Erythrocyte Glut1 Triggers Dehydroascorbic Acid Uptake in Mammals Unable to Synthesize Vitamin C

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SUMMARY

Of all cells, human erythrocytes express the highest level of the Glut1 glucose transporter. However, the regulation and function of Glut1 during erythropoiesis are not known. Here, we report that glucose transport actually decreases during human erythropoiesis despite a >3-log increase in Glut1 transcripts. In contrast, Glut1-mediated transport of L-dehydroascorbic acid (DHA), an oxidized form of ascorbic acid (AA), is dramatically enhanced. We identified stomatin, an integral erythrocyte membrane protein, as regulating the switch from glucose to DHA transport. Notably though, we found that erythrocyte Glut1 and associated DHA uptake are unique traits of humans and the few other mammals that have lost the ability to synthesize AA from glucose. Accordingly, we show that mice, a species capable of synthesizing AA, express Glut4 but not Glut1 in mature erythrocytes. Thus, erythrocyte-specific coexpression of Glut1 with stomatin constitutes a compensatory mechanism in mammals that are unable to synthesize vitamin C.

INTRODUCTION

Glucose provides a key supply of energy and carbon for all living organisms and its transport is a universally conserved property. Vertebrate glucose transporters belong to the Glut family of multimembrane-spanning facilitative transporters. The first identified protein of this family, Glut1, is a type 2 integral membrane protein composed of 12 transmembrane domains that delineate six extracellular loops (Mueckler et al., 1985). Fourteen Glut isoforms have now been identified in the human genome but Glut1 is the main functional transporter of glucose in most transformed cells as well as in various hematopoietic cell lineages (Mueckler, 1994, 1985). The Glut1 transporter is also crucial for facilitating glucose transport in the brain; a haploinsufficiency of Glut1 results in infantile seizures, delayed development, and microcephaly (Seidner et al., 1998).

Glut1 also transports L-dehydroascorbic acid (DHA), an oxidized intermediate of ascorbic acid (AA) (Bianchi and Rose, 1986; Rumsey et al., 1997; Vera et al., 1993). DHA entry via Glut family transporters was initially investigated because of the structural similarities between DHA and glucose. Once transported into the cell, DHA is immediately reduced to AA allowing a recycling of ascorbate (May, 1998). The uptake of AA into cells is mediated by a distinct class of transporters; sodium-dependent vitamin C transporters (SVCT1 and SVCT2) whose expression profiles differ from those of Glut family members (Tsukaguchi et al., 1999). AA is essential for maintaining plasma and tissue reductive capacity, removing superoxide via its own oxidation into DHA.

Of all cell lineages, the human erythrocyte expresses the highest level of the Glut1 transporter, harboring greater than 200,000 molecules per cell. Moreover, in the context of the red cell membrane, Glut1 accounts for 10% of the total protein mass (Helgerson and Carruthers, 1987; Mueckler, 1994). Here, we show that in erythrocytes, Glut1 preferentially transports DHA rather than glucose. The switch from glucose to DHA transport is associated with an induction of stomatin, an integral erythrocyte membrane protein. Accordingly, in a patient with overhydrated hereditary stomatocytosis (OHSt), a rare genetic disorder of red cell membrane permeability wherein stomatin is only present at low levels, DHA transport is decreased by 50% while glucose uptake is significantly increased. Erythrocyte-specific Glut1 expression and DHA transport are specific traits of the few vitamin C-deficient mammalian species, encompassing only higher primates, guinea pigs and fruit bats. Indeed, adult mice erythrocytes do not harbor Glut1 and do not transport DHA. Rather, Glut4 is expressed on their RBC. Thus, the concomitant induction of Glut1 and stomatin during erythroid differentiation constitutes a compensatory mechanism in mammals that are unable to synthesize the essential AA metabolite.

RESULTS

Glut1 Expression Increases during Human Erythropoiesis

Erythropoietin (EPO)-stimulated erythropoiesis of human CD34+ progenitors resulted in the appearance of erythroid progenitors,

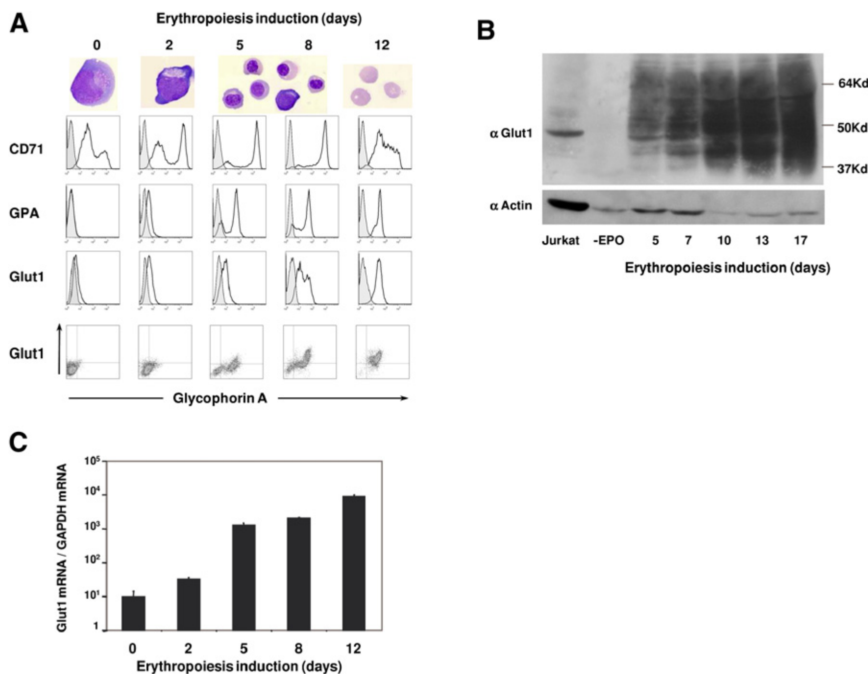

Figure 1. Glut1 Is a Late Marker of Erythrocyte Differentiation

CD34+ progenitors were isolated from UC and amplified in the presence of SCF, IL-3 and IL-6 prior to induction of erythropoiesis by addition of rEPO (noted as Day 0).

(A) At the indicated time points, cell morphology was monitored by May-Grünwald-Giemsa staining of cytocentrifuged smears. Expression of the erythrocyte-associated cell surface markers, the CD71 transferrin receptor and glycophorin A (GPA) was evaluated with the appropriate fluorochrome-conjugated mAbs. Surface Glut1 expression was determined using an eGFP-tagged HTLV receptor-binding domain (H_{RBD}) fusion protein (Kim et al., 2004) that specifically binds this transporter (Manel et al., 2003a, 2005). Control stainings are presented as shaded histograms.

