K-Cl cotransport modulation by intracellular Mg in erythrocytes from mice bred for low and high Mg levels

LUCIA DE FRANCESCHI,1 EMMA VILLA-MORUZZI,2 LAURA FUMAGALLI,3 CARLO BRUGNARA,4 FRANCO TURRINI,5 ROLAND MOTTA,6 EMANUELA VEGHINI,3 CRISTINA CORATO,3 SETH L. ALPER,7 AND GIORGIO BERTON3

Departments of 1Clinical and Experimental Medicine and 3Pathology, Section of General Pathology, University of Verona, Verona, Italy; 2Department of Experimental Pathology, University of Pisa, Pisa, Italy; 4Departments of Laboratory Medicine and Pathology, Children’s Hospital, Harvard Medical School, Boston 02115, and 7Laboratory of Research Genetic on Models Animaux, Centre National de la Recherche Scientifique, Orleans, France

Received 8 March 2001; accepted in final form 16 May 2001

De Franceschi, Lucia, Emma Villa-Moruzzi, Laura Fumagalli, Carlo Brugnara, Franco Turrini, Roland Motta, Emanuela Veghini, Cristina Corato, Seth L. Alper, and Giorgio Berton. K-Cl cotransport modulation by intracellular Mg in erythrocytes from mice bred for low and high Mg levels. Am J Physiol Cell Physiol 281: C1385–C1395, 2001.—Mg is an important determinant of erythrocyte cation transport system(s) activity. We investigated cation transport in erythrocytes from mice bred for high (MGH) and low (MGL) Mg levels in erythrocytes and plasma. We found that K-Cl cotransport activity was higher in MGL than in MGH erythrocytes, and this could explain their higher mean corpuscular hemoglobin concentration, median density, and reduced cell K content. Although mouse KCC1 protein abundance was comparable in MGL and MGH erythrocytes, activities of Src family tyrosine kinases were higher in MGH than in MGL erythrocytes. In contrast, protein phosphatase (PP) isoform 1α (PP1α) enzymatic activity, which has been suggested to play a positive regulatory role in K-Cl cotransport, was lower in MGH than in MGL erythrocytes. Additionally, we found that the Src family kinase c-Fgr tyrosine phosphorylates PP1α in vitro. These findings suggest that in vivo downregulation of K-Cl cotransport activity by Mg is mediated by enhanced Src family kinase activity, leading to inhibition of the K-Cl cotransport stimulator PP1α.

K-Cl cotransport, a high-turnover, gradient-driven transmembrane transport system, which plays an important role in the erythrocyte dehydration observed in patients that are homozygous for HbS or β-thalassemia (11, 39). The human and the murine proteins responsible for erythropoietic K-Cl cotransport activity have been recently cloned (28, 46). The murine erythroid K-Cl cotransport (mKCC1) is 96% identical at the amino acid level to its human counterpart (hKCC1), and both polypeptides display consensus sequences for phosphorylation by casein kinases and protein kinase C (28, 46, 53).

Studies with inhibitors of serine/threonine protein phosphatases (PPs) and kinases suggest that K-Cl cotransport activity is regulated by a cycle of phosphorylation and dephosphorylation and that the serine/threonine phosphatase PP1 is a positive regulator of the K-Cl cotransport (9, 10, 35, 49, 52). Among multiple PP1 isoforms, PP1α, PP1γ, and PP1δ are found in virtually all mammalian cells, but little is known about their possible differential functions (17, 51, 57).

We have shown that genetic deficiency of Fgr and Hck, two tyrosine kinases of the Src family, results in elevated K-Cl cotransport activity in mouse erythrocytes, indicating a role for these kinases in the regulation of erythroid K-Cl cotransport (23). On the basis of the well-established role of serine/threonine phosphatase(s) (PP1 and/or PP2A) in activating K-Cl cotransport, we proposed that Fgr and Hck are among the kinases responsible for the negative regulation of these phosphatases (23).

