An Iron-Regulated Ferric Reductase Associated with the Absorption of Dietary Iron

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The ability of intestinal mucosa to absorb dietary ferric iron is attributed to the presence of a brush-border membrane reductase activity that displays adaptive responses to iron status. We have isolated a complementary DNA, Dcytb (for duodenal cytochrome b), which encoded a putative plasma membrane di-heme protein in mouse duodenal mucosa. Dcytb shared between 45 and 50% similarity to the cytochrome b561 family of plasma membrane reductases, was highly expressed in the brush-border membrane of duodenal enterocytes, and induced ferric reductase activity when expressed in *Xenopus* oocytes and cultured cells. Duodenal expression levels of Dcytb messenger RNA and protein were regulated by changes in physiological modulators of iron absorption. Thus, Dcytb provides an important element in the iron absorption pathway.

Iron is essential for a large number of biological processes ranging from O_2 transport to DNA synthesis and electron transport. Therefore, iron acquisition is a fundamental requirement in almost all living organisms. At physiological pH and in the presence of oxygen, iron exists predominantly in the highly insoluble ferric Fe(III) form, whereas iron transport systems take up the ferrous Fe(II) ion, which is very unstable and quickly oxidizes to ferric iron. To overcome this problem, specialized transmembrane electron transport systems have evolved, known collectively as ferric or ferric-chelate reductases. These redox systems use intracellular reducing cofactors to reduce ferric Fe(III) to the ferrous Fe(II) form at the extracellular surface, thus allowing the cell to take up the ferrous iron. A number of genes encoding plasma membrane ferric reductases have been cloned and characterized in yeast (*1*), plants (*2*), and bacteria (*3*), but none have so far been described in mammalian systems.

In mammals, iron is taken up by the proximal small intestinal epithelium, primarily the duodenum, where it is known that ferrous iron is more efficiently absorbed than ferric iron. A divalent cation transporter (DCT1), also known as Nramp2 and DMT1 (*4–6*), has now been shown to be responsible for the uptake of ferrous iron from the lumen into the mucosa. However, because most dietary nonheme iron is in form of ferric iron complexes, these must be reduced to yield ferrous ions before iron can be successfully transported by DCT1. A brush-border surface ferric reductase enzymic activity has been demonstrated, both in the duodenal mucosa itself and in cultured intestinal cells (*7, 8*). The enzyme has been partially purified and is associated with heme containing b-type cytochrome (*9*). By using a subtractive cloning strategy designed to identify intestinal genes involved in iron absorption, we isolated a previously unidentified gene encoding a cytochrome b–like molecule, which we named Dcytb (for duodenal cytochrome b) (Fig. 1) (*10, 11*).

Dcytb Is a Homolog of Cytochrome b561

A BLAST search (*12*) of the SwissProt database revealed that the predicted protein sequence of Dcytb was most similar to sheep cytochrome b561 (accession number p49447) [41% identical, 54% similar (over a 218–amino acid region)]. Weak homology was also found to mitochondrial cytochrome b from various species. Interestingly, no significant homology was found between Dcytb and previously described ferric reductases from plant or yeast origin. The NH_2-terminal region of Dcytb was virtually identical to that of a hemoprotein or cytochrome b558, called p30 (SwissProt accession number G546819), isolated from rabbit neutrophil plasma membranes (*13*). No full-length protein or cDNA sequence for p30 has been reported. p30 is known to be 30 kD in size, but the physiological function of the molecule is not known. An alignment of Dcytb and p30 revealed that there was only one change in 20 residues (Fig. 1), a Gly (GGN) to Val (GUN) at position 6. Therefore, p30 is either an isoform or the rabbit homolog of Dcytb. The sizes of b561, p30, and Dcytb proteins are similar, and both b561 and p30 are thought to be di-heme proteins and reside in the plasma membrane. Cytochrome b561 functions as a transmembrane electron shuttle between the cytoplasm, where ascorbate acts as a reducing cofactor, and the inside of chromaffin granules, where the electron is accepted by semidehydroascorbic acid (*14*). It has been suggested that His pairs 48 and 118 and 84 and 157 of cytochrome b561 are potential heme ligands (*14*). An alignment of Dcytb with b561 sequences from a number of species revealed that these His residues were conserved in Dcytb, suggesting that Dcytb may also bind two heme groups (Fig. 1). Cytochrome b561 and Dcytb share a similar membrane topology, with six predicted transmembrane spanning regions (Fig. 1). Putative binding sites for the cytochrome b561 substrates [ascorbic acid and semidehydroascorbic acid (*14*)] were found to be partially conserved in Dcytb (Fig. 1, boxed regions), suggesting that Dcytb might react with one or more of these compounds.