(B) Total Glut1 protein levels were evaluated in progenitor cells following induction of erythropoiesis by immunoblot analysis. CD34+ cells expanded in the absence of rEPO as well as Jurkat cells were added as controls. Protein loading was monitored by actin staining.

(C) Glut1 transcripts were quantified at the indicated time points during erythropoiesis by qRT-PCR. cDNAs were amplified with Glut1-specific primers in triplicate samples and normalized to GAPDH. The means ± SD are shown on semilogarithmic graphs.

as monitored by cell morphology. Markers used to assess the progression of erythropoiesis included glycophorin A (GPA) and the transferrin receptor CD71 (Figure 1A). Glut1 was not detected on immature progenitors but was induced on cells of the basophilic erythroblast stage, increasing further on more mature acidophilic erythroblasts (cells with light cytoplasm in upper panel, Figure 1A). Double staining for GPA and Glut1 showed that GPA expression preceded that of Glut1 but both markers then remained elevated throughout the differentiation process (Figure 1A, bottom panel). Glut1 expression was confirmed by western blot analysis using a Glut1 specific pAb, demonstrating that surface Glut1 expression was concordant with total protein levels (Figure 1B). Indeed, Glut1 was not detected on CD34+ cells expanded in the absence of EPO. The relative mobility of Glut1 increased during erythropoiesis, consistent with a loss of glycosylation as previously reported (Mueckler, 1994). The "smear-like" pattern of Glut1 is specific for the erythroid process as a single sharp band was detected in Jurkat cells (Figure 1B). Moreover, Glut1 mRNA, present only at the limit of detection in immature progenitors, increased by 2-logs following 5 days of EPO-induced differentiation and increased by an additional log at day 12 (Figure 1C). Thus, Glut1 expression in differentiating erythroblasts is highly regulated at the mRNA level and its expression is characteristic of progenitors that have progressed through the basophilic erythroblast stage.

DHA Transport Is Preferentially Augmented during Human Erythropoiesis

Given the function of Glut1 as a sugar transporter (Mueckler et al., 1985), we first assessed whether the observed increase in Glut1 on differentiating erythroblasts was associated with enhanced glucose transport. Glucose transport kinetics were monitored using the nonhydrolyzable 2-deoxy-D[1-^{3}H]glucose (2-DG) analog. Deoxyglucose is trapped in erythrocytes following its phosphorylation to deoxyglucose-6-phosphate whereas the nonphosphorylated deoxyglucose can be imported as well as exported via Glut1. Transport and accumulation can therefore be discriminated by performing uptake assays for extremely short periods (Vera et al., 1995). Importantly, during time points wherein uptake at RT was in the linear range, 15–60 s, 2-DG uptake was drastically reduced between day 0 and day 8 of erythroid differentiation (Figure 2A). Moreover, upon more extended analysis of 2-DG uptake between 1 and 1200 s, the slope of 2-DG transport was significantly decreased upon erythroid differentiation (Figure 2A). Indeed, glucose uptake decreased throughout the 12 day erythroid differentiation (Figure 2B).

This result was surprising and prompted us to test the transport of the second Glut1 substrate, DHA (Bianchi and Rose, 1986; Rumsey et al., 1997; Vera et al., 1993). Importantly, DHA uptake was enhanced during erythropoiesis, as assessed in transport assays performed between 15 and 60 s (Figure 2A). This increase was significant by day 8 and was verified in extended time course assays ranging from 1 to 1200 s (Figure 2A). Indeed, changes in DHA uptake were inversely proportional to glucose uptake (Figures 2A and 2B).

To address the question of whether DHA uptake is competed by glucose during erythropoiesis, we measured the effect of cold glucose on the uptake of radiolabeled DHA during the erythroid differentiation period. Prior to addition of erythropoietin, cold glucose significantly inhibited DHA uptake, as expected for a competing ligand (Figure 2C). However, by day 8 of differentiation, a time point at which Glut1 had been upregulated on the vast

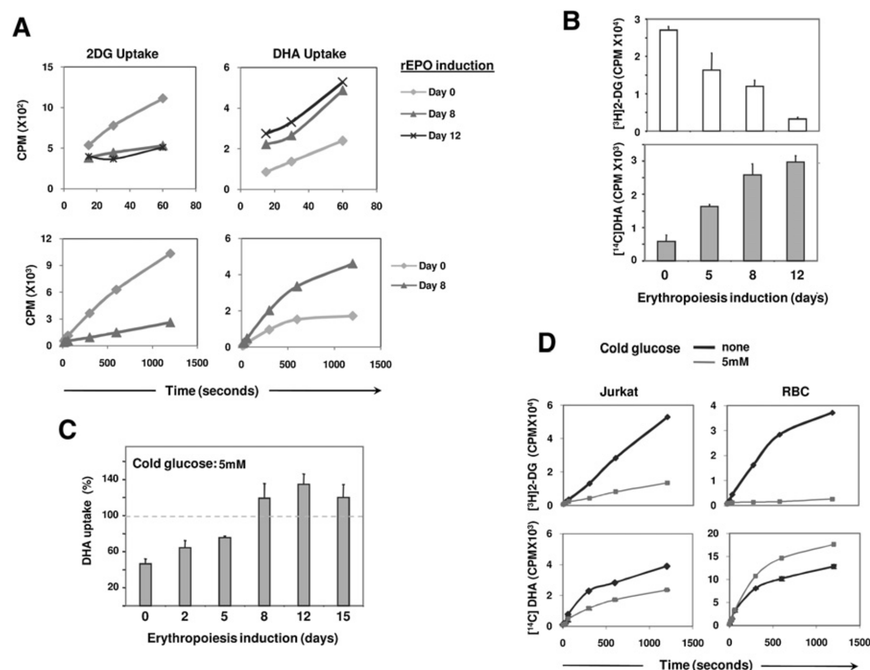

Figure 2. Induction of Glut1 on Human Erythrocytes Results in Enhanced DHA, but Not Glucose, Uptake

(A) To assess glucose and DHA transport kinetics during erythropoiesis, progenitors obtained after 0, 8, and 12 days of ex vivo rEPO stimulation were incubated with either the nonhydrolyzable glucose analog 2-deoxy-D[1-^{3}H]glucose (2-DG) or [^{14}C]DHA for 15, 30, and 60 s at RT (upper panels). At days 0 and 8 of erythroid differentiation, [^{3}H]2-DG and [^{14}C]DHA uptake was monitored for 1, 15, 30, 60, 300, 600, and 1200 s (lower panels).

(B) Glucose and DHA transport in erythroid progenitors obtained on days 0, 5, 8, and 12 of erythropoiesis were assayed during a 10 min uptake at RT.

(C) [^{14}C]DHA uptake was monitored for 10 min at RT in progenitors differentiated for 0, 2, 5, 8, 12, and 15 days, in the absence or presence of 5 mM cold glucose. At each time point, DHA uptake in the absence of glucose was defined as 100%, and the relative incorporation in the presence of glucose is shown.