Accumulating evidence suggests that Mg is an important regulator of erythrocyte functions. The effect of Mg deficiency on membrane function and erythrocyte metabolism has been studied in animal models of dietary Mg deficiency (2, 20, 22, 27, 43, 48, 55). Mg deficiency in hamsters increases erythrocyte susceptibility to free radical injury and, in rats, leads to signif-
significant reduction in erythrocyte glutathione (GSH), which is reversed on administration of vitamin E or d-propanolol (27, 43). K-Cl cotransport is activated by oxidative stress (11, 44). We have reported that Mg deficiency exacerbates anemia in patients and in mouse models of sickle cell disease and β-thalassemia, (2, 19, 20, 22, 44). Dietary Mg supplementation can increase erythrocyte Mg content, ameliorate anemia, and lead to inhibition of K-Cl cotransport, which is pathologically elevated in these diseases. Mouse strains have been bred to select for high (MGH) and low (MGL) Mg levels in erythrocytes and plasma. These strains were developed by bidirectional selective breeding from outbred populations consisting of F2 segregant hybrids among four inbred strains (C57BL/6, DBA/2, C3H/eB, and AKR) and extending over 18 generations of inbreeding (1, 8, 16, 30–34, 42). Although these strains were developed to study the role of Mg in the stress response (33), they can be also exploited to evaluate other Mg-dependent cellular functions (1, 32).

We have used these mice to study the role of high and low erythrocyte Mg levels on erythrocyte susceptibility to oxidative stress and on activities of cation transport pathways implicated in cell volume regulation. We have found that K-Cl cotransport activity is elevated in MGL erythrocytes, in parallel with decreased erythrocyte enzymatic activity of Src family kinases. In addition, we have evaluated the expression and activity of PP1α in MGH and MGL mouse erythrocytes. We provide evidence that PP1α activity varies inversely with cell Mg content and is higher in MGL erythrocytes. These findings suggest that inhibition of erythrocyte dehydration and K-Cl cotransport by elevated Mg is due to reduced PP1α activity likely resulting from modulation of Src family kinase activities. The capability of Src family kinases to downregulate PP1α activity is indeed supported by our finding that c-Fgr phosphorylates PP1α in vitro.

METHODS

Drugs and chemicals. NaCl, KCl, NaNO₃, okadaic acid (OA), staurosporine, N-ethylmaleimide (NEM), dithiothreitol (DTT), ouabain, bumetanide, nystatin, sulfamic acid, A-21387 ionophore, tris(hydroxymethyl)aminomethane (Tris), and 3-(N-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma Chemical (St. Louis, MO). MgCl₂, Mg(NO₃)₂, dimethyl sulfoxide, and n-butyl phthalate were purchased from Fisher Scientific. Choline chloride was purchased from Calbiochem-Boehringer (San Diego, CA). Bovine serum albumin fraction V was purchased from Boehringer Mannheim (Mannheim, Germany). d-[1-¹⁴C]glucose (specific activity 53.4 mCi/mmol) was obtained from Amersham International (Little Chalfont, UK). Recombinant c-Fgr and Lyn were kind gifts of Dr. A. M. Brunati (Dept. of Biological Chemistry, University of Padova, Padua, Italy) (14, 15). Antibodies against KCC1 were characterized in a previous study (53). All solutions were prepared using double-distilled water.

Animals and design of the study. Animals were bred in the Centre National de la Recherche Scientifique. Bidirectional selective breeding over 18 generations from a heterogeneous outbred population of F2 segregant hybrids among the four inbred strains C57BL/6, DBA/2, C3H/eB, and AKR (33) followed the method of Biozzi et al. (8). Assortive mating of mice with the lowest erythrocyte Mg values produced the MGL strain, and assortive mating of mice with the highest erythrocyte Mg values produced the MGH strain (33). For each strain, 12–16 pairs were culled at each generation, and erythrocyte and plasma Mg levels were determined on 80 of their offspring taken at random (1, 32, 33). Plasma and erythrocyte Mg levels assayed as described elsewhere (20, 44) are presented in Fig. 1. Although we could not assay free cytosolic Mg, it has been previously demonstrated that total Mg is strictly correlated with free cytosolic Mg (50).

