Research Article

A Single Amino Acid Substitution in the Renal Betaine/GABA Transporter Prevents Trafficking to the Plasma Membrane

Christopher R. Day, Sashana S. Gordon, Cherissa L. Vaughn, and Stephen A. Kempson

Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Medical Science Building 385, 635 Barnhill Drive, Indianapolis, IN 46202-5120, USA

Correspondence should be addressed to Stephen A. Kempson; skempson@iupui.edu

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One response to hypertonic stress in the renal medulla and MDCK cells is the upregulation of betaine transporter (BGT1) synthesis, followed by trafficking to the plasma membrane (PM) and an increase in betaine transport. Upregulation of BGT1 was enhanced by inhibitors of phosphatases PP1 and PP2A and was attenuated by inhibitors of protein kinase C, suggesting an important role for phosphorylation reactions. This was tested using mutants of BGT1 tagged with EGFP. The PM trafficking motifs of BGT1 reside near the C terminus, and truncation at lysine⁵⁶⁰ resulted in a protein that remained intracellular during hypertonic stress. This K560Δ mutant colocalized with endoplasmic reticulum (ER). Substitution of alanine at Thr⁴⁰, a putative phosphorylation site, also prevented trafficking to the PM during hypertonic stress. Live-cell imaging showed that T40A was not retained in the ER and colocalized with markers for Golgi and endosomes. In contrast, substitution of aspartate or glutamate at Thr⁴⁰, to mimic phosphorylation, restored normal trafficking to the PM. HEK293 cells transfected with K560Δ or T40A mutants had 10% of the GABA transport activity of native BGT1, but normal transport activity was restored in cells expressing T40E. Normal BGT1 trafficking likely requires phosphorylation at Thr⁴⁰ in addition to C-terminal motifs.

1. Introduction

The hypertonicity of interstitial fluid in the renal medulla, more than 1,000 mosmol/kg in humans, is essential for excretion of concentrated urine and for conserving water for the body. Mammalian cells in a hypertonic environment will lose water rapidly and shrink which, if prolonged, leads to misfolding and aggregation of proteins, cell cycle delay, apoptosis, and necrosis [1–3]. One cellular defense against hypertonicity is intracellular accumulation of organic compounds termed osmolytes which do not interfere with normal cell functions. As the intracellular osmolyte concentrations increase, the cell volume also increases due to osmotic entry of water, and the intracellular ionic strength is reduced. The process is slow, up to 24 h, because gene expression is involved. In renal medullary cells, which are chronically

exposed to hypertonic stress, both plasma membrane transporters for osmolytes (e.g. betaine, *myo*-inositol, and taurine) and intracellular enzymes for synthesis of other osmolytes (principally sorbitol and glycerophosphorylcholine) are transcribed and synthesized. Upregulation of a heat shock protein (HSP70) helps to protect cells from the damaging effects of high concentrations of urea [4]. These processes are driven by a transcription factor (termed TonEBP or NFAT5) that accumulates in the nucleus during onset of hypertonic stress [5–8]. Activation of transcription is likely due to signaling by multiple kinase pathways [9–12].

Little is known about the protein trafficking steps that are required for insertion of newly synthesized osmolyte transporters in the plasma membrane of renal medullary cells. Control of this process may be another critical step in upregulation of osmolyte accumulation during hypertonic

stress [13]. Phosphorylation steps appear to be required for successful targeting of some plasma membrane transport systems such as aquaporin-2 [14], anion exchanger 1 [15], a urea transporter [16], and NKCC [17] in kidney cells during hypertonic stress. This study is focused on hypertonic upregulation and trafficking of the renal betaine/GABA transporter (BGT1). The primary role of BGT1 in the kidney is to transport the osmolyte betaine, but it can also accept GABA as a substrate [18], and GABA has been used extensively in this laboratory to monitor the transport activity of BGT1 [19, 20]. The primary structure of BGT1 contains potential phosphorylation sites on intracellular loops [21], and there is some evidence for regulation *in situ* by protein kinase C [22, 23]. Our goal was to determine the potential importance of phosphorylation in the initial upregulation of BGT1 transport activity during adaptation to hypertonic stress.

2. Methods

Cell lines were purchased from American Type Culture Collection, Rockville, MD, USA. Madin Darby canine kidney (MDCK, #CCL-34) cells were used between passages 15 and 40 and were grown in a 1:1 mixture of DMEM-Ham's F12 containing 10% bovine calf serum penicillin (100 IU/mL) and streptomycin (100 µg/mL). Cultures were maintained in an atmosphere of 5% CO₂ in air. The same medium was used for growth of human embryonic kidney (HEK293, #CRL-1573) cells. Cells were grown on coverglasses for microscopy and in plastic 24-well plates for transport studies. The transport activities of BGT1, amino acid transport system A (ATA2), and taurine (TauT) in MDCK cells were measured as Na⁺-dependent uptakes of [³H]GABA, [¹⁴C]methylaminoisobutyric acid, and [³H]taurine, respectively, by whole cells as described previously [19, 20, 24, 25].