(D) The effect of 5 mM cold glucose on [^{3}H]2-DG and [^{14}C]DHA transport was monitored in Jurkat cells and peripheral blood RBC in uptake assays performed for 1, 15, 30, 60, 300, 600, and 1200 s.

All data are presented as mean CPMs of triplicate samples. Error bars represent SD.

majority of differentiating erythroblasts (Figure 1A), DHA uptake was not inhibited by the addition of a 10-fold higher concentration of glucose (Figure 2C). Moreover, the importance of a Glut transporter in the uptake of both glucose and DHA was demonstrated by a 70%–80% transport inhibition following treatment of progenitors with cytochalasin B (CytB), a molecule that abrogates Glut1 function by directly binding to its sugar export site (Hebert and Carruthers, 1992) (Figure S1 available online). Therefore, the presence of Glut1 on differentiating erythrocytes results in an increased DHA transport that is not sensitive to competition by physiological levels of extracellular glucose.

Our results, showing a differential uptake of glucose and DHA, were initially difficult to reconcile with previous research in *Xenopus* oocytes and mammalian cell lines indicating that DHA and glucose competitively bind Glut1 (Rumsey et al., 1997; Vera et al., 1993, 1995). We therefore assessed whether glucose competitively inhibits DHA uptake in mature erythrocytes. As expected, cold glucose competitor inhibited the uptake of the radio-labeled glucose analog in RBC (Figure 2D and Figure S2). RBC uptake of the nonmetabolizable glucose analog, 3-*O*-methylglucose, was also inhibited by the addition of cold glucose, under conditions of uptake extending for more than 60 s (data not shown). Additionally, as anticipated from previously published studies (Rumsey et al., 1997; Vera et al., 1995), cold glucose inhibited radio-labeled DHA uptake in nonerythroid cells. In striking contrast, cold glucose did not diminish DHA transport in RBC; uptake was actually higher than that detected in its absence (Figure 2D and Figure S2). The increased uptake of DHA in the presence of glucose is likely a reflection of an increased reduction of DHA to AA, as the latter form is sequestered (May et al., 2001). Notably though, our finding that cold glucose does not inhibit DHA accumulation in mature erythrocytes, even at short 15–60 s time points wherein transport is linear (Figure 2A), indicates that glucose is not an inhibitor of the DHA transport process in mature human erythrocytes.

Because glucose did not compete with DHA for transport, we next assessed whether the transports of both these solutes in RBC were mediated by a Glut-type glucose transporter. In agreement with previous reports (Helgerson and Carruthers, 1987; Mueckler, 1994), CytB inhibited glucose as well as DHA transport in RBC by > 90%. This CytB effect was specific as neither glucose nor DHA transport was decreased by the related non-Glut binding molecule, cytochalasin D (Figure S3). Nonetheless, as CytB can interfere with glucose transport by other Gluts, these data left open a potential role for other Glut molecules in the differential glucose/DHA uptake described here. It was therefore important to study the expression, as well as changes in the expression profiles, of all glucose-transporting Gluts during erythropoiesis. To this end, we designed primers allowing amplification of the different Glut mRNAs and monitored the levels of their transcripts during ex-vivo erythropoiesis. As presented in Figure 3A, qRT-PCR analyses of Glut1–8 and 10–14 demonstrated Glut1 to be the only mRNA to be significantly upregulated following rEPO stimulation of CD34 progenitors. These data strongly suggest a specific role for Glut1 in the differential glucose/DHA transport that we observed during human erythropoiesis as well as in mature RBC.

Stomatin Regulates Glut1 Transport

As shown above Glut1-mediated DHA uptake in human erythrocytes is distinctive in that it is not competed by glucose. Thus, either differences in Glut1 itself and/or differences in Glut1 partners account for the specific inability of glucose to inhibit DHA uptake in erythrocytes. Notably, the N-glycosylation modification of Glut1 at Asn45 has been shown to be distinctive of erythrocytes (Mueckler, 1994). Nevertheless, mutation of this site did

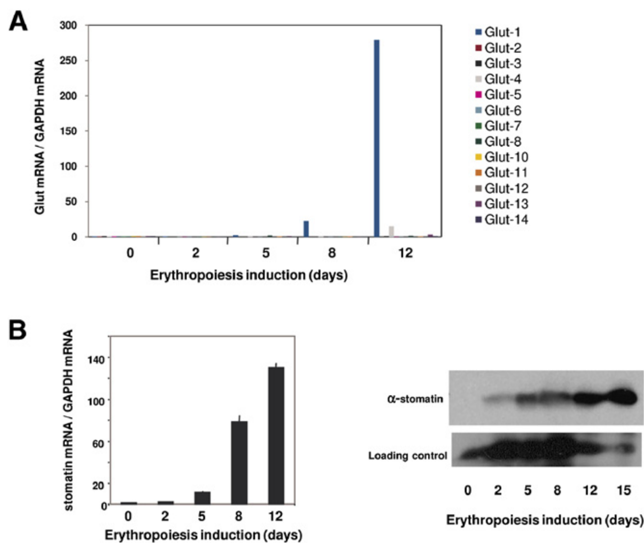

Figure 3. Glut1 and Stomatin Transcripts Are Specifically Increased during Human Erythropoiesis

(A) Transcripts of Glut1–8 and 10–14 were assessed in CD34+ progenitor cells differentiated toward the erythroid lineage for 0, 2, 5, 8, and 12 days by qRT-PCR. cDNAs were amplified with primers specific for the various Gluts in triplicate samples and normalized to GAPDH.

(B) Stomatin transcripts were assessed by qRT-PCR at the same time points, and means ± SD are shown. Stomatin protein levels were monitored by immunoblot analysis.

not alter either glucose or DHA transport. Moreover, mutation of a second site, Q161, known to negatively affect glucose uptake, similarly inhibited DHA uptake (Figure S4).

In RBC, the only Glut1 partner that has been identified is stomatin (Zhang et al., 1999). The latter protein is expressed at high levels with a reported 10^5 molecules per erythrocyte. The regulation of stomatin expression during hematopoiesis has not been extensively studied but it has been reported that CD34-differentiated erythroid progenitors become stomatin-immunopositive (Fricke et al., 2005). Here, we determined that stomatin transcripts, present at only extremely low levels in nondifferentiated progenitors, increased by 10-fold following 5 days of EPO-induced erythropoiesis, a time point at which basophilic erythroblasts were detected. Upon a further 7 days of differentiation, stomatin transcripts were augmented by a 100-fold and stomatin protein, not detected in primitive progenitors, was upregulated upon erythroid differentiation (Figure 3B).

