Available online at www.sciencedirect.com
SCIENCE DIRECT
Blood Cells, Molecules, and Diseases 36 (2006) 347–354
BLOOD CELLS, MOLECULES, & DISEASES
www.elsevier.com/locate/ybcmd

A novel R416C mutation in human DMT1 (SLC11A2) displays pleiotropic effects on function and causes microcytic anemia and hepatic iron overload

Steven Lam-Yuk-Tseung [a], Clara Camaschella [b], Achille Iolascon [c], Philippe Gros [a,*]

[a] *Department of Biochemistry, McGill Cancer Center and Center for Host Resistance, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, Canada H3G 1Y6*
[b] *Università Vita-Salute, Istituto Scientifico San Raffaele, Milan, Italy*
[c] *Department of Biochemistry and Biomedical Technologies, University Federico II, CEINGE, Advanced Biotechnologies, Naples, Italy*

Submitted 9 January 2006; revised 23 January 2006
Available online 3 April 2006
(Communicated by H.F. Bunn, M.D., 23 January 2006)

Abstract

A patient suffering from microcytic anemia and hepatic iron overload was found to be compound heterozygote for polymorphisms in the iron transporter *DMT1* (*Nramp2*, *SLC11A2*), including a 3-bp deletion ($DMT1^{delCTT}$) in intron 4 that partially impairs splicing and an amino acid substitution ($DMT1^{C1246T}$, R416C) at a conserved residue in transmembrane domain 9 of the protein. The functional properties and possible contribution to disease of the DMT1 R416C mutation were studied in independent mutants at that position (R416C, R416A, R416K, R416E) expressed in LLC-PK_1 kidney cells. Non-conservative substitutions at R416 (C, A, E) cause multiple functional deficiencies including defective protein processing, loss of transport activity, impaired cell surface targeting, and recycling through endosomes, concomitant with retention of the transporter in the endoplasmic reticulum. Conversely, a conservative isoelectric substitution (R416K) was less vulnerable, resulting in a functional transporter that was properly processed and targeted to the cell surface and to recycling endosomes. We propose that $DMT1^{C1246T}$ (R416C) represents a complete loss-of-function, and that a quantitative reduction in DMT1 expression is the cause of the microcytic anemia and iron overload in the patient.

Keywords: DMT1; *Nramp2*; *SL2HA2*; Microcytic anemia; Iron overload; Iron transport

Introduction

The mechanisms by which nutritional iron is acquired, redistributed, and recycled involve a number of structurally and functionally distinct membrane transporters including the *Divalent Metal Transporter 1* (*DMT1*, also known as *Nramp2* or *SLC11A2*) [1]. DMT1 is an integral membrane phosphoglycoprotein expressed at the brush border of the duodenum, where it imports dietary iron in absorptive epithelial cells [2]. DMT1 is also ubiquitously expressed in recycling endosomes of many cell types and is abundant in erythroid precursors, where it is required for transport of transferrin-associated iron into the cytoplasm [3]. DMT1 requires a proton gradient to transport Fe^{2+} as well other divalent cations (Mn^{2+}, Co^{2+}, Zn^{2+}) in a pH-dependent manner [4]. Much of our knowledge of the function of DMT1 in vivo comes from studies of rodent models of microcytic anemia and iron deficiency such as *mk* in mice and *Belgrade* in rats, that are both caused by the same naturally occurring mis-sense mutation in DMT1 (G185R) [5,6]. A similar but more severe phenotype has recently been reported for a mouse mutant with a complete inactivation of *DMT1* ($DMT1^{-/-}$) [7]. Furthermore, studies involving tissue-specific inactivation of *DMT1* have demonstrated the critical role of the transporter in intestinal iron absorption and in erythroid iron utilization but suggested a less critical role of DMT1 in liver iron uptake [7].

In humans, Priwitzerova et al. first reported a human patient homozygote for a mutation in DMT1 ($DMT1^{G1285C}$). The young female patient is the product of a consanguineous union who suffered from severe congenital hypochromic microcytic anemia and iron overload [8,9]. The phenotype is similar to that

* Corresponding author. Fax: +1 514 398 2603.
E-mail address: philippe.gros@mcgill.ca (P. Gros).

1079-9796/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcmd.2006.01.011

of *DMT1* mouse mutants with the notable exception of elevated hepatic iron stores that are specific to the human patient. The mutation has a dual effect, partially impairing splicing of exon 12 of *DMT1* (1285G>C) and introducing an amino acid substitution (E399D) in the remaining properly spliced transcript found in the patient. We recently showed that the E399D mutation does not in itself affect expression, function, or targeting of the DMT1 protein [10–12], and thus reduced DMT1 function in this patient is likely caused by a quantitative reduction in *DMT1* mRNA levels due to improper splicing.