Regulation of Dcytb by Iron Status

Iron-deficiency anemia and chronic anemia caused by ineffective erythropoiesis are common disorders of iron metabolism affecting Western populations and are potent stimulators of iron absorption. Homozygous hpx mice (which lack circulating transferrin) develop chronic anemia because of a failure to mobilize iron to the erythron (*15*). Compared to heterozygotes, homozygous hpx mice have an expanded erythron and greatly increased iron absorption (*16*). By using Northern and Western blotting (*17, 18*), we confirmed that Dcytb protein and mRNA levels were highly up-regulated in the duodenal mucosa from homozygous hpx mice, as compared with the heterozygotes (Fig. 2, A and D), indicating that chronic anemia up-regulated Dcytb levels. Iron deficiency induced by feeding mice an iron-deficient diet also strongly increased Dcytb mRNA levels and protein expression in duodenal extracts (Fig. 2, B and C) (*17*). Three major transcripts of ~1, ~4, and >5 kb in size were detectable by Northern blot analysis (Fig. 2C, arrows), indicative of alternate splicing or the presence of unprocessed pre-mRNA species. Relative expression of Dcytb mRNA in other tissues from mice fed a normal diet was lower (Fig. 2C). We did not test the effect of iron deficiency in all other tissues; however, there was no

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detectable effect of iron deficiency on mRNA levels in ileal extracts (Fig. 2D).

Exposure to hypoxia (0.5 atm) for 1 to 3 days is another stimulator of iron absorption (and ferric reductase), which acts independently of body iron stores (*19*). Dcytb protein and mRNA levels both increased in the hypoxic mouse duodenum (Fig. 2, B and D). The effect of hypoxia on Dcytb expression was less marked in the ileum (Fig. 2D), as is the case for intestinal ferric reductase activity (*7*). As a control, we analyzed how levels of

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                  1                                                50   TM 1                               TM 2    100
Human Dcytb                               MA MEGYWRFLAL LGSALLVGFL SVIFALVWVL HYREGLGWDG  SALEFNWHP VLMVTGFVFI QGIAIIVYRL
Mouse Dcytb             .......... .......... ..MA.MEGYRGFLGL LVSALLVGFL SVIFVLIWVL HFREGLGWNG .SGLEFNWHP VLAVTGFVFI QGIAIIVYRL
Rabbit P30                                       MEGYRGFLVL LVSALLVGFL
Bovine b561       MWRTLTCTLT AGGSAASSRL SMEGPASPAR APGALPYYVA FSQL..LGLI VVAMTGAWLG MYRGGIAW.E .SALQFNVHP LCMIIGLVFL QGDALLVYRV
Ovine b561        .......... .......... .MEGPASPAP APGALPYYVA FSQL..LGLT VVAMTGAWLG MYRGGIAW.E .SALQFNVHP LCMVIGLVFL QGDALLVYRV
Porcine b561      .......... .......... .MESPAGRTP APGALPYYVA FSQL..LGLT VVAVTGAWLG AYRGGIAW.E .SALQFNVHP LCMIIGLVFL QGDALLVYRV
Human b561        .......... .......... .MEGGAA.AA TPTALPYYVA FSQL..LGLT LVAMTGAWLG LYRGGIAW.E .SDLQFNAHP LCMVIGLIFL QGNALLVYRV
Mouse b561        .......... .......... .....MEHSS ASVLLHCRTM WPSPSCLGLT VVAVTGAWLG LYRGGIAW.E .SSLQFNVHP LCMVIGMIFL QGDALLVYRV
Xenopus b561      .......... .......... ..MENALSSQ NLGFMPYLVA GSQI..LGIA NLAITGAWLA QLQRRFLW.S .GPLQFNVHP LCMVLGMVFL CGEALLVYRV
C.elegans b561    .......... ......MSLL FDPGFVILRE DQSVKLFNII LVMSQVFGGL AVLLVTIWMS KFESGFAWNE DPDKEFNYHP TFMIMGMVFL FGEALLVYRV

                  101                  TM 3                        150         TM 4                               200
Human Dcytb       PWTWKCSKLL MKSIHAGLNA VAAILAIISV VAVFENH         NVNNI ANMYSLHSWV GLIAVICYLL QLLSGFSVFL LPWAPLSLRA FLMPIHVYSG
Mouse Dcytb       PWTWKCSKLL MKSIHAGLNA VAAILAIISV VAVFEYH... .....NVQKV PHMYSLHSWV GLTALILYIQ QLVVGFFVFL LPWAPPSLRA IVMPIHVYSG
Bovine b561       ..FRNEAKRT TKVLHGLLHV FAFVIALVGL VAVFEHH... .....RKKGY ADLYSLHSWC GILVFALFFA QWLVGFSFFL FPGASFSLRS RYRPQHVFFG
Ovine b561        ..FRNEAKRT TKVLHGLLHV FAFVIALVGL VAVFEHH... .....RKKGY ADLYSLHSWC GILVFALFFA QWLVGFSFFL FPGASFSLRS RYRPQHVFFG
Porcine b561      ..FRNEAKRT TKILHGLLHV LAFVIALVGL VAVFDYH... .....RKKGI ADLYSLHSWC GILVFVLFLA QWLVGLGFFL FPGASFSLRS RYRPQHVFFG
Human b561        ..FRNEAKRT TKVLHGLLHI FALVIALVGL VAVFDYH... .....RKKGY ADLYSLHSWC GILVFVLYFV QWLVGFSFFL FPGASFSLRS RYRPQHIFFG
Mouse b561        ..FRREAKRT TKILHGLLHV FAFIIALVGL VAVFDYH... .....KKKGY ADLYSLHSWC GILVFVLYFV QWLVGFSFFL FPGASFSLRS RYRPQHIFFG
Xenopus b561      ..FRHETKRS TKILHGVLHI MALVISLVGV IAVFQYH... .....QANGY PDMYSLHSWC GIVTFTLYIL QWIIGFSLFF IPGVAFTYRS QFKPLHEFFG
C.elegans b561    ..FRNERKKF SKTLHVILHS CVLVFMLMAL KAVFDYHNLH KDPSGNPAPI VNLVSLHSWI GLSVVILYFA QYIVGFITYF FPGMPIPIRQ LVMPFHQMFG