These data show that stomatin expression is induced under conditions wherein DHA uptake increases and becomes insensitive to glucose competition. In this regard, it is notable that the association of Glut1 with stomatin has been reported to decrease Glut1-mediated glucose uptake by 30%–70%, at least in some cell types (Kumar et al., 2004; Zhang et al., 2001). It was therefore of interest to determine whether, in contrast to its role in glucose uptake, stomatin may function to enhance Glut1-mediated DHA uptake. To this end, stomatin was introduced into A431 cells, a human cell line expressing only low levels of stomatin. As shown in Figure 4A, introduction of stomatin did not significantly alter cell surface or total Glut1 levels. Indeed, no correlation between stomatin and Glut1 levels was detected in nonerythroid cells (data not shown). Notably though, Glut1 associated with stomatin in transfected cells as demonstrated by their coimmunoprecipitation (Figure S5). Transport of both 2-DG and DHA into A431 cells were linear at time points ranging from 15 to 300 s (Figure S6). We therefore assessed concomitant glucose and DHA uptake at 30 and 300 s and determined that stomatin expression effectively decreases glucose transport to 70%–80% of control levels while enhancing DHA transport by 130%–200% (Figure 4A and data not shown). These data indicate a role for stomatin in the relative efficacy of DHA and glucose transport.

To verify that the role of stomatin in DHA transport was mediated by Glut1, siRNAs directed against the Glut1 3′UTR were transfected into A431-stomatin cells. This resulted in a 50%–70% decrease in both total and cell surface Glut1 expression without modulating stomatin expression (Figure 4B). To test whether DHA and glucose transport were similarly affected by Glut1 silencing in the presence of stomatin, uptake assays were performed at 30 and 300 s. Indeed, both DHA and glucose transport were similarly decreased in siRNA-treated A431-stomatin cells, by means of 67% for 2-DG and 64% for DHA in a representative experiment (Figure 4B and data not shown). Thus, the effects of stomatin on DHA and glucose transport in these A431-transfected cell are mediated via Glut1.

Stomatin expression is diminished in erythrocytes from patients with OHSt. In this rare genetic disorder of red blood cell membrane permeability, the dramatically reduced stomatin expression is an indirect consequence of a mutation that has not yet been identified (Delaunay, 2004). We found that surface and total Glut1 levels were equivalent on control and RBC from two different OHSt patients (Figure 4C). As previously reported, the reduction in stomatin expression between OHSt patients is variable, as seen in Figure 4C. Notably, glucose and DHA transport in these RBC was consistent with that observed in the stomatin-overexpressing cell line: At 10 min, glucose uptake was significantly higher in the OHSt RBC as compared to control RBC while there was a concomitant 40% reduction in DHA transport (Figure 4C). Moreover, under the same conditions, DHA uptake was also lower in OHSt RBC from a second patient as compared to an RBC sample with equivalent reticulocyte counts (sickle cell anemia; data not shown). Altogether, these observations demonstrate that stomatin inversely regulates the relative transports of glucose and DHA by Glut1.

Glut4 but Not Glut1 Is Expressed in Adult Murine Erythrocytes

Sugar transport in RBC has been shown to be significantly higher in humans than in other species, and this is assumed to be due to a higher Glut1 density on human RBC (Lowe and Walmsley, 1986). Unexpectedly, Glut1 was not detected in RBC from mice over 25 days of age even though expression was readily detectable in newborn murine erythrocytes (Figure 5A). As this result was very surprising, it was important to determine whether the loss of detectable Glut1 protein was due to changes in Glut1 transcription. To this end, Ter119+ splenic erythroid precursors were purified and Glut1 transcripts monitored by qRT-PCR. Glut1 mRNA levels decreased by more than 10-fold between day 2 and day 5 of life and were not detectable in adult

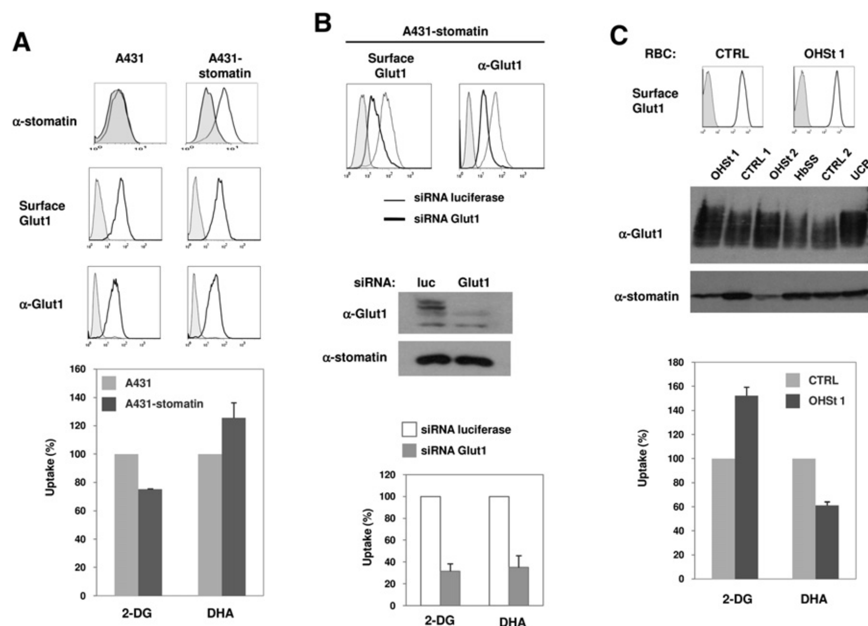

Figure 4. Stomatin Negatively Modulates Glut1-Mediated Glucose Uptake and Enhances DHA Uptake

(A) Stomatin and Glut1 levels in A431 cells and A431 cells stably transfected with a stomatin expression vector (A431-stomatin) were analyzed by intracellular staining with the appropriate Abs. Control IgG staining is shown as shaded histograms. Surface Glut1 was detected using the eGFP-tagged H_{RBD} fusion protein. [^{3}H]2-DG and [^{14}C]DHA transport were assessed concurrently in the parental and stomatin-transfected A431 cell lines (30 s at RT), and data from triplicate samples ± SD are shown.

(B) The A431-stomatin cell line was transfected with an irrelevant siRNA (luciferase; luc) or a mixture of Glut1 specific siRNAs. Surface and total Glut1 levels were monitored 72 hr posttransfection. Shaded histograms show nonspecific staining, and gray and black lines show Glut1 staining following transfection of luc and Glut1 siRNAs, respectively. Glut1 and stomatin were also monitored by immunoblotting. Transport was measured over 30 s as above, and uptake in luc siRNA-transfected cells was defined as 100%. Error bars represent SD.