Determination of hematologic parameters. Blood was collected from ether-anesthetized mice by retroorbital venipuncture into heparinized microhemocrit tubes. Hb concentration was determined by the spectroscopic measurement of the cyanomet derivative. Hematocrit was determined by centrifugation in a microhemocrit centrifuge. Distribution of cell volume, Hb concentration, and reticulocyte count were determined using a hematology analyzer (ADVIA 120, Bayer Diagnostics, Tarrytown, NY) (3, 19). Density distribution curves and median erythrocyte density were obtained according to the method of Biozzi et al. (8). Assortive mating of mice bred for high (MGH) and low (MGL) Mg levels. Values obtained in the independent experiments were 1.104 ± 0.105 and 0.854 ± 0.100 (SD) mmol/l for MGH and MGL, respectively (P < 0.05).

Fig. 1. A: plasma Mg levels in mice bred for high (MGH) and low (MGL) Mg levels. Values obtained in the independent experiments were 1.104 ± 0.105 and 0.854 ± 0.100 (SD) mmol/l for MGH and MGL, respectively (P < 0.05). B: erythrocyte (RBC) Mg content in MGH and MGL mice. Values obtained in the independent experiments were 11.4 ± 1.5 and 8.6 ± 2.7 mmol/kg Hb for MGH and MGL, respectively (P < 0.05).

AJP-Cell Physiol • VOL 281 • OCTOBER 2001 • www.ajpcell.org
Characterization of K-Cl cotransport.

K-Cl cotransport polyepitope was analyzed by immunoblot using antibodies to the NH₂ terminus of mKCC1 (53). Packed erythrocytes were quickly spun down with a refrigerated microcentrifuge and resuspended to the original cell number in PBS-G prewarmed at 37°C. Samples were taken at different time points, and GSH was measured as previously described (4, 47). To assess hexose monophosphate shunt (HMS) function, [14C]CO₂ production from [1-14C]glucose was measured as described by Pescamore et al. (47).

Measurements of cation content and maximal transport rates of Na-K pump and Na-K-2Cl cotransport in MGH and MGL mouse erythrocytes. Erythrocyte Na and K contents were determined as previously described (11, 13, 20, 44, 45) after five washes in 172 mM choline chloride, 1 mM MgCl₂, and 10 mM Tris-MOPS (pH 7.4 at 4°C, choline washing solution) (11, 13, 20, 44, 45). Rates of Na-K pump and Na-K-2Cl cotransport activity were measured in cells containing equal amounts of Na and K by the nystatin technique, whereby the cells are loaded with “nystatin solution” (77 mM NaCl, 77 mM KCl, and 55 mM sucrose). The Na-K pump activity was estimated as the 1 mM ouabain-sensitive fraction of Na efflux into a medium containing 165 mM choline chloride and 10 mM KCl (11, 13, 20, 44, 45). Na-K-2Cl cotransport activity was estimated as (10 μM) bumetanide-sensitive Na efflux into medium containing 174 mM choline chloride and 1 mM ouabain (11, 13, 20, 44, 45).

Characterization of K-Cl cotransport. K-Cl cotransport polyepitope was analyzed by immunoblot using antibodies to the NH₂ terminus of mKCC1 (53). Packed erythrocytes were washed three times with ice-cold isotonic NaCl and then lysed in ice-cold hypotonic medium as previously described (53). After three washes in the same medium, erythrocyte ghosts were solubilized in SDS-PAGE sample buffer and subjected to electrophoresis in 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and, after they were blocked with 5% bovine serum albumin in Tris-buffered saline, pH 7.4 (TBS), washed twice with RIPA buffer, once with TBS, and once with kinase buffer (20 mM HEPES, pH 7.4, 10 mM MnCl₂, and 1 mM DTT), and then incubated in kinase buffer containing 2 μM [γ-32P]ATP (Amersham International). After 10 min of incubation at room temperature, the reaction was stopped with heated (95°C) SDS sample buffer, and samples were subjected to electrophoresis in 10% SDS-polyacrylamide gels. Proteins in the gel were transferred to nitrocellulose, and blots were processed for autoradiography by exposure to X-Omat AR films.

The radioactivity incorporated in the immunoprecipitated kinase was allowed to decay until 1-h exposures of the blots to X-Omat AR film produced no signal, and then the gels were processed as immunoblots. Primary antibodies were coupled with horseradish peroxidase by using a commercially available kit (Amersham International). Antibody binding was revealed by ECL with exposure times <15 min.

