Overexpression of stomatin depresses GLUT-1 glucose transporter activity

JIN-ZHONG ZHANG,1 WAFA ABBUD,1 RAINER PROHASKA,2 AND FARAMARZ ISMAIL-BEIGI1
1Departments of Medicine and of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106-4951; and 2Institute of Medical Biochemistry, University of Vienna, Vienna Biocenter, A-1030 Vienna, Austria

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Zhang, Jin-Zhong, Wafa Abbud, Rainer Prohaska, and Faramarz Ismail-Beigi. Overexpression of stomatin depresses GLUT-1 glucose transporter activity. Am J Physiol Cell Physiol 280: C1277–C1283, 2001.—We showed previously that GLUT-1 glucose transporter is associated with stomatin (band 7.2b) in human red blood cell membranes and in Clone 9 cells. We show here that in a mixed population of stably transfected cells, overexpression of either murine or human stomatin resulted in 35–50% reduction in the basal rate of glucose transport. Moreover, there was a correlation between increased expression of stomatin and depression in the rate of glucose transport. In two clones chosen for further study, the ~10% and ~70% reduction in basal rate of glucose transport was associated with increases in stomatin mRNA and protein expression without a detectable change in GLUT-1 content in plasma membranes of either clone. In the clone overexpressing high levels of stomatin, immunoprecipitated GLUT-1 was associated with a large amount of stomatin as a coimmunoprecipitant. Employing extracts of cells overexpressing human stomatin, we found that stomatin bound to the glutathione-S-transferase (GST) fusion protein containing the COOH-terminal 42-amino acid segment of GLUT-1 but not to GST alone or a GST fusion protein containing the 66-amino acid central loop of GLUT-1. Rat stomatin cDNA was cloned by RT-PCR and found to be highly homologous to mouse (97%) and human (86%) stomatins. These results suggest that overexpression of stomatin results in a depression in the basal rate of glucose transport by decreasing the “intrinsic” activity of GLUT-1, probably through protein-protein interaction.

GLUT-1-glutathione-S-transferase fusion protein; transfection

FACILITATIVE, Na+-independent transport of glucose across the plasma membrane of mammalian cells is mediated by a family of glucose transporter proteins (13, 15). In most mammalian cells, glucose transport is rate limiting for glucose metabolism, and an increase in the rate of glucose metabolism induced by hormones and other stimuli necessitates a stimulation of glucose transport (13). The acute stimulation of glucose transport associated with no change in the total cellular GLUT content is mediated by either “translocation” or “activation” of glucose transporters (13). For example, translocation of GLUT-4 transporters from intracellular compartments to the plasma membrane accounts in large part for the enhancement of glucose transport in response to insulin (3, 15, 26). In contrast, the acute stimulation of glucose transport mediated by GLUT-1 in response to a number of stimuli and physiological perturbations appears to be mediated mainly through activation of GLUT-1 transporters preexisting in the plasma membrane; the mechanisms underlying this response are less well understood (20, 21, 32). Interestingly, a similar conclusion was reached in recent studies on insulin-mediated stimulation of glucose transport where it was found that an activation step is necessary before the translocated GLUT-4 molecules manifest an increase in their transport activity (9, 10, 27).

While the molecular nature of activation itself remains undefined, the concept of activation can be operationally defined as 1) an increase in the number of functionally active sites by a release of inhibition, or “unmasking” of sites, or 2) an increase in the catalytic turnover number of previously functional sites by an increase in their “intrinsic” activity (13). Irrespective of the underlying mechanism, a depression in the basal rate of glucose transport followed by its stimulation would be registered as an exaggerated response, i.e., a higher degree of activation. Nevertheless, the relationship, if any, between mechanisms underlying the control of basal rate of glucose transport and activation of transporters is not known.