(C) Surface Glut1 levels on control and OHSt patient RBC were compared. Total Glut1 and stomatin levels were assessed in erythrocytes from two OHSt patients (OHSt1 and OHSt2), a patient with sickle cell anemia (HbSS), two adult controls (CTRL1, CTRL2), and an UC sample by immunoblotting. [^{3}H]2-DG and [^{14}C]DHA transport in control and OHSt1 RBC were assessed concurrently during a 10 min uptake; uptake in control RBC was defined as 100%, after normalizing all values for AA diffusion. Data from triplicate samples ± SD are shown.

progenitors (Figure 5B). Thus, in disparity with human erythrocytes, Glut1 is lost on murine RBC during postnatal development and this decrease is regulated at the transcriptional level.

The data presented above raised the question as to the identity of the transporter responsible for glucose transport in adult murine RBC. We determined that glucose uptake in adult murine RBC is mediated via a Glut-type transporter as it was effectively abrogated by the specific inhibitor CytB but not by the related CytD molecule (Figure 5C). DHA uptake, on the other hand, was present only at the limits of detection in murine RBC and was not affected by CytB (Figure 5C). Glucose, but not DHA, transport is therefore mediated by a Glut-facilitated process in murine RBC.

To determine which Glut molecule is expressed in murine erythrocytes, Ter119+ splenic precursors were purified and primers were designed to amplify distinct Glut molecules. Neither Glut2, 3, nor 5 mRNAs were detected by qRT-PCR, performed using validated primer pairs (data not shown). In contrast, Glut4 mRNA was readily detected and increased during the postnatal period (Figure 6A).

We therefore compared Glut1 and Glut4 protein expression in peripheral RBC isolated from mice of different ages. Importantly, as described above, Glut1 expression in peripheral RBC decreased significantly following birth and was undetectable by 25 days of age (Figure 6B). In marked contrast, Glut4 was detected in RBC at all ages, albeit at significantly lower levels in adult as compared to neonatal mice (Figure 6B). Given the high levels of Glut4 mRNA in splenic Ter119+ precursors, it was somewhat surprising to detect lower protein levels in peripheral RBC. However, this correlated with an RBC glucose uptake that decreased with age (Figure 6C). Notably though, uptake remained CytB-sensitive (Figure 5C), in agreement with a transport mediated by Glut4. Significantly, Glut4 protein was not detected in human RBC, whether neonatal or adult (data not shown). Altogether these data demonstrate the presence of a second glucose transporter in murine RBC.

Glut1 Expression and Associated DHA Uptake Is Specific to Erythrocytes of Species Unable to Synthesize AA

The marked disparity in Glut1 expression and DHA uptake between murine and human erythrocytes raised the following question: What is the nature of the selective pressure maintaining Glut1 expression and DHA uptake in humans? We hypothesized that DHA uptake by human erythrocytes could be linked to the inability of humans to synthesize the reduced form of DHA, AA, from glucose. Of the >4000 species of mammals, it appears that only man, other higher primates, guinea pigs and fruit bats are unable to synthesize AA from glucose.

Importantly, we detected Glut1 expression on human, guinea pig and fruit bat erythrocytes but not on any other mammalian RBC tested including rabbit, rat, cat, dog and chinchilla (Figure 7A). Notably, the eGFP-tagged $HTLV_{RBD}$ fusion protein binds Glut1 from all mammalian species tested and is specifically increased/decreased by Glut1 overexpression and siRNA transfection, respectively (Figure S7; Kinet et al., 2007; Manel et al., 2003a). Furthermore, Glut1 expression was specifically associated with DHA uptake; glucose transport was equivalently efficient in murine and guinea pig RBC whereas DHA was only efficiently transported by the latter (Figure 7B). As the chinchilla is phylogenetically closer to the guinea pig (*Hystricognathi* suborder of the *Rodentia* order) than the guinea pig is to man, the common

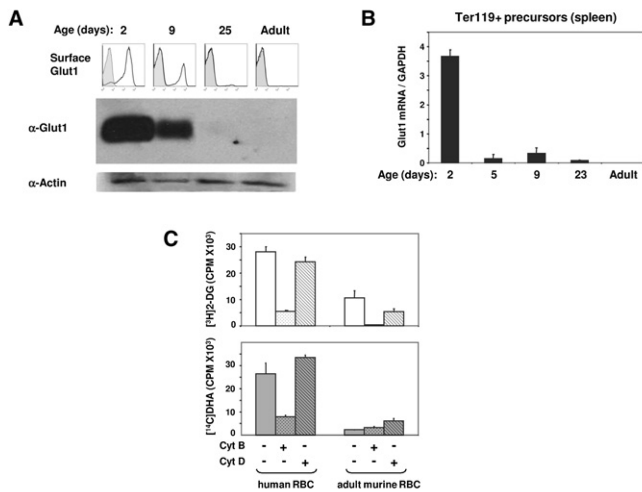

Figure 5. Glut1 Expression and Associated DHA Uptake Is Not a Feature of Adult Murine Erythrocytes

(A) Glut1 protein levels in peripheral RBC obtained from 2, 9, and 25 day-old and adult mice were assessed by immunoblotting. Surface Glut1 in all samples was monitored by cytometry, and shaded histograms represent control staining.

(B) Glut1 transcripts were assessed in Ter119+ splenic erythroid progenitors isolated from mice of different ages, as indicated, by qRT-PCR. cDNAs were amplified with primers specific for Glut1 and duplicate samples were normalized to GAPDH with error bars representing the SD.

(C) Human and murine RBC were pretreated in the absence or presence of the Glut inhibitor CytB or the related CytD molecule for 30 min. $[^3H]$2-DG and $[^{14}C]$DHA uptake were then assayed for 10 min at RT. Relative uptakes ± SD are presented, with glucose and DHA uptake in nontreated human erythrocytes defined as 100%.

profile of the latter two species is compatible with a selection for erythrocyte Glut1 in mammals with defective AA synthesis.

The relationship between erythrocyte Glut1 expression and loss of AA synthesis potential was further studied in primates. Primates belonging to the *Haplorrhini* suborder (including prosimian tarsiers, new world monkeys, old world monkeys, humans and apes) have lost the ability to synthesize AA whereas primates in the *Strepsirrhini* suborder (including lemurs) are reportedly able to produce this vitamin (Nakajima et al., 1969; Pollock and Mullin, 1987). Notably, Glut1 was detected on all tested erythrocytes of primates within the *Haplorrhini* suborder, including long-tailed macaques (*Macaca fascicularis*), rhesus monkeys (*Macaca mulata*), baboons (*Papio anubis*) and magot monkeys (*Macaca sylvanus*) (Figure 7C). In marked contrast, Glut1 was not detected on lemur (*Varecia variegata rubra*) RBC (Figure 7C). Moreover, although DHA uptake in human and magot RBC was similar, the level of transport in RBCs from 3 different lemur species was less than 10% of that detected in higher primates (Figure 7C). The ensemble of these data reveals erythrocyte Glut1 expression and associated DHA uptake to be specific attributes of vitamin C-defective primates of the *Haplorrhini* clade.