                  201   TM 5                                  TM 6      250                                           300
Human Dcytb       IVIFGTVIAT ALMGLTEKLI FSLR DPAYS TFPPEGVFVN TLGLLILVFG ALIFWIVTRP QWKRPKEPNS TILHPNGGTE QGARGSMPAY SGNNMDKSDS
Mouse Dcytb       LLLFGTVIAT VLMGVTEKLF FVLK.RPSYH SFPPEGVFTN TLGLLILVFG ALIFWIVTRP QWKRPREPGS VPLQLNGGNA DRMEGAIAIS SAHSMDAADA
Bovine b561       AAIFLLSVAT ALLGLKEALL FELG..TKYS MFEPEGVLAN VLGLLLATFA TVILYILTRA DWKRPLQAEE QALSMD.... .......... .FKTLTEGDS
Ovine b561        AAIFLLSVAT ALLGLKEALL FELG..TKYS TFEPEGVLAN VLGLLLAAFA TVVLYILTRA DWKRPLQAEE QALSMD.... .......... .FKTLTEGDS
Porcine b561      AAIFLLSVGT ALLGLKEALL FQLG..TKYS AFESEGVLAN VLGLLLVAFG AVVLYILTRA DWKRPLQAEE QALSMD.... .......... .FKTLTEGDS
Human b561        ATIFLLPVGT ALLGLKEALL FNLG..GKYS AFEPEGVLAN VLGLLLACFG GAVLYILTRA DWKRPSQAEE QALSMD.... .......... .FKTLRQGDS
Mouse b561        ATIFLFSAGH SLLGLKEALL FKLG..SKYS TFEPEGVVAN VLGLLLVCFG VVVLYILAQA ALERGSQAEE QALSMD.... .......... .FKTLTEGDS
Xenopus b561      RALFLSSIAT SLLGLTEKMF ......SEYS SHPAEGILVN SLGVLLVVFG AVIAYILTRE DWRRPPLPEE QALSMD.... .......... .FKTLTEGDS
C.elegans b561    VLIFIFVSIT VAMGISERAA WKHTCWTKEG QMCAQQATSS FVGVFTFLYT VCVLLLVLNP RWKRQSLPEE EGLHHLTSSH SMSD

                  301                   324
Human Dcytb       ELNSEVAARK RNLALDEAGQ RSTM
Mouse Dcytb       ESSSEGAARK RTLGLADSGQ RSTM
Bovine b561       PSSQ...... .......... ....
Ovine b561        PSSQ...... .......... ....
Porcine b561      PSSQ...... .......... ....
Human b561        PGSQ...... .......... ....
Mouse b561        PTSQ...... .......... ....
Xenopus b561      PTDQ...... .......... ....
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Fig. 1. Multiple sequence alignment of the mouse and human Dcytb with the NH_2-terminus of the rabbit hemoprotein cytochrome b558 (p30) and seven cytochrome b561 sequences from different species. The sequence data for the cytochromes were obtained from the SwissProt database, and their alignment with Dcytb was determined with the program PileUp (GCG, Madison, Wisconsin). Sequences are shown in single-letter code (*28*) with the six predicted transmembrane domains (TM1 through TM6) highlighted in blue. The four conserved His residues proposed as heme ligands are highlighted in red, and the regions of b561 thought to be related to substrate binding or recognition are boxed.