In previous studies we have found that GLUT-1 is associated with stomatin in Clone 9 and HepG2 cells and in human red blood cell (RBC) plasma membranes (31). Stomatin is a 31-kDa integral membrane protein named after stomatocytosis, a rare human hemolytic anemia (7, 25). The protein was first identified in human RBC membranes as a major component of band 7 (band 7.2b) (11, 29). Subsequently, it was shown that stomatin is expressed in a wide variety of cells and tissues (8, 11, 29). cDNA cloning and amino acid se-

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Address for reprint requests and other correspondence: F. Ismail-Beigi, Clinical and Molecular Endocrinology, Case Western Reserve Univ., Cleveland, OH 44106-4951 (E-mail: fxi2@po.cwru.edu).
quence analysis have suggested that the protein is a monotopic membrane protein with ~92% of its amino acids being intracellular (12, 18). More recently, it has been demonstrated that stomatin is a homooligomer composed of 9–12 identical subunits (23). The absence of the protein in RBCs of some patients is associated with a massive leak of Na⁺ and a corresponding stimulation of Na⁺-K⁺-pump activity (24, 25), although the phenotype was absent after genetic disruption of the gene in mice (33). Stomatin is expressed in an appropriate temporal and spatial manner to participate in a putative vertebrate mechanotransduction complex (14), and a stomatin homologue, unc-1, has been reported to control the sensitivity of neurons to volatile anesthetics in Caenorhabditis elegans (17). Nevertheless, although the wide distribution of stomatin suggests an important role for the protein, its physiological function remains unknown.

We have recently reported our preliminary findings that overexpression of murine stomatin in a mixture of stably transfected Clone 9 cells results in a decrease in the basal rate of glucose transport (31). Clone 9 cell is a rat liver cell line that expresses GLUT-1 as the only glucose transporter isoform and in which glucose transport is rate limiting for glucose metabolism. In the present study we verify and extend these observations. A report of some of our findings has been presented (30).

MATERIALS AND METHODS

Materials. Polyclonal antibody (RAM-ST) directed against the NH₂-terminal region of mouse stomatin (VSQRIIPESF-RENSKTELGC; starting at position 10) and monoclonal antibody (GARP-50) directed against the NH₂-terminal peptide of human stomatin (AQRLPDSFKDSPSKG; starting at position 12) were prepared by Hiebel-Dirschmied et al. (11). Rabbit anti-GLUT-1 IgG was purchased from Chemicon (Temecula, CA). Reagents for cell culture, RT-PCR, and secondary antibodies were obtained from GIBCO BRL (Gaithersburg, MD). The ECL (enhanced chemiluminescence) Western blotting detection kit and 3-O-[3H]methyl-D-glucose (3-OMG) were from Amersham Life Science (Arlington Heights, IL). [α-32P]dCTP was purchased from NEN (Boston, MA). The random primed DNA labeling kit and Flag gene transfection kit were from Roche (Indianapolis, IN). Sulfo-NHS-SS-biotin was purchased from Pierce (Rockford, IL). pGEX-6P-2 plasmid and glutathione-S-transferase (GST) purification modules were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Other reagents were from Sigma (St. Louis, MO).

Cell culture, construction of plasmids, and transfection of Clone 9 cells. Clone 9 and BAC1 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM containing 10% calf serum (19). Human stomatin cDNA flanked by its 5'- and 3'-untranslated regions was subcloned into pcDNA3 expression vector (12). Construction of murine stomatin cDNA in pcDNA3 was carried out as described previously (31). After transfection and selection with G418 (31), mixtures of transfected cells and individual clones were employed for further study.

Glucose transport assay. Cytochalasin B-inhibitable 3-OMG uptake was carried out as described previously (20).

RNA isolation and Northern blots. Cytoplasmic RNA was isolated by employing a Nonidet P-40-containing buffer (19). Northern blots were probed with mouse stomatin cDNA that had been 32P labeled by the random priming method.

SDS-PAGE and Western blotting. Cell lysates and Western blots were prepared as described previously (31). Rabbit anti-GLUT-1 IgG was used at a 1:3,000 dilution. Anti-human stomatin monoclonal antibody (GARP-50) was used at a 1:50 dilution. Anti-mouse stomatin polyclonal antibody (RAM-ST) was used at a 1:500 dilution. Secondary antibodies were a 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit or anti-mouse IgGs.

Isolation of plasma membranes. Membrane fractions enriched with plasma membranes were isolated from Clone 9 cells transfected with plasmid expressing stomatin and from cells transfected with the empty vector; cell surface biotinylation with sulfo-NHS-SS-biotin was employed as described previously (20).