Recently, some of us reported a second human patient who was compound heterozygote for two novel mutations in *DMT1* [13]. The patient is a 5-year-old male who suffers from severe hypochromic microcytic anemia and who has developed hepatic iron overload shortly after birth. Hematological values and iron metabolism indices for this patient and for the patient reported by Priwitzerova et al. were similar: both had low hemoglobin, low mean corpuscular volume, low mean corpuscular hemoglobin, high serum iron and serum ferritin, and elevated transferrin saturation. However, the degree of anemia was more severe in the child patient who required blood transfusions until erythropoietin treatment sufficiently ameliorated the anemia to allow transfusion independence. The patient possessed two novel mutations in *DMT1*: a 3-bp deletion (del CTT) in intron 4 and a C to T transition at nucleotide 1246 ($DMT1^{C1246T}$) in exon 13 that results in an Arg to Cys substitution at position 416 (R416C) of DMT1. The CTT deletion disrupts normal splicing of *DMT1* pre-mRNA by affecting the consensus splicing acceptor site of intron 4, causing a partial (30–35%) skipping of exon 5 [13]. This truncated DMT1 variant lacks 40 amino acids, including an entire transmembrane domain (TM2), and appears to represent a loss-of-function that is inherited in a fully recessive manner. In contrast, the effects of the R416C mutation on DMT1 expression, targeting, and function are unknown. Previous studies by our group have suggested that R416 may be critical for DMT1 function [14]. An alanine substitution mutant at R416 (R416A) failed to restore growth under metal limiting conditions to a yeast mutant deleted for two of the three endogenous *DMT1* homologs (*smf1/smf2*) despite robust membrane expression of this mutant. The R416A variant may not be stably expressed in transfected CHO cells precluding its functional analysis.

The effects of the R416C mutation on DMT1 function, including a possible contribution to the iron metabolism disorder seen in this patient, remain unknown and were studied. We have created independent mutations at R416 and analyzed the effect on protein expression, glycosylation, targeting, and function in transfected LLC-PK_1 cells.

Materials and methods

Construction, expression, and functional characterization of DMT1 mutants

A mouse *DMT1* isoform II (non-IRE) cDNA backbone modified by insertion of a hemagglutinin epitope (HA) tag in the predicted extracytoplasmic loop defined by the TM7-TM8 interval (DMT1-HA) was used for mutagenesis [15]. Mutations at R416 were constructed using a recombinant polymerase chain reaction (PCR) strategy [14], utilizing primers 5′-GTGATCCTGACATGTTCTATCGCCATC-3′ (R416C), 5′-GATCCTGACCAAGTCTATCGCCATC-3′ (R416K), and 5′-GATCCTGACCGAGTCTATCGCCATC-3′ (R416E). Mutants were introduced into the plasmid vector pCB6 as *Sac*I/*Eco*RI fragments. Construction of the R416A mutation in a DMT1 construct bearing two c-Myc epitope tags at the carboxyl terminus has been described earlier (R416A-myc) [14]. R416A-myc was introduced into DMT1-HA as a *Sac*I/*Eco*RI fragment. All mutants were transfected and stably expressed in porcine LLC-PK_1 kidney cells as previously described [14]. DMT1 transport of Co^{2+} was measured using a calcein fluorescence quenching assay (pH 5.0, 20 μM final metal concentration) [10]. Initial rates of metal transport were calculated from the initial slopes of the fluorescence quenching curves.

Quantification of cell surface expression by ELISA

Quantification of the proportion of DMT1-HA molecules expressed at the cell surface was by an ELISA we have previously described [16]. Briefly, cells were grown to confluency in 48-well plates and fixed with 4% paraformaldehyde (30 min). Cells were washed (PBS containing 1 mM $MgCl_2$, 0.1 mM $CaCl_2$), blocked (5% nonfat milk in PBS), and incubated first with anti-HA Ab (1:500, 90 min) and then with donkey anti-mouse-HRP secondary antibody (1:4000, 1 h). For total DMT1-HA expression, cells were permeabilized (0.1% Triton X-100 in PBS, 30 min) prior to blocking and incubation with anti-HA antibody. Peroxidase activity was measured with an HRP substrate (0.4 mg/ml *o*-phenylenediamine dihydrochloride), according to conditions from the commercial supplier (Sigma). Background absorbance readings from non-specific binding of secondary antibody and non-specific binding of primary antibody to untransfected cells were subtracted for each sample. Cell surface readings were normalized to total DMT1-HA values for each clone expressed as a percentage.

Immunofluorescence

GFP plasmids were kind gifts of Dr. D. Williams (University of Toronto; GFP-Syntaxin 13) and Dr. S. High (University of Manchester, Manchester, United Kingdom; GFP-Sec61). LLC-PK_1 cells grown on coverslips were transfected with GFP plasmids, fixed with 4% paraformaldehyde, permeabilized (5% non-fat milk, 0.2% saponin in PBS), and incubated (1 h, 20°C) with anti-DMT1 NT polyclonal antibody (1:300) [17]. Coverslips were then washed, incubated with goat anti-rabbit-Cy3 (1:2000) for 1 h, and mounted on glass slides.

Results

The C to T transition at nucleotide 1246 of *DMT1* causes an Arg to Cys substitution (R416C) in the 9th predicted membrane-spanning segment of DMT1 (Fig. 1A). Although R416 is