For assay of Src family kinase activities in Mg-depleted cells, wild-type mouse erythrocytes (C57B6/J) were incubated for 15 min at 37°C with the ionophore A-23187 (10 μM) and EDTA (2 mM) as previously described (24–26, 37). Under these conditions, intracellular Mg is reduced to <1 μM (24–26, 37). Mg-depleted cells were washed three times with 0.1% albumin-supplemented incubation medium and then four times in Mg-free choline washing solution and were then lysed for tyrosine kinase assays.

Analysis of activity and expression of PP1α. Erythrocytes were washed three times in choline washing solution and then lysed as described by Bize et al. (9, 10) with slight modifications. Cells were lysed by freezing and thawing in isosmotic conditions in a buffer containing 80 mM NaCl, 80 mM KCl, 10 mM Tris-MOPS, pH 7.4, 0.1% β-mercaptoetha-
RESULTS

In vitro phosphorylation of PP1 by c-Fgr tyrosine kinase. Rabbit muscle PP1 was purified as described by Tognarini and Villa-Moruzzi (54) and Villa-Moruzzi and co-workers (56, 57), and rat spleen c-Fgr (a kind gift of Drs. A. M. Brunati and L. A. Pinna, Dept. of Biological Chemistry, University of Padova) as described by Brugnati et al. (14, 15). Two microliters of c-Fgr (50–100 ng/ml total proteins) and 1.5–3 μl of the PP1 preparation (5 mg/ml total proteins) were incubated in a total volume of 20 μl containing 50 mM Tris, pH 7.4, 0.01 mM ATP, 5 mM MgCl₂, 1 mM sodium orthovanadate, 10 mM OA, and 10 μCi of [γ-³²P]ATP. After incubation for 30–60 min at 30°C, the reaction was stopped with heated (95°C) SDS sample buffer, and samples were subjected to electrophoresis in 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and subjected to autoradiography. The radioactivity incorporated in the immunoprecipitated kinase or PP1 was allowed to decay until a 1-h exposure of the blot no longer produced any signal on X-Omat AR film. The blots were then processed as immunoblots with anti-PP1α antibodies (54, 57), with ECL exposures of the blot no longer producing any signal on X-Omat AR film. The radioactivity incorporated in the immunoprecipitated kinase or PP1 was allowed to decay until a 1-h exposure of the blot no longer produced any signal on X-Omat AR film. The blots were then processed as immunoblots with anti-PP1α antibodies (54, 57), with ECL exposures of the blot no longer producing any signal on X-Omat AR film.

Statistical analysis. Values are means ± SD. For each group of mice, comparisons of separate variables were performed using two-tailed Student’s t-test. Comparisons of more than two groups were performed by one-way ANOVA followed by Tukey’s test for post hoc comparison of means.

Table 1. Hematologic data, ATP, 2,3-DPG, and cation content in MGH and MGL mouse erythrocytes.

<table>
<thead>
<tr>
<th></th>
<th>MGH</th>
<th>MGL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct, %</td>
<td>44.7±0.3(12)</td>
<td>41.1±1.0*(12)</td>
</tr>
<tr>
<td>Cell Hb, g/dl</td>
<td>12.9±0.7(12)</td>
<td>13.6±0.4(12)</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>58.6±0.1(12)</td>
<td>52.4±0.3*(12)</td>
</tr>
<tr>
<td>MCHC, g/dl</td>
<td>28.9±0.2(12)</td>
<td>33.1±0.9*(12)</td>
</tr>
<tr>
<td>D50</td>
<td>1.096±0.002(12)</td>
<td>1.106±0.003*(12)</td>
</tr>
<tr>
<td>Reticulocyte, %</td>
<td>1.9±0.5(10)</td>
<td>2.2±0.4(10)</td>
</tr>
<tr>
<td>ATP, mmol/g Hb</td>
<td>5.16±0.49(6)</td>
<td>3.9±0.42(1)</td>
</tr>
<tr>
<td>2,3-DPG, mmol/RBC</td>
<td>9.9±0.09(6)</td>
<td>8.3±1.02* (6)</td>
</tr>
<tr>
<td>Cell Na, mmol/kg Hb</td>
<td>57.7±11.1(10)</td>
<td>51.2±5.01(10)</td>
</tr>
<tr>
<td>Cell K, mmol/kg Hb</td>
<td>497.9±47.8(10)</td>
<td>289.4±61.5(10)</td>
</tr>
<tr>
<td>Cell Na + K, mmol/kg Hb</td>
<td>557.4±43(10)</td>
<td>340.76±32.3(10)</td>
</tr>
</tbody>
</table>