Immunoprecipitation. Lysates from Clone 9 cells overexpressing the mouse stomatin and from cells transfected with the empty vector were prepared and immunoprecipitated with anti-GLUT-1 monoclonal antibody as described previously (31).

Construction and expression of GST-GLUT-1 fusion proteins. Rat GLUT-1 cDNA fragments encoding the 66-amino acid central loop and 42-amino acid COOH-terminal segment of GLUT-1 were generated by PCR using the rat GLUT-1 cDNA as template (1). The resulting PCR products were cloned into pGEX-6P-2 and sequenced. Subsequent steps were performed according to the manufacturer’s protocol using GST purification modules. Lysates of Clone 9 cells overexpressing human stomatin were prepared as described previously (31) and were incubated with the column at 25°C for 10 min and then eluted by the addition of glutathione.

RT-PCR and sequencing of rat stomatin cDNA. In vitro reverse transcription reaction was carried out with the use of cytoplasmic RNA isolated from Clone 9 cells and random primers. PCR was performed with primers based on the mouse stomatin cDNA sequence (8). The upstream oligo was located 90 nt 5' of the translation start site and had the following sequence: 5'-tagagttgggacctcgagcag-3'. The downstream oligo was located at position 1228 to 1250 (305 nt after the stop codon) and had the following sequence: 5'-ttgacggtgctctgggact. The resulting PCR product was cloned into pCR2.1-TOPO (Invitrogen), and both strands were sequenced. The above PCR reaction was repeated three different times, yielding the same result upon DNA sequencing.

GenBank/European Molecular Biology Laboratories. The nucleotide sequence of the 1250-bp segment of rat stomatin cDNA including 61 bp of 5'-untranslated region and 305 bp of 3'-untranslated region has been submitted to GenBank/European Molecular Biology Laboratories.

Statistical analysis. Results are expressed as means ± SE. Student’s unpaired two-tailed t-test was used, and P < 0.05 was considered significant (22).

RESULTS

We have previously reported that overexpression of mouse stomatin in a mixture of stably transfected Clone 9 cells resulted in a 30 ± 3% reduction in the basal rate of glucose transport (31). In the present study, this finding was verified in an independent transfection experiment, in which we found a 35 ± 3% reduction in basal rate of glucose transport (P < 0.05) (Fig. 1). In addition, transfection with a plasmid containing the human stomatin cDNA resulted in a 50 ±
3% reduction in the rate of glucose transport in a mixture of stably transfected cells compared with either nontransfected cells or cells stably-transfected with the empty vector ($P < 0.05$) (Fig. 1).

We next isolated individual clones of cells that were stably transfected with the plasmid expressing the murine form of stomatin. In 12 individual clones, the basal rate of glucose transport was decreased by $\frac{10}{10} \text{ to } \frac{80}{80} \%$. There was a significant correlation between the cellular content of stomatin determined by Western blotting and the depression of glucose transport (Fig. 2).

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**Fig. 1.** Depression of basal rate of glucose uptake in Clone 9 cells transfected with mouse or human stomatin cDNA. Clone 9 cells were transfected with pcDNA3 containing mouse or human stomatin cDNA and selected with G418. Cells transfected with empty vector served as control. Cytochalasin B (CB)-inhibitable glucose uptake was measured with 3-O-[^H]methyl-d-glucose (3-OMG).

**Fig. 2.** Correlation between the expression of stomatin and rate of glucose uptake in individual clones of Clone 9 cells stably transfected with a plasmid expressing mouse stomatin. The content of stomatin in the lysate of individual clones was measured by Western blot with anti-stomatin antibody directed against mouse and rat stomatin sequence. CB-inhibitable glucose uptake was measured in parallel in triplicate with 3-OMG. The experiment was repeated, and the results were averaged. All data are normalized against values obtained in Clone 9 cells stably transfected with the empty vector assayed in parallel (shown at 1.0, 1.0). Clones $A$ and $H$, transfected with the plasmid containing stomatin cDNA, were selected for further study.