DISCUSSION

Here, we show that erythrocyte Glut1 is unique to those few mammalian species that are unable to synthesize AA from glucose. These species include humans and other higher primates as

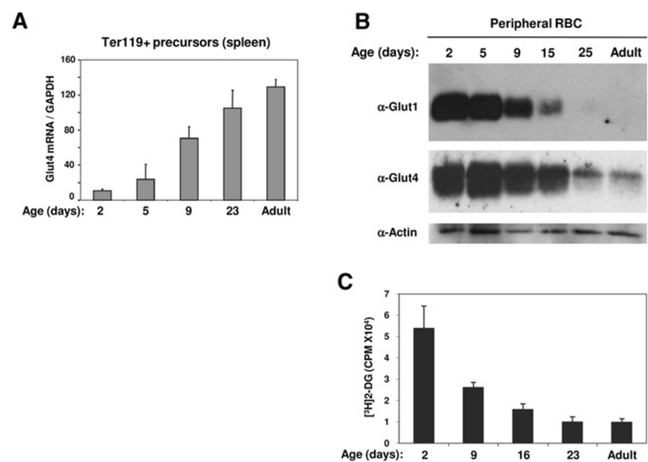

Figure 6. Murine Erythrocytes Express the Glut4 Glucose Transporter

(A) Glut4 mRNA levels were assessed in splenic Ter119+ erythroid progenitors isolated from mice of different ages, as indicated, by qRT-PCR. cDNAs from purified Ter119+ cells were amplified with primers specific for Glut4 and normalized to GAPDH. Means ± SD are shown.

(B) Glut1 and Glut4 protein levels in peripheral RBC obtained from mice of different ages were monitored by immunoblotting.

(C) Glucose uptake was monitored in peripheral RBC obtained from mice of different ages. $[^3H]$2-DG (2 μCi) uptake was performed for 10 min at RT. Data are presented as mean CPMs ± SD of triplicate samples.

well as guinea pigs and fruit bats. Moreover, in mice, a species that naturally synthesizes AA, we identified Glut4 as a glucose transporter in RBC. To our knowledge, this is the first evidence of a glucose transporter other than Glut1 in erythrocytes, irrespective of the species. The previous assumption that Glut1 expression is maintained on murine erythrocytes was based on extrapolations of human RBC data. Interestingly, previous reports on postnatal changes in Glut1 expression in rat heart, skeletal muscle and brown adipose tissue are strikingly similar to that reported here in murine erythrocytes, with a loss of expression by 20 days of age (Santalucia et al., 1999). Our identification of Glut4 on murine erythrocytes is nonetheless surprising because this glucose transporter is insulin-sensitive (Huang and Czech, 2007). Importantly though, the Glut4-mediated transport rate for glucose is significantly higher than that for DHA, both in the absence and presence of insulin (Rumsey et al., 2000). Therefore, it remains to be determined to whether the absence of a Glut-dependent DHA uptake in murine erythrocytes is due to the absence of a transmittable insulin signal in these cells and/or to the significantly lower Vmax of Glut4 for DHA as compared to glucose.

AA is a vital substance that is produced in the livers of most mammals (Chatterjee et al., 1961). The absence of AA production in humans, due to an inactive L-gulono-γ-lactone oxidase (GLO), the enzyme that catalyzes the terminal step of L-ascorbic acid biosynthesis (Burns, 1957), has been described by some scientists as a "species inborn metabolic error" (Stone, 1966). Indeed, supplementation of human diets with exogenous vitamin C has significantly reduced the incidence of scurvy. There has been much debate spurred by several scientists, including Linus Pauling, as to the appropriate daily recommended vitamin C allowance. The fact that endogenous synthesis of AA in

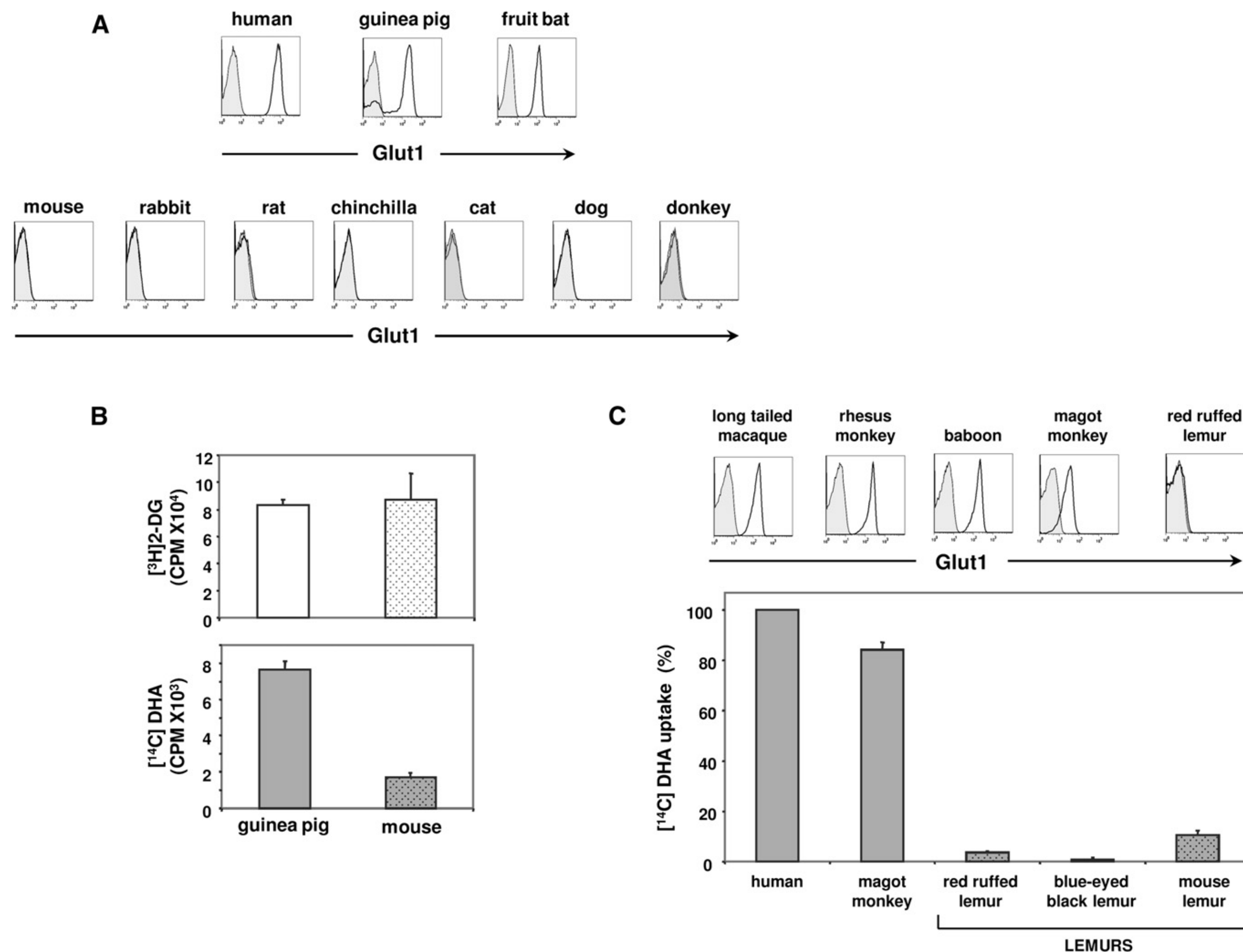

Figure 7. Erythrocyte DHA Uptake Is Specific to Mammals with Defective AA Synthesis and Requires Persistent Glut1 Expression