Values are means ± SD of number n of experiments in parentheses. MGH and MGL, high and low Mg level, respectively; Hct, hematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular Hb concentration; D50, median erythrocyte density; 2,3-DPG, 2,3-diphosphoglycerate; RBC, red blood cell. *P < 0.05, †P < 0.005 vs. MGH.
puscular Hb concentration is shown in Fig. 2B, bottom (3). The MGL erythrocyte corpuscular Hb concentration was rightward shifted with corresponding increase in mean cell Hb concentration (mean corpuscular Hb concentration, Table 1) and decrease in cell K content (Table 1). These data indicate a dehydrated state of MGL erythrocytes compared with MGH erythrocytes. The reduced K content of MGL erythrocytes can explain the decrease in erythrocyte osmotic fragility in MGL (T50%L = 110 ± 10.4 mosM, P < 0.05, n = 10) compared with MGH (T50%L = 152 ± 21.3 mosM, n = 10) erythrocytes. Other clear differences in erythrocytes of the two mouse strains included reduced levels of ATP and 2,3-DPG in MGL cells (Table 1).

Redox state of MGH and MGL mouse erythrocytes. Measurement of reduced GSH revealed decreased levels in MGL compared with MGH erythrocytes (2.07 ± 0.38 vs. 2.22 ± 0.29 mmol/l, P < 0.05, n = 6). Similarly, HMS was slightly decreased in MGL mouse erythrocytes compared with MGH erythrocytes (0.73 ± 0.31 vs. 0.98 ± 0.10 mmol·10^8 cells^{-1}·h^{-1}, P < 0.05, n = 6).

Under unstressed conditions, MGL erythrocytes showed little modification of reductive metabolism. To evaluate reductive capability of MGL erythrocytes, we measured the kinetics of reduction of GSH after addition of the sulphydryl group oxidant diamide (4, 47). As shown in Fig. 3, the kinetics of GSH reduction was slower in MGL than in MGH erythrocytes. However, after 60 min of recovery, GSH levels were indistinguishable in the two cell types, suggesting that MGL erythrocytes can reach normal steady-state GSH levels, despite their decreased maximal reductive capability (Fig. 3).


![Fig. 3. Time dependence of reduced glutathione (GSH) regeneration in MGH and MGL erythrocytes after treatment with diamide (0.1 mM) to oxidize GSH. Values are expressed as percentage of basal GSH levels measured before diamide treatment. Results are from 1 of 3 experiments with similar results.](http://ajpcell.physiology.org/)

To compare membrane transport activities in erythrocytes from MGH and MGL mice, we examined activities of three major erythrocyte K transporters. As shown in Fig. 4, the maximal rate of the Na-K pump was similar in MGH and MGL erythrocytes. In contrast, Na-K-2Cl and K-Cl cotransport activities varied in the two cell types (Fig. 4). Na-K-2Cl cotransport was significantly lower in MGL than in MGH erythrocytes, whereas K-Cl cotransport was markedly higher in MGL than in MGH erythrocytes. These findings indicate that the different Mg content of the two strains is associated with different transport activities in vivo.

Regulation of K-Cl cotransport activity in MGH and MGL erythrocytes. K-Cl cotransport can be activated by cell swelling or by drugs that inhibit protein phosphorylation. Examination of the time dependence of K-Cl cotransport activity induced by swelling provides information on the functional state of the transporter and its regulation (2, 9, 10, 35, 49, 52). The lag time for K-Cl cotransport activation after exposure of the cells to hypotonic medium (Fig. 5B) was markedly different in MGH and MGL erythrocytes (12.3 ± 2.1 and 8.3 ± 1.6 min, respectively, P < 0.05, n = 3), indicating that the different erythrocyte Mg contents may regulate the activation-deactivation cycle of K-Cl, which is dependent on the phosphorylation/dephosphorylation regulatory mechanisms.