**Fig. 3.** Overexpression of stomatin in clones $A$ and $H$ of Clone 9 cells. **A:** CB-inhibitable glucose uptake measured with 3-OMG. **B:** Northern blot of RNA isolated from clones $A$ and $H$ probed with mouse stomatin cDNA. Asterisk denotes a possible exogenous transcript terminating at SV40 poly(A) signal, rather than BGH poly(A) signal, of the pcDNA3 plasmid. **C:** Western blots developed with an anti-stomatin polyclonal antibody directed against mouse (and rat) stomatin.

We next isolated individual clones of cells that were stably transfected with the plasmid expressing the murine form of stomatin. In 12 individual clones, the basal rate of glucose transport was decreased by $\sim 10 \text{ to } >80\%$. There was a significant correlation between the cellular content of stomatin determined by Western blotting and the depression of glucose transport (Fig.
2. Among the 12 clones, 7 clones showed a 50% or greater depression in the basal rate of glucose transport, and no clone had a rate higher than that of control cells transfected with the empty vector. Two of the clones transfected with the plasmid containing stomatin cDNA (Fig. 2, clones A and H) and a mixture of cells stably transfected with the empty vector were chosen for further study. Clones A and H exhibited, respectively, a \(~10\%\) and \(~70\%\) reduction in their rate of glucose transport compared with cells transfected with the empty vector (Fig. 3A). A dramatic increase in the content of mouse stomatin mRNA was observed in clone H as determined by Northern blotting; a lesser increase was also observed in clone A (Fig. 3B). The endogenous mRNA transcript of rat stomatin is \(~2.8\) kb, while the plasmid-derived transcript of mouse stomatin (devoid of most of its 3′-untranslated region) is \(~1.5\) kb (16). Western blot analysis performed with an anti-stomatin antibody that recognizes both the mouse and rat stomatin (see below) revealed that the content of stomatin was increased \(~1.8 \pm 0.1\)-fold in clone H (Fig. 3C). These results verify that the overexpression of stomatin mRNA and protein in the clones, especially in clone H, was associated with a significant depression in the rate of glucose transport.

The depression in the rate of glucose transport in cells overexpressing stomatin can be mediated by a variety of mechanisms, including decreased expression of GLUT-1 or retention of GLUT-1 intracellularly rather than its transfer to the plasma membrane. To examine this issue, we determined the content of GLUT-1 in cell lysates and plasma membranes of control and stomatin-overexpressing cells. As shown in Fig. 4, there was no detectable change in the content of GLUT-1 in either postnuclear lysates or plasma membranes isolated from control or stomatin-overexpressing cells. In repeated experiments, the content of GLUT-1 in plasma membranes of clones A and H was, respectively, \(0.9 \pm 0.1\) and \(0.95 \pm 0.1\) of that present in control cells (normalized to 1.0) \((n = 4, P > 0.4)\). Hence, the marked depression in GLUT-1-mediated glucose transport observed in clone H was not associated with any change in the expression of GLUT-1 or in its localization. 

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**Fig. 4.** Content of glucose transporter GLUT-1 in cell lysates and plasma membranes from clones A and H overexpressing stomatin and from control cells transfected with the empty vector. The content of GLUT-1 in lysates (postnuclear lysate 20 μg protein/lane) and plasma membranes (10 μg protein/lane) was measured by the Western blot technique with anti-GLUT-1 antibody.

**Fig. 5.** Increased association of stomatin with GLUT-1 in cells overexpressing stomatin. GLUT-1 was immunoprecipitated (IP) with anti-GLUT-1 monoclonal antibody. The amount of GLUT-1 immunoprecipitated (A) and the amount of stomatin coimmunoprecipitated with GLUT-1 (B) were revealed by Western blotting. 

**Fig. 6.** In vitro binding of stomatin to the COOH-terminal segment of GLUT-1. The central loop and COOH-terminal segments of GLUT-1, expressed as glutathione-S-transferase (GST) fusion proteins, were immobilized on glutathione-Sepharose 4B. Cell lysates from Clone 9 cells overexpressing human stomatin were incubated with the above-described columns. After elution, proteins were fractionated by SDS-PAGE and analyzed by Western blotting. A: blotting with anti-GST antibody. B: blotting with anti-human monoclonal antibody.
localization at the cell surface. These findings suggest that the transport function of GLUT-1 transporters in the plasma membrane had been negatively modulated in these cells.