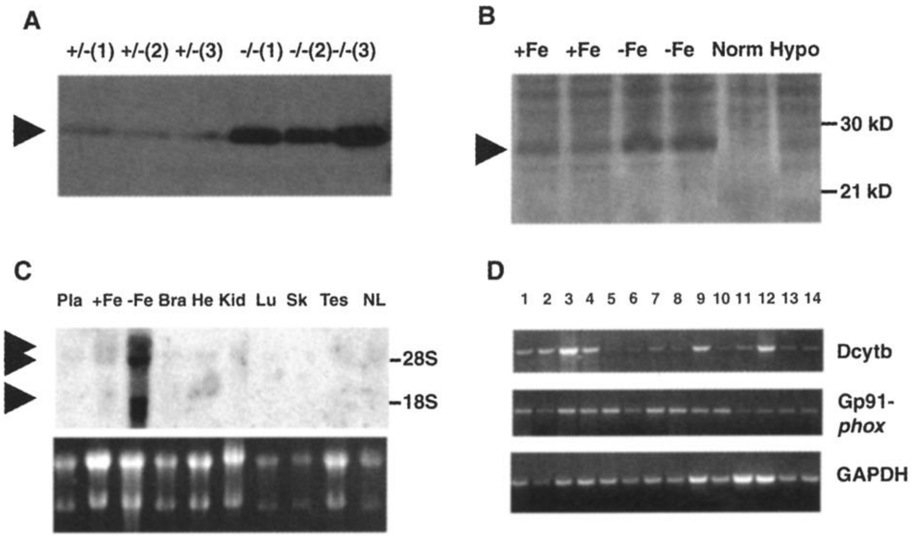

Fig. 2. Expression and regulation of Dcytb in mouse tissues. (**A**) Western blot analysis of Dcytb protein levels in duodenal extracts from three individual hpx mice (right three lanes), compared with three individual heterozygotes (left three lanes). (**B**) Western blot analysis of duodenal extracts from two individual iron-replete mice (+Fe), two individual iron-deficient mice (–Fe), one individual mouse fed a normal diet (Norm), and one mouse fed a normal diet but exposed to 24 hours of hypoxia (Hypo). Ten micrograms of protein was loaded in each lane in (A) and (B). (**C**) Northern blot analysis showing Dcytb mRNA expression in the following mouse tissues: Pla, placenta; +Fe, iron-replete duodenum; –Fe, iron-deficient duodenum; Bra, whole brain; He, heart; Kid, kidney; Lu, lung; Sk, skeletal muscle; Tes, testis; and NL, neonatal liver. Arrows indicate the three main transcripts of Dcytb, and the positions of the 18*S* and 28*S* ribosomal RNA bands are indicated. Lower panel shows the ethidium bromide–stained RNA gel before blotting. (**D**) Reverse transcription–PCR analysis of the effect of hypoxia, hypotransferrinemia, and iron deficiency on Dcytb, gp91-*phox*, and GAPDH mRNA expression in mouse intestine. Lanes 1 through 4 show duodenal extracts from mice fed a normal diet: lane 1, no hypoxia; lanes 2, 3, and 4, mice exposed to 6, 24, and 72 hours of hypoxia, respectively. Lanes 5 through 8 show ileal extracts from mice fed a normal diet: lane 5, no hypoxia; lanes 6, 7, and 8, mice exposed to 6, 24, and 72 hours of hypoxia, respectively. Lanes 9 and 10 show duodenal extracts from homozygous and heterozygous hpx mice, respectively. Lanes 11 and 12 show duodenal extracts from mice fed an iron-replete diet and an iron-deficient diet, respectively. Lanes 13 and 14 show ileal extracts from mice fed an iron-replete diet and an iron-deficient diet, respectively.

another mammalian membrane reductase, the neutrophil oxidoreductase gp91-*phox*, was regulated in the intestine by changing iron status (Fig. 2D). gp91-*phox* is not thought to reduce ferric iron complexes, despite homology to the yeast ferric reductases FRE1 and FRE2 (*20*). In contrast to Dcytb mRNA, which showed a marked gradient of expression between normal duodenal and ileal tissues, gp91-*phox* mRNA levels were constant. In addition, there was no increase in duodenal expression of gp91-*phox* mRNA in mice exposed to hypoxia (Fig. 2D), in hypotransferrinemic mice (Fig. 2D), or in mice with iron deficiency (Fig. 2D). Thus, the expression and regulation of Dcytb by iron status in the intestine is similar to the ferric reductase activity measured in intestinal fragments (*7*), as would be expected for a protein involved in iron acquisition.

Dcytb Is Located in the Brush Border and Functions as a Ferric Reductase

We next investigated whether Dcytb protein was present in an appropriate subcellular location to have a role in reduction of dietary ferric iron (*18*). In the duodenum of iron-deficient mice, Dcytb mRNA was mostly localized in the mature enterocytes of the upper villus region, with the crypt cells being negative for Dcytb (Fig. 3A). The upper villus region, populated largely by mature enterocytes, is the site of the highest iron-transporting activity (*21*). Dcytb protein was highly expressed in brush-border membrane vesicles prepared from the duodenal mucosa but not from those prepared from ileal mucosa (Fig. 3B). This is consistent with the decreased reductase activity associated with this region of the intestine (*7*). Immunostaining for Dcytb mouse duodenal mucosa (Fig. 3C) provided further evidence that Dcytb was present in the brush-border membrane, although some cytoplasmic staining was observed within enterocytes. No staining was present in the crypts, and expression in the brush border of enterocytes was visible along the entire length of villi from the crypt-villus junction to the villus tip. Staining was more intense in sections from mice fed an iron-deficient diet. This staining pattern is similar to that observed with DCT1 in iron-deficient rat duodenum (*22*). Thus, Dcytb was in the region of the intestine most active in the absorption of dietary iron (duodenum) and at the appropriate subcellular location (brush-border membrane) to have a role in reduction of dietary iron.