The sensitivity of K-Cl cotransport to drugs affecting protein phosphorylation state was also examined (Fig. 6). OA, an inhibitor of serine/threonine phosphatases (PP1 and PP2), induced a marked decrease in K-Cl
cotransport in MGH and MGL erythrocytes (17, 35). In contrast, staurosporine and NEM, both known activators of erythrocyte K-Cl cotransport (2, 35, 38), enhanced K-Cl cotransport activity in MGH erythrocytes but failed to increase further the already elevated K-Cl cotransport activity of MGL erythrocytes. Thus MGL erythrocyte K-Cl cotransport exhibited apparently maximal activation in the basal state.

Activities and expression of Src kinases in MGH and MGL mouse erythrocytes. Mouse erythrocytes express the Src family members Fgr, Hck, and Lyn (23). We implicated Fgr and Hck in the negative regulation of K-Cl cotransport activity with our demonstration of K-Cl cotransport activation in erythrocytes from $hck^{+/-}/fgr^{+/-}$ double-null mice (23). We therefore hypothesized that variation in erythrocyte Mg concentration might regulate Src kinase activities. To evaluate this hypothesis, wild-type mouse erythrocytes were depleted of Mg to <1 μM by treatment with the ionophore A-21387 and EDTA (24–26). Such Mg depletion increased erythrocyte K-Cl cotransport activity by 64.3 ± 7.2% ($n = 4$; not shown). The differences in the magnitude of activation of K-Cl cotransport between in vivo MGL mouse erythrocytes (Figs. 4 and 6) and in vitro control mouse erythrocytes depleted of Mg are not easy to explain. In MGL erythrocytes, prolonged Mg depletion may cause alterations of additional K-Cl cotransport regulatory components that are not affected during the in vitro manipulation of wild-type mouse erythrocytes (32, 33).

As shown in Fig. 7A, the activity of Fgr and Lyn was greatly reduced in Mg-depleted erythrocytes, whereas kinase polypeptide abundance assessed by immunoblot differed only minimally. In addition, activity of Fgr and Lyn, as well as of Hck (data not shown), was higher in MGH than in MGL erythrocytes, whereas kinase polypeptide abundance assessed by immunoblot did not differ (Fig. 7B). These findings suggest that erythrocyte Mg content modulates Src family kinase activity in vivo and that enhanced Src kinase activities in MGH erythrocytes may downregulate K-Cl cotransport.

Expression and activity of PP1α in MGH and MGL mouse erythrocytes. The differential inhibition of K-Cl cotransport activity by the PP1 inhibitors OA and calyculin A has implicated PP1 as an activator of K-Cl cotransport (see the introduction). The PP1α isoform was recently demonstrated to be inhibited by tyrosine phosphorylation in v-Src-transfected cells (57). As shown in Fig. 8, PP1α activity and protein are more abundant in the cytosolic than in the membrane fraction in erythrocytes from MGH and MGL mice. However, whereas protein expression was similar in both erythrocyte fractions from the two strains of mice, PP1α activity in cytosol and membrane fractions was higher in MGL than in MGH erythrocytes. We conclude that regulation of the K-Cl cotransport by Mg is directly correlated with the regulation of PP1α activity.
In the presence of high Mg levels, activities of PP1α and K-Cl cotransport are downregulated. 

In vitro phosphorylation of PP1α by the c-Fgr tyrosine kinase. The above-described findings, together with the evidence that K-Cl cotransport is upregulated in double-knockout hck2/2/fgr2/2 erythrocytes (23), suggest that Src family kinase activity decreases K-Cl cotransport activity via inhibition of PP1α activity. We therefore tested the ability of Src family kinases to tyrosine phosphorylate PP1α in intact cells and, by analogy with v-Src (57), in vitro.