(A) Erythrocyte Glut1 expression profiles in mammals defective in AA synthesis and those capable of synthesizing this essential carbohydrate are shown in the top and bottom panels, respectively.

(B) $[^3H]$2-DG and $[^{14}C]$DHA uptakes in murine and guinea pig erythrocytes were performed for 10 min at RT. Mean CPM ± SD of triplicate samples are shown.

(C) Surface Glut1 expression was monitored on erythrocytes from primates of the *Haplorrhini* suborder; long-tailed macaques, rhesus monkeys, baboons, and magot monkeys, as well as on a lower primate from the *Strepsirrhini* suborder, a red-ruffed lemur. Relative DHA uptakes in triplicate samples ± SD are shown with uptake in human erythrocytes defined as 100%.

ascorbate-synthesizing mammals is significantly higher than the 1 mg/kg recommended exogenous supplements for humans has echoed this question (Pauling, 1970). For example, in goats, the liver produces AA at a striking rate of 200 mg/kg/day (Chatterjee, 1973; Stone, 1979). Moreover, upon inactivation of GLO in mice and rats, the amount of exogenous vitamin C that is required to obviate symptoms and obtain appropriate plasma levels is greater than 80 mg/kg and 300 mg/kg, respectively (Horio et al., 1985; Maeda et al., 2000; Mizushima et al., 1984).

The lower AA requirements of humans appear linked to the persistence of Glut1 expression on their erythrocytes. Indeed, this trait appears to have coevolved with AA production deficiency. Thus, although guinea pigs and humans independently lost the ability to synthesize AA 40–50 and 20–25 million years ago, respectively (Nishikimi et al., 1994, 1992), erythrocytes from both species exhibit Glut1 expression whereas AA-synthesizing species more closely related to guinea pigs do not. Moreover, within the primate order, the loss of AA-synthesizing potential, due to mutations in GLO, has been tracked to the split of the *Strepsirrhini*/*Haplorrhini* primate suborders (Nakajima et al., 1969; Pollock and Mullin, 1987). We show here that AA-producing *Strepsirrhini* primates, encompassing the lemur families, do not harbor Glut1 on their RBC whereas AA-defective *Haplorrhini* species, including man and higher primates, are distinguished by high Glut1 erythrocyte expression.

May and colleagues have shown that erythrocyte uptake of DHA and its rapid intracellular conversion to AA is crucial for ascorbate recycling, providing a critical export source of this reducing agent (May, 1998). The rapid uptake and reduction of DHA by erythrocytes is also likely to account for the extremely low plasma concentration of DHA (<2 μM) (Dhariwal et al., 1991; Evans et al., 1982; May, 1998). Our experiments, showing

that Glut1 is highly expressed on differentiating erythroblasts, suggest that DHA can be effectively removed from the bone marrow by erythroid cells that have progressed to the basophilic stage. The high DHA uptake by circulating erythrocytes would then allow the AA redox molecule to be efficiently transported throughout the body.

The association of Glut1 with stomatin (Zhang et al., 2001) favors the movement of Glut1 into lipid rafts (Kumar et al., 2004; Rubin and Ismail-Beigi, 2003), resulting in a lower glucose uptake (Rubin and Ismail-Beigi, 2003). Notably, we show here that this phenomenon is promoted during erythropoiesis due to a 2-log increase in stomatin mRNA levels. We further document a role for stomatin in the distinctive transport properties of erythrocyte Glut1 by the observation that the low level of stomatin in OHSt patient erythrocytes is associated with an altered balance of glucose/DHA uptake.

The function of stomatin in RBC remains an enigma (Zhu et al., 1999) and stomatin does not appear to be directly involved in the increased passive leak of monovalent cations in OHSt. However, it is now known that stomatin is a monotopic cholesterol-binding, structural/scaffolding protein like caveolin-1. It forms large oligomeric complexes associated with cholesterol-rich membrane domains that may include various channels and transporters (Salzer et al., 2007). Stomatin is expressed in a variety of tissues including brain, liver, kidney, gut and muscle (Stewart et al., 1992) and other stomatin-like proteins (SLPs) have been detected in diverse organs (Morrow and Parton, 2005). It is notable that SLPs and other members of the prohibitin (PHB) superfamily are conserved from bacteria through humans. Extensive experiments in *Caenorhabditis elegans* indicate a role for the SLPs, Unc-1, Unc-24, and Mec-2, in mediating sensitivity to volatile anesthetics and mechanosensation (Morgan et al., 2007) and experiments in stomatin knock-out mice indicate that stomatin itself may serve a similar function (Sedensky et al., 2006). Moreover, mice with mutations in SLP-3 show defective mechanotransduction (Wetzel et al., 2007). The mechanisms via which SLPs affect these processes have not been completely elucidated but it has been recently shown that MEC-2 and podocin both function by binding cholesterol, an interaction that favors association with ion-channel complexes (Huber et al., 2006).

The low level of DHA transport observed in murine erythrocytes, strongly argues against a role for stomatin in inducing DHA uptake via Glut4, at least in these cells. Importantly though, in skeletal muscle, Glut4 associates with another PHB family protein, flotillin-1. This association occurs via a Cbl-CAP complex (Baumann et al., 2000; Kimura et al., 2001) and following insulin stimulation, flotillin-1/Glut4 complexes are translocated to the plasma membrane (Fecchi et al., 2006). Notably, flotillin-1 is a major component of erythrocyte lipid rafts (Salzer and Prohaska, 2001). It is therefore tempting to speculate that flotillin-1 may regulate membrane Glut4 expression in murine RBC. Oligomeric, raft-associated proteins of the PHB superfamily, such as stomatin, flotillin-1 and Unc-1 and −24, may therefore condition the differential recruitment of channel and transporter glycoproteins to membrane microdomains, allowing diverse cell types to adjust to variable metabolic requirements.