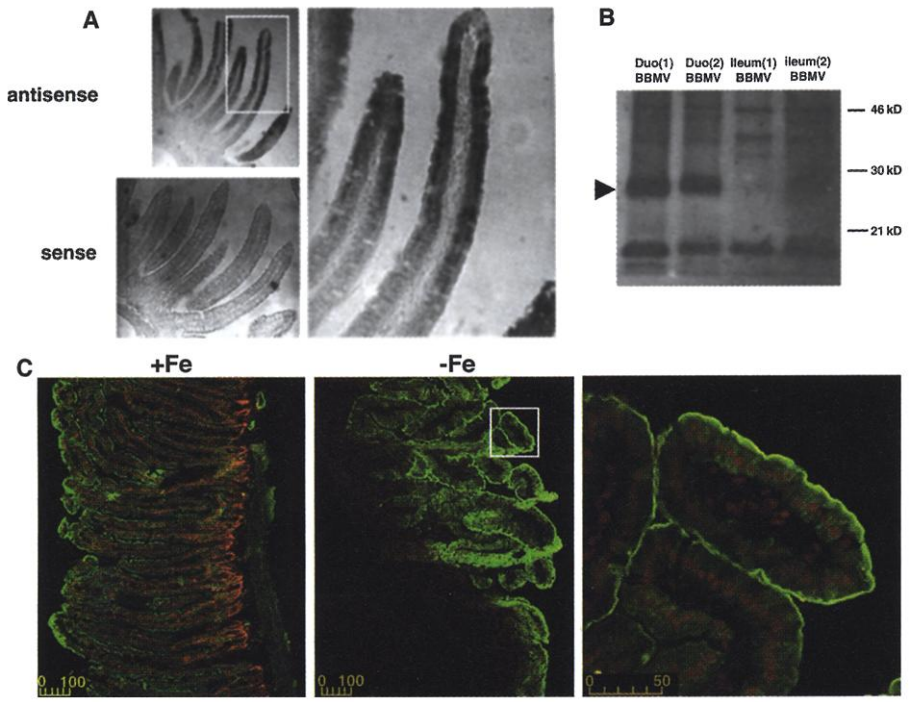

Fig. 3. Dcytb localizes to the brush-border membrane. (**A**) In situ hybridization with sense and antisense probes for Dcytb on sections from a wild-type CD-1 mouse fed an iron-deficient diet for 4 weeks. The right panel shows the antisense image at a higher power magnification. (**B**) Western blot analysis of brush-border membrane vesicles. Expression of Dcytb in brush-border membrane vesicles prepared from the duodenum of two individual mice fed a normal diet (left two lanes) and in vesicles prepared from the ileum of two individual mice fed a normal diet (right two lanes); 10 μg of protein were loaded in each lane. Arrow indicates Dcytb protein. (**C**) Low-power (×10) immunofluorescence image of mouse duodenal mucosa [from an iron-replete (+Fe) (left) and an iron-deficient (–Fe) animal (middle)] stained with a Dcytb antibody. Box represents the area of the higher power image (right) from the iron-deficient animal (×40). Nuclei are stained with propidium iodide (red), and Dcytb is visualized with a FITC-labeled secondary antibody (green).

To establish whether expression of Dcytb itself was capable of reducing ferric complexes, we microinjected *Xenopus* oocytes with Dcytb cRNA (*23*). A five- to sixfold increase in ferric reductase activity was observed in oocytes injected with Dcytb cRNA, as compared to either water- or mock-injected oocytes (Fig. 4A). We also transfected cell lines derived from intestinal cells (HuTu-80 and CaCo-2) with Dcytb (*23*). We measured ferric reductase activity in whole cells and in membrane-enriched fractions, using many substrates known to react with the reductase found in the duodenal mucosa. In intact HuTu-80 cells incubated with MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; thiazole blue], there was a significant ($P < 0.001$) increase in MTT reductase activity in cells transfected with Dcytb at all time points (Fig. 4B). Treatment with Dcytb antibody significantly ($P < 0.001$) reduced MTT reductase in transfected cells after 60 min and also inhibited reductase activity in untransfected cells after incubation for 45 ($P < 0.01$) and 60 min ($P < 0.05$). In CaCo-2 cells, ferric reductase activity was measured in a plasma membrane plus microsome and cytosol fraction by using ferric iron [Fe(III)NTA_2] (NTA, nitrilotriacetic acid) as the substrate (Fig. 4C). Ferric reductase activity was significantly ($P < 0.001$) higher in membranes prepared from cells transfected with Dcytb, as compared to untransfected cells (Fig. 4C). Treatment of transfected cells with Dcytb antibody blocked the increase in ferric reductase activity seen on transfection ($P < 0.05$).

Nitroblue tetrazolium (NBT) is an electron acceptor that produces a water-insoluble, intensely blue reduced product, enabling localization of redox reactions (*23*). NBT reductase was increased in hypotransferrinemic mouse duodenal mucosa (Fig. 4D) and in iron-deficient mice (*24*), paralleling previously described changes in ferrireductase activity (*7*). This activity was blocked by preincubation of tissue slices with a polyclonal antibody to Dcytb (Fig. 4E) but not with preimmune serum. The antibody was also found to inhibit the reduction of Fe(III)/NTA (1:2) by mouse duodenal fragments by up to 60% of the control incubation (no serum present), 61.1 ± 1.6; 1% preimmune serum present during incubation, 58.2 ± 5.1; and 1% anti-Dcytb serum present during incubation, 23.3 ± 1.9 (results are mean ± SEM, $n = 3$; units are pmol/min/mg of tissue; $P < 0.003$). These results indicate that Dcytb is responsible for the iron-regulated ferric reductase activity observed in the duodenum.