Tyrosine phosphorylation of PP1α, assayed by antiphosphotyrosine immunoblots, proved to be undetectable in erythrocytes from C57BL6, MGL, and MGH mice (not shown). Partially purified rat spleen c-Fgr exhibited autophosphorylation when incubated in standard kinase buffer (Fig. 9A, lane 1). However, addition of partially purified rabbit muscle PP1 containing different PP1 isoforms (56) resulted in phosphorylation of two polypeptides that migrated as bands of ~38 and 32 kDa (lanes 5–8). Incubation of the PP1 preparation in the same kinase buffer without c-Fgr revealed the absence of any phosphorylated proteins (lane 4), demonstrating the absence of contaminating kinase activity in the PP1 preparation. As shown in Fig. 9B, immunoblot of the same protein fractions with anti-PP1α antibodies revealed comigration of the 38-kDa phosphoprotein with authentic PP1α (compare lane 4 with other lanes). An additional immunoreactive band of ~33/34 kDa may represent a proteolytic product (57) and migrated above the phosphorylated 32-kDa polypeptide (lane 6). Because a 33-kDa proteolytic fragment of PP1α loses the COOH-terminal tyrosine undergoing phosphorylation (57), it is likely that the phosphorylated 32-kDa polypeptide represents a contaminant of the PP1 preparation we used. We conclude that c-Fgr phosphorylates PP1α in vitro, as does v-Src (57).

DISCUSSION

By comparing MGH and MGL mice, we show in this report that Mg content regulates erythrocyte membrane cation transport and hydration state in vivo. Although activity of the Na-K pump did not differ between MGH and MGL erythrocytes, Na-K-2Cl cotransport activity and the K-Cl cotransport activity were inversely altered as a function of erythrocyte Mg content. Enhanced K-Cl cotransport activity in MGL erythrocytes is likely responsible for erythrocyte dehydration in this mouse cell line. A role for Na-K-Cl...
In vitro studies clearly implicated Mg content as an important determinant of erythrocyte membrane transport activities (24, 25). For example, Mg was shown to be an essential cofactor for the Na-stimulated phosphorylation of the Na-K pump (24). However, activity of the Na-K pump ATPase in MGH and MGL erythrocytes did not differ. An Mg-to-ATP concentration ratio near 1 is optimal for Na-K pump activity, with inhibition observed at higher and lower ratios (26). Hence, the comparable erythrocyte Na-K pump activities in the two mouse strains may be explained with the observation that, in MGL erythrocytes, both ATP and Mg contents are decreased so that the optimal ratio for the function of the Na-K-ATPase is maintained (24). Similar parallel changes were evident in 2,3-DPG (Table 1).

Na-K-2Cl cotransport activity was reduced in MGL mouse erythrocytes, indicating that erythrocyte Mg content modulates this cation transport system in vivo. Previous in vitro studies have demonstrated a strong dependence of Na-K-2Cl cotransport on intracellular Mg content (24–26, 37). It has been suggested that Mg regulates Na-K-2Cl cotransporter activity by controlling NKCC1 polypeptide phosphorylation (24–26, 37). The serine/threonine phosphatases PP1 and PP2A may participate in the negative regulation of Na-K-2Cl cotransport activity (25). Very recently, PP1 was shown to bind directly to NKCC1 (5). Because Src family kinases may inhibit PP1 activities (56, 57), the elevated Na-K-2Cl cotransport activity of MGH erythrocytes may result from higher Src kinase activities, which would downregulate phosphatases and so activate the Na-K-2Cl cotransporter. Although we propose that Src kinases regulate PP1/2A activity, we found that erythrocytes from fgr−/−hck−/− double-knockout mice did not exhibit altered Na-K-2Cl cotransport activity (23). Mg also regulates Lyn kinase activity (Fig. 7). Erythrocytes express additional kinases such as p72syk (29), further encouraging studies of possible...
MGL mouse erythrocytes show increased density and mean corpuscular Hb concentration and decreased K content (Table 1). Because erythrocyte Na content was similar in both strains, the water and K loss observed in MGL mouse erythrocytes likely results from activation of K-Cl cotransport (Fig. 4). Factors other than erythrocyte Mg level also modulate the function of the K-Cl cotransport, such as cell age (11, 38, 45). However, reticulocyte count was similar in MGL and MGH mice (Table 1). Thus, in MGL mice, the increased activity of the K-Cl cotransport is not related to an increased number of circulating young erythrocytes or to oxidative damage due to the presence of free α-globin chain, as in β-thalassemia, or the presence of abnormal Hbs, such as in homozygous HbS or Hbc disease, but rather to reduction in erythrocyte Mg content. Moreover, we recently showed in mouse and human erythrocytes that in vivo changes in erythrocyte Mg content can modulate K-Cl cotransport activity (2, 19, 44).

