as cerebral venous sinuses, and is associated with significant morbidity and mortality. The majority of patients in a flare up will be in a hypercoaguable state and therefore young patients should be treated aggressively with rehydration therapy, prophylactic heparin, as well as conventional treatment for their IBD to try and minimise the risk of thrombosis.