Conclusions

We identified a mammalian plasma membrane b-type cytochrome with ferric reductase activity from the duodenal mucosa. Unlike previously described ferric reductases

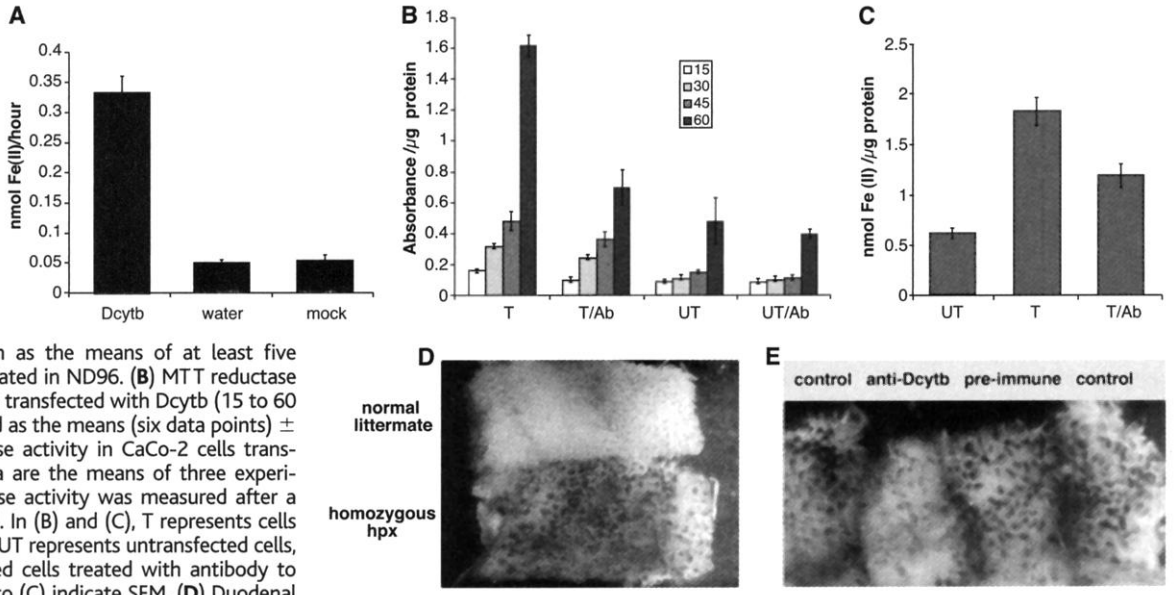

Fig. 4. Dcytb expression induces ferric reductase activity. (**A**) Reductase activity in *Xenopus* oocytes injected with Dcytb cRNA, water, and an unrelated cRNA. The formation of ferrous iron was calculated by measuring the optical density of the oocyte incubation buffer at 562 nm (*23*). Results are shown as the means of at least five individual oocytes incubated in ND96. (**B**) MTT reductase activity in HuTu-80 cells transfected with Dcytb (15 to 60 min). Data are presented as the means (six data points) ± SEM. (**C**) Ferric reductase activity in CaCo-2 cells transfected with Dcytb. Data are the means of three experiments ± SEM. Reductase activity was measured after a 5-min incubation period. In (B) and (C), T represents cells transfected with Dcytb, UT represents untransfected cells, and T/Ab are transfected cells treated with antibody to Dcytb. Error bars in (A) to (C) indicate SEM. (**D**) Duodenal slices from a normal littermate (top) and hypotransferrinemic (hpx) mice after incubation with NBT to localize reductase activity, demonstrating that NBT reductase is induced in a similar fashion to Dcytb and ferrireductase activity. (**E**) NBT reductase in hypotransferrinemic mouse duodenum is blocked by preincubation with antiserum to Dcytb. Control tissues (left and right) were incubated along with tissue slices incubated with serum (anti-Dcytb and preimmune).

from plants (*2*) and yeast (*1, 25, 26*), Dcytb appears to lack any conventional NADH (reduced form of nicotinamide adenine dinucleotide), NADPH (reduced form of nicotinamide adenine dinucleotide phosphate), or flavin binding motifs that would allow these cofactors to act as intracellular electron donors. The lack of sequence homology with yeast and plant sequences indicates that Dcytb evolved as a ferric reductase independently. Cytochrome b561 receives an electron from ascorbate (*27*) and does not appear to require other components. Dcytb may also use ascorbate or, like gp91-*phox*, associate with several other proteins to form an active complex. Dcytb was highly expressed in the brush-border membrane of duodenal enterocytes and was capable of reduction of ferric iron complexes in both *Xenopus* oocytes and cultured cells. Antibody-blocking experiments support the notion that Dcytb is responsible for the physiological reductase activity present in the duodenal mucosa. Dcytb mRNA and protein levels were up-regulated by several independent stimulators of iron absorption, including chronic anemia, iron deficiency, and hypoxia. The lack of a definable iron-responsive element within the mRNA sequence of Dcytb is unusual for a protein of iron metabolism and points to iron-dependent regulation by other mechanisms, including transcription. The isolation of Dcytb provides an important clue as to how dietary ferric iron is absorbed, and it identifies an iron-regulated mechanism by which ferrous iron could be supplied to the divalent cation transporter DCT1/Nramp2. Such a mechanism would be particularly important in iron deficiency.