The competitive binding of DHA and glucose to Glut1 described in nucleated cells (Rumsey et al., 1997; Vera et al., 1995) was not observed in human erythrocytes. It has been hypothesized that erythrocyte-specific factors modulate Glut1-mediated sugar translocation across cell membranes (Cloherty et al., 1996; Leitch and Carruthers, 2006). The importance of stomatin in modulating the relative efficacy of glucose/ DHA transport in human erythrocytes implies a unifying explanation: The high coexpression of Glut1 with stomatin negatively modulates glucose uptake while enhancing DHA transport, thereby allowing DHA uptake under physiological conditions where glucose is present at a >3-log higher molarity. The persistence of erythrocyte Glut1 expression is a unique trait of vitamin C-defective mammals, resulting in a massive expression of DHA transporters on the most abundant cell in the circulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Isolations

RBC were obtained from mouse, rat, donkey, guinea pig, macaque, magot monkey, baboon, lemur, and chinchilla blood in accordance with local animal facility regulations. RBC were obtained from two patients with OHSt after parental or personal informed consent, aged 8 and 29 years (patients OHSt1 and 2, respectively). RBC from a patient with sickle cell anemia was used to control for increased reticulocyte counts. Healthy human RBC were obtained from the *Etablissement Français du Sang*. CD34+ cells and neonatal RBC were isolated from umbilical cord blood (UC) obtained after informed consent. CD34+ cells were isolated using EasySep (StemCell) or Milteyni selection kits. Murine erythroid progenitors were isolated from spleen cells at the indicated time points. Cells were labeled with a rat α−murine Ter119 mAb and selected with α−rat IgG magnetic beads (Dynal). 293T, HeLa, and A431 cells were grown in DMEM with high glucose (4.5 g/l) and 10% fetal bovine serum (FBS) while Jurkat cells were grown in RPMI with 10% FBS.

In Vitro Erythroid Differentiation

CD34+ cells (5×10^5 cells/ml) were expanded in StemSpan media (StemCell) supplemented with 5% FBS, 25 ng/ml rhuSCF, 10 ng/ml rhuIL-3 and 10 ng/ml rhuIL-6 (Peprotech) at 37°C. Erythropoiesis was induced at day 7 by addition of 3 IU/ml recombinant erythropoietin (rhuEPO). Cytokines were supplemented every 3 days.

Flow Cytometry

CD71 and Glycophorin A expression were monitored by incubating cells on ice for 20 min with the appropriate fluorochrome-conjugated mAbs (Beckman Coulter). Background fluorescence was measured using isotype-matched Abs. Surface Glut1 expression was monitored as previously described (Manel et al., 2003b; Swainson et al., 2005) by binding to its ligand, the receptor-binding domain of a recombinant envelope glycoprotein from the human T lymphotrophic virus (HTLV) (Manel et al., 2003a, 2005) fused to EGFP (H_{RBD}EGFP) (Kim et al., 2004). Intracellular Glut1 and stomatin levels were determined using an α−carboxy terminal Glut1 pAb (generously provided by A. Carruthers) and an α−stomatin mAb (GARP-50). Intracellular staining was performed following fixation (2% PFA) and permeabilization (0.5% saponin). The secondary Ab was a FITC-conjugated goat α−rabbit IgG and a PE-conjugated goat α−mouse IgG (Sigma), respectively. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) and data analyses were performed using CellQuest Pro (Becton Dickinson) or FlowJo (Tree Star) softwares.

Expression Vectors and siRNA Transfections

A431 cells were transfected with the human stomatin cDNA inserted in pEF-Puro.PL3 as described (Umlauf et al., 2004). Transfection of A431-stomatin cells with the indicated siRNAs (2 μl each at 100 μM) was performed using lipofectamine 2000 (Invitrogen). The modified sequences of the three synthetic siRNAs, complementary to the *Glut1* 3′ UTR, and the luciferase siRNA are included in the Supplemental Data.

Quantitative Analysis of Human and Murine Gluts and Stomatin mRNAs

Total RNA was isolated from differentiating human and murine erythroid progenitors at the indicated time points using the GenElute mammalian total RNA kit (Sigma-Aldrich). Quantitative PCR of cDNAs was performed using the Quantitect SYBR green PCR Master mix (QIAGEN) with 2 μl of cDNA and 500 nM primers in 20 μl. Primers for human and murine Gluts, human stomatin and human/murine GAPDH are noted in the Supplemental Data. All primers were designed within a single exon allowing the efficacy of all primer pairs to be verified on genomic DNA samples and standard curves for each amplification product were established on genomic DNA. Amplification of all Gluts, stomatin and GAPDH cDNAs were performed using the LightCycler 2000 (Roche). Cycling conditions comprised a denaturation step for 15 min at 95°C, followed by 40 cycles of denaturation (95°C for 15 s), annealing (59°C for 20 s) and extension (72°C for 15 s). After amplification, melting curve analysis was performed with denaturation at 95°C for 5 s and continuous fluorescence measurement from 70°C to 95°C at 0.1°C/s. Each sample was amplified in duplicate.

Glucose, AA, and DHA Uptakes

Cells (1–25 × 10^6) were incubated in serum/glucose-free RPMI for 30 min. Under conditions where cells were treated with inhibitors, cells were incubated in the presence or absence of CytB or CytD (100 μM) for 30 min in 500 μl of serum/glucose-free RPMI prior to uptake analyses. Cells were then washed and resuspended in 50 μl of the same media. For glucose competition assays, 5 mM cold glucose was added to the media. Glucose uptake was initiated by addition of labeled 2-deoxy-D[1-^{3}H]glucose (Amersham) to a final concentration of 0.5 μM (2 μCi) (1 Ci = 37 GBq). Transport assays were performed for 1, 15, 30, 60, 300, 600, and 1200 s at RT, as indicated. AA and DHA uptake analyses were initiated by addition of [^{14}C]-AA to a final concentration of 500 μM (0.2 μCi; specific activity: 8.5 mCi/mmol) (PerkinElmer). For DHA uptake assays, ascorbate oxidase (2 U/ml) (Calbiochem) was added to the media. Transport was terminated by addition of 20-volumes of ice-cold medium. Cells were then solubilized in 500 μl of 0.1% SDS. ^{3}H and ^{14}C incorporation were counted by liquid scintillation.

Immunoblots

Nonboiled cell lysates were electrophoresed on SDS-10% acrylamide gels, transferred and probed with a C-terminal Glut1 pAb (1:10,000), a C-terminal Glut4 pAb (ab654, Abcam), a stomatin mAb (GARP50) or an actin Ab followed by a peroxidase-conjugated α-rabbit or α–mouse Ig, as indicated.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and seven figures and can be found with this article online at http://www.cell.com/cgi/content/full/132/6/1039/DC1/.

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