Our previous observation that GLUT-1 and stomatin coimmunoprecipitate with each other from samples of RBC membranes and from lysates of Clone 9 cells (31) prompted us to determine whether the association between the two proteins is altered in cells overexpressing stomatin. To do this, we immunoprecipitated GLUT-1 from clone H and control cells and then analyzed the amount of stomatin in the pellet (Fig. 5). In clone H overexpressing stomatin, a higher amount of stomatin is associated with GLUT-1.

We next performed experiments to identify the regions of interaction between these two proteins. As an initial step, we focused on cytoplasmic domains of GLUT-1 (15). GLUT-1 has a relatively large intracellular central loop (66 amino acids) and a 42-amino acid cytoplasmic COOH-terminal domain (15). Stomatin also has a relatively large intracellular COOH-terminal domain that comprises most of the molecule (8, 14). We therefore determined whether either of these domains of GLUT-1 interacts with stomatin. To perform these studies, we prepared GST-fusion proteins containing these two regions of GLUT-1. Postnuclear lysates prepared from a mixed population of Clone 9 cells overexpressing human stomatin were incubated with columns containing the above fusion proteins or with the GST protein alone. After washing, eluted proteins were analyzed by Western blotting with the use of either anti-GST antibody (Fig. 6A) or anti-human stomatin antibody (Fig. 6B). Stomatin interacted with the COOH-terminal domain of GLUT-1 but not with either the central loop of GLUT-1 or GST alone.

DISCUSSION

These findings are of interest because the COOH-terminal domain of GLUT-1 has been reported to play an important role not only in localization of the transporter to the plasma membrane but also in the control of its transport activity (4, 5, 28). For example, deletion of the 42-amino acid COOH-terminal segment of GLUT-1 results in 95% loss of its function (5). In addition, while truncation of the COOH-terminal 24 amino acids has no effect on GLUT-1 expression and function, deletion of 25 (or more) residues results in a profound loss of activity (16). The COOH-terminal 42-amino acid peptide of GLUT-1 also contains at its COOH terminus a binding site for PDZ-domain-containing proteins (2, 6); however, the function of such binding is not known. Further studies are necessary to delineate the specific regions of interaction between GLUT-1 and stomatin and to determine the physiological significance of these sites in the control of GLUT-1 function.

It was important to determine the nucleotide and amino acid sequences of the rat stomatin because only the sequences of mouse and human stomatins have been described (8, 12). The cDNA of rat stomatin was amplified by RT-PCR as described in MATERIALS AND METHODS. Mouse stomatin sequence was from Muraoka et al. (16), and human stomatin sequence was from Rajaram et al. (17). Asterisks indicate conserved residues in all sequences in the alignment; colons indicate conserved substitutions; and periods indicate semiconserved substitutions. The peptide employed for generation of anti-mouse stomatin antibody (16) was identical to the sequence of the rat (amino acids 10–30: VQSQRRIPESFRENKTELGAC).
sequence (8). The generated cDNA included 90 bp of 5'–untranslated region and 305 bp of 3'–untranslated region of stomatin. The open reading frame was 284 amino acids in length (Fig. 7). At the level of the protein, rat and mouse stomatins are 97% homologous, and rat and human stomatins have an 86% homology. Likewise, there is an 88% homology between the murine and human stomatins.

The results of our studies suggest that overexpression of stomatin results in a depression of GLUT-1 function. This conclusion is based on the novel findings that the two proteins exhibit an affinity for each other and that overexpression of stomatin and the reduction in the basal rate of glucose transport are associated with no change in the cellular content of GLUT-1 or the content of GLUT-1 in plasma membranes. We also found that in cells overexpressing stomatin, there is a higher degree of association between the two proteins. The results are most consistent with the hypothesis that association of stomatin with GLUT-1 results in a decrease in the intrinsic activity of GLUT-1, suggesting that stomatin potentially represents a "GLUT-1-masking" protein. As such, stomatin represents the first GLUT-1-binding protein that appears to modulate masking protein. As such, stomatin represents the first GLUT-1-binding protein that appears to modulate GLUT-1 activity by stomatin

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