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11. Duodenal mucosa from a hpx mouse was used as the target tissue to isolate Dcytb. A subtracted cDNA library was constructed as previously described (*10*). Using this strategy, we isolated a 114–base pair (bp) cDNA fragment (representing base pairs 205 to 319 of Dcytb). Using RACE (rapid amplification of cDNA ends) polymerase chain reaction (PCR), we isolated a 1100-bp cDNA for Dcytb, which appeared to encode the entire protein of 284 amino acids and had a predicted molecular mass of ~30 kD. Further analysis of expressed sequence tags (ESTs) indicated an additional two amino acids (Met and Ala) at the NH_2-terminus, giving a predicted protein of 286 amino acids. A genomic sequence (GenBank accession number AC007969) containing the human Dcytb open reading frame was found by searching the High-Throughput Genomic Sequences (HTGS) database with the mouse sequence. In addition, a fully sequenced cDNA clone from a human small intestine library containing the full-length human Dcytb cDNA [pME18S-FL/Dcytb (GenBank accession number AK027115)] was found by searching EST databases.
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17. Male mice (6 to 9 weeks old) of the CD-1 strain were used for experimental manipulations of iron status. Hypoxia, iron deficiency, and iron loading were carried out as described previously (*10*). Hypotransferrinemic mice were bred and maintained as described previously (*16*). Animal procedures were approved by the U.K. Home Office. Total RNA was extracted from tissues, and Northern blotting was carried out as previously described (*10*). The above-described 114-bp fragment of Dcytb was used as the probe. We carried out reverse transcription on 10 µg of total RNA from each tissue, using 100 U of Superscript II (Gibco) and 100 nM random hexamers (Pharmacia) and following the manufacturer's instructions. PCR was performed in a PTC 200 thermal cycler (MJ Research, Waltham, MA) with 0.5 U of Taq polymerase (Supertaq, HT Biotechnology, Cambridge), 200 µM deoxynucleoside triphosphate (Fermentas, Hanover, MD), and gene-specific primers (sequences available on request) at a concentration of 10 µM. Products were compared after 20, 25, and 30 cycles. The products shown in Fig. 2D are after 20 [GAPDH (glyceraldehyde phosphate dehydrogenase) and Dcytb] and 30 cycles (gp91-*phox*). The data shown were reproducible in independent experiments on individual mice from each group. In situ hybridizations were carried out as previously described (*10*).
18. To generate antisera to Dcytb, we synthesized the peptides [Cys]-DAESSSEGAARKRTLGLADSGQRSTM (peptide 1) and [Cys]-KRPREPGSVPLQLNGGNADRME (peptide 2), corresponding to the COOH-terminus and amino acids 223 to 244, respectively, of mouse Dcytb (*28*). Peptides 1 and 2 were then injected into two individual rabbits (coded 834 and 835, respectively), and a mix of both peptides 1 and 2 was injected into a third rabbit (coded 836) (Sigma-Genosys). Western blots were performed with 836 antiserum, whereas immunohistochemical and antibody-blocking experiments were performed with 834 antiserum. For both Western blots and immunohistochemistry, 834 antiserum was found to work well, whereas 836 worked only on Western blots and did not block reductase activity; 835 antiserum was not tested as a function-blocking antibody and worked weakly (compared to 834) in Western blotting and immunostaining experiments. Duodenal protein samples (mucosal scrapes) from hypotransferrinemic mice were prepared with Trisol (Gibco), following manufacturer's instructions. We prepared intestinal brush-border membrane vesicles from mucosa of the duodenum and ileum of normal mice, using a well-established procedure that, in our hands [(*9, 29*), and references therein], yields highly enriched brush-border membranes. Western blotting was performed as

described (*10*). Membranes were incubated for 1 hour with Dcytb antisera (antiserum 836) at a dilution of 1:100. Detection of the primary antibody was performed with the Westernbreeze system (Invitrogen). For immunohistochemistry, sections (5 μm) were cut from frozen tissue blocks at −20°C and placed on glass slides, fixed for 10 min at room temperature in acetone, and allowed to air-dry. Blocking was performed in 1% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min. Sections were incubated with primary Dcytb antibody (antiserum 834) diluted 1:100 for 1 hour in blocking buffer. After extensive washing in PBS, sections were incubated for 30 min with a fluorescein isothiocyanate (FITC)–conjugated anti-rabbit secondary (DAKO, Carpinteria, CA) and then washed extensively with PBS. Sections were counterstained with propidium iodide and mounted. Images were captured on an MRC1024 confocal microscope (Bio-Rad). As a negative control, we processed parallel sections incubated with preimmune sera in the same way. No signal was obtained with preimmune serum.