Examination of MGL erythrocyte oxidative metabolism indicated a moderately higher susceptibility of MGL erythrocytes to oxidative stress (Fig. 2). This oxidative vulnerability may influence the K-Cl cotransport activity, despite normal GSH levels. This conclusion is consistent with the failure of NEM to further activate the already elevated K-Cl cotransport activity in MGL erythrocytes. It is also supported by the fact that the activity of K-Cl cotransport in mouse erythrocytes is only slightly modified by treatment with sulfhydryl group donors such as DTT or N-acetylcysteine (data not shown).

Present models of K-Cl cotransport regulation propose that activity is dependent on a phosphorylation-dephosphorylation cycle (9, 10, 52, 53). To evaluate whether erythrocyte Mg content may control the phosphorylation state of the transporter in vivo, we carried out experiments with OA, an inhibitor of PP1 and PP2A, and staurosorpin, a broad-specificity serine/threonine and tyrosine kinase inhibitor (9, 10, 35, 49, 52). Inhibition of K-Cl cotransport by OA in MGH and MGL erythrocytes shows that PP1/2A-dependent dephosphorylation reactions activate K-Cl cotransport in both strains.

Whereas the protein kinase inhibitor staurosorpin and the sulfhydryl reducing agent NEM enhanced K-Cl cotransport in MGH erythrocytes, the high basal cotransport activity of MGL erythrocytes was not further activated by these agonists. This result may reflect a common stimulatory pathway for staurosorpin, NEM, and low Mg, or it may merely reflect a maximum level of K-Cl cotransport activity in mouse erythrocytes.

Several observations support a role for Src family kinases in regulating erythroid K-Cl cotransport. K-Cl cotransport is positively regulated by a dephosphorylation reaction mediated by the serine/threonine phosphatase PP1 (9, 10, 35, 49, 52). Src family kinases, as well as other tyrosine kinases, phosphorylate and inhibit PP1 activity. PP1α is tyrosine phosphorylated and inhibited in cells overexpressing v-Src (54, 57). We implicated Src family kinases in regulation of K-Cl cotransport activity with our demonstration that K-Cl cotransport is activated in erythrocytes lacking Hck and Fgr kinases (23). High Src family kinase activity in MGH erythrocytes correlated with decreased activity of PP1α. Conversely, the reduced Src family kinase activity detected in MGL erythrocytes was accompanied by enhanced PP1α activity. Furthermore, although we were unable to detect tyrosine phosphorylation in vivo, we showed that c-Fgr phosphorylates rabbit muscle PP1α in vitro (Fig. 9).

Taken together, these findings suggest that Src family kinases act as upstream negative regulators of PP1 and that their increased activity downregulates K-Cl cotransport activity. In vivo modulation of K-Cl cotransport activity by Mg may thus be explained by regulation of Src family kinase activities, which consequently affect PP1 function (Fig. 10). We conclude that Src family kinases and PP1 play a coordinated role in regulation of K-Cl cotransport and erythrocyte volume. These phosphotransferases therefore represent new targets for the pharmacological correction of erythrocyte dehydration in sickle cell disease, β-thalassemia, and other pathological conditions.

The authors thank Angela Siciliano and Patrizia Pattini for excellent technical assistance.

This work was supported by Telethon Grants E792 and E491 (G. Berton) and E1112 (E. Villa-Moruzzi) and National Institutes of Health Grants HL-15157 (C. Brugnara and S. L. Alper) and DK-50422 and HL-64885 (C. Brugnara). S. L. Alper was an Established Investigator of the American Heart Association.

REFERENCES