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23. *Xenopus* oocytes were prepared for injection as previously described (*5*). To synthesize Dcytb cRNA, we linearized the mouse Dcytb/pcDNA3.1 construct with Avr II and cRNA transcribed from the T7 RNA polymerase promoter site, using a commercial kit (Ambion, Austin, TX). Oocytes were injected with 25 ng of cRNA. Controls were injected with the same volume of water or 25 ng of an unrelated cRNA encoding a membrane protein. Reductase assays were carried out on individual oocytes 48 hours after injection. Oocytes were incubated for 10 hours in the dark in 50 μl of either standard Barths solution (pH 7.4) or buffer ND96 [98 mM NaCl, 2.0 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM Hepes (pH 6.0), with tris base] containing 100 μM $FeNTA_2$ (100 μM ferric chloride: 200 μM nitrilotriacetic acid) and 1 mM ferrozine. Formation of ferrous iron was calculated by measuring the optical density of the oocyte incubation buffer at 562 nm. Results were consistent in experiments performed on batches of oocytes harvested from three individual frogs. HuTu-80 and CaCo-2 cells were obtained from the American Type Culture Collection. Both cell types were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum and antibiotics [penicillin G (3 mg/liter) and streptomycin (5 mg/liter)] supplemented with nonessential amino acids (Sigma). Human Dcytb was excised from the original vector [pME18S-FL/Dcytb (GenBank accession number AK027115)] with Eco RI and Xho I and subcloned into the pcDNA3.1myc/his(+) mammalian expression vector (Invitrogen). Cells grown to ~40% confluence in 10-cm plates were transfected with ~2 μg of DNA, using the transfection reagent Effectene, following manufacturer's instructions (Qiagen, Valencia, CA). Stable cell lines were obtained by selection in G418 sulfate (50 μg/ml) (Calbiochem, Darmstrad, Germany) for 10 days. Expression of Dcytb was confirmed by Western blotting. The MTT assay is based on the reduction of a yellow, water-soluble monotetrazolium salt, MTT, to an insoluble purple formazan. HuTu-80 cells (mixed population of Dcytb-expressing cells and untransfected cells) were seeded in 96-well plates (1×10^4/ml) and left to grow for 48 hours. On the day of the assay, the medium was removed, and cells were incubated with Dcytb antibody (834 antiserum) in PBS or with PBS alone for 15 min. Solutions were removed and replaced with culture medium containing 20 μl of MTT solution (5 mg/ml) and incubated at 37°C for varying times. The medium and MTT solution were then removed, and 200 μl of dimethyl sulfoxide was added to each well. Absorbance was read at 540 nm. Data are the average of six wells, and the experiment was repeated three times with similar results. Ferric reductase assays were carried out on CaCo-2 cells as follows. Cells were harvested and homogenized in PBS with a glass Dounce-type homogenizer and centrifuged for 10 min at 10,000*g* to remove mitochondria, nuclei, and intact cells. The supernatant (corresponding to microsomes, plasma membrane, and cytosol) was incubated with 10 μM NADH in 50 mM Hepes (pH 7.4) with 100 μM $FeNTA_2$ and 1 mM bathophenanthroline at 37°C for up to 2 hours. Absorbance was read at 540 nm versus blanks lacking cells. For antibody-blocking experiments, cells were preincubated for 15 min at room temperature with antibody to Dcytb (834 antiserum diluted 1:50). The experiment shown was representative and has been repeated with the same result. For NBT assays, the duodenum was removed, opened lengthwise, and rinsed with 150 mM NaCl. Slices (full width of duodenum by 1 to 2 mm) were taken ~1 cm from the pylorus and incubated for 5 min at 37°C in 200 μl of 1 mM NBT in incubation buffer [125 mM NaCl, 3.5 mM KCl, and 16 mM Hepes/NaOH (pH 7.4)]. After incubation, tissues were rinsed twice with 150 mM NaCl and photographed with a Polaroid Microcam and a dissecting microscope. Inhibition of NBT reductase was demonstrated by preincubation of the tissue for 15 min with 200 μl of preimmune or antiserum to Dcytb (834 antiserum diluted 1:100 in incubation buffer), rinsing once in 200 μl of incubation buffer, then incubating and washing as above. The experiment shown was representative of at least three similar results. The mouse Dcytb construct used in oocyte experiments began MEGYRG, whereas in later experiments in cultured cells, we used a human Dcytb construct, which started MAMEGYRG (*28*). Both constructs induced reductase activity in transfected cells and gave a 30-kD protein by Western blotting. We do not at present know which methionine is used as the start of translation.
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28. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Spins in the Vortices of a High-Temperature Superconductor

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Neutron scattering is used to characterize the magnetism of the vortices for the optimally doped high-temperature superconductor $La_{2-x}Sr_xCuO_4$ ($x = 0.163$) in an applied magnetic field. As temperature is reduced, low-frequency spin fluctuations first disappear with the loss of vortex mobility, but then reappear. We find that the vortex state can be regarded as an inhomogeneous mixture of a superconducting spin fluid and a material containing a nearly ordered antiferromagnet. These experiments show that as for many other properties of cuprate superconductors, the important underlying microscopic forces are magnetic.

Many of the practical applications of type-II superconductors rely on their ability to carry electrical currents without dissipation even in magnetic fields greater than the Meissner field, below which the superconductor excludes magnetic flux entirely. In such high magnetic fields, superconductors are in a mixed state or "vortex lattice," comprising an array of cylindrical inclusions (vortices) of normal material in a superconducting matrix. Vortex lattices have two magnetic aspects. The first is that there are magnetic field gradients due to the inhomogeneous flux penetration; each vortex allows a magnetic flux quantum to penetrate, and the magnetic field

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