BGT1 tagged at the N terminus with enhanced green fluorescent protein (EGFP, Clontech) was previously shown to behave and function identically to native BGT1 when expressed in MDCK cells [19, 26]. Using the Prosite database (ExPASy Proteomics Server, Swiss Institute of Bioinformatics), four potential phosphorylation sites for PKC were identified on cytoplasmic regions of BGT1. These sites (T40, T235, S418, and S564) were highly conserved in rat, mouse, human, and dog BGT1, based on multiple sequence alignments. Single mutations were created at each site by substituting the neutral amino acid alanine (A) in EGFP-BGT1. Additional substitutions at T40 were made with glutamate (E) or aspartate (D) to add a charged group to mimic phosphorylation at this site [27]. Lastly, native EGFP-BGT1 was terminated at K560 by introducing a stop codon to produce a mutant (K560Δ), which lacked the basolateral plasma membrane targeting motifs near the C terminus of BGT1, namely, the 8 residues in positions 565–572 [28]. The same sequence is needed also for exit from the endoplasmic reticulum [28], so the K560Δ mutant will be expected to colocalize with a marker for this organelle, as a positive control. Mutations were performed by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) in the laboratories of Dr. G. Burckhardt (University of Göttingen, Germany) and Dr. S. Rhodes (Indiana University

School of Medicine, IN, USA) and were confirmed by DNA sequencing.

After plating on 25 mm coverglasses for 24 h, subconfluent MDCK cells were cotransfected with EGFP-BGT1 (or mutant) and one of several organelle markers each tagged with mCherry (red fluorescence, Clontech). The endoplasmic reticulum marker was calnexin, and the Golgi marker was the N-terminal 45 amino acids (135 nucleotides) of human sialyltransferase (SiT). The endosomal marker was RhoB-GTPase from Clontech. All were tagged with mCherry in the laboratory of Dr. M. Davidson (Florida State University, Tallahassee, FL, USA). The GeneJammer transfection reagent was used according to the protocol provided by the manufacturer (Stratagene), and cells were returned to the CO₂ incubator for 24 h before further use. Following transfection, the coverglasses were placed in growth medium lacking phenol red to minimize background fluorescence and made hypertonic (500 mOsm) by NaCl addition for 4 h. The coverglasses were processed in the same medium for live-cell epifluorescence imaging using an ISS ALBA workstation (ISS Inc. Champaign, IL, USA) equipped with an Olympus IX71 inverted microscope. EGFP was imaged with excitation at 488 nm and emission at 500–550 nm. Excitation and emission for mCherry were 587 nm and 610 nm, respectively.

In a separate study, some batches of MDCK cells grown on coverglasses were transfected with either native EGFP-BGT1 or the T40A mutant and were subjected to hypertonic stress (500 mOsm). After 5 h, the cells were prepared for immunohistochemistry and confocal microscopy by fixation in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated for 60 min with primary antibodies to furin (Santa Cruz Biotechnology, 1:100 dilution), an endoprotease used as a marker for the trans-Golgi network [29]. This was followed by 30 min incubation with goat anti-rabbit IgG conjugated to Cy5 (1:200 dilution, Jackson ImmunoResearch, West Grove, PA, USA). Excitation and emission for Cy5 were 650 and 670 nm, respectively.

Delivery of EGFP-BGT1 or mutants to the plasma membrane was assessed by two methods. First, by expression in HEK293 cells which lack endogenous BGT1. After transfection overnight, the cells were checked for transfection efficiency by visual inspection of fluorescence, followed by incubation in hypertonic (450 mOsm) growth medium for 6 hr to induce trafficking of the expressed protein to the plasma membrane [19]. A 6 h exposure to osmolality of 450 mOsm was chosen, because HEK293 cells proved to be less tolerant of hypertonic stress than MDCK cells. Plasma membrane localization of the expressed transport protein was determined indirectly based on measurement of Na⁺-dependent [³H]GABA uptake by whole cells. Second, the same mutants were expressed in MDCK cells for direct visualization of subcellular distribution by confocal microscopy following overnight hypertonic stress (500 mOsm) and paraformaldehyde fixation.

Transport data are expressed as the mean ± SD of at least three separate experiments, and within each transport experiment the mean value (*n* = 1) was derived from triplicate measurements. Where appropriate, different groups

FIGURE 1: Incubation of MDCK cells for 3 h in hypertonic (Hyp) medium containing calyculin (0–30 nM), an inhibitor of protein serine/threonine phosphatases, stimulated transport activity of BGT1 but not amino acid transport system ATA2 or taurine transport (TauT). Data are mean $\pm$ SD of 3–7 experiments. *Significantly different ($P < 0.05$) from isotonic (Iso) controls.

were compared by Student's *t*-test or by analysis of variance and Tukey's test for multiple comparisons. All images are representative of several cells on each coverglass.

3. Results and Discussion

When phosphorylation reactions were enhanced in MDCK cells by use of inhibitors of serine and threonine phosphatases PP1 and PP2A [17], this was a dose-dependent stimulation of BGT1 transport suggesting that phosphorylation steps were required for upregulation of BGT1 transport during hypertonic stress. For example, calyculin applied when MDCK cells were switched to hypertonic medium (500 mOsm) for 3 h caused an increase in Na⁺/GABA cotransport that was significant at 3 nM calyculin (Figure 1). Note that there was no significant upregulation in the untreated controls (0 nM), because a 3 h exposure to hypertonicity is not sufficient to upregulate BGT1 transport activity. Although plasma membrane insertion of some BGT1 proteins can be detected by 2 h hypertonicity [19], a period of more than 10–12 h is required

in order to measure significant upregulation of whole cell transport activity [30]. Notably, there was no effect of calyculin on the transport activity of either ATA2 and TauT which, like BGT1, are located in the basolateral plasma membrane of MDCK cells.

Use of okadaic acid, an alternative phosphatase inhibitor in MDCK cells [31], produced similar findings. Within 5 h after switching MDCK cells to hypertonic medium containing okadaic acid, there was significant stimulation of Na⁺/GABA cotransport in response to 0.3 μ M okadaic acid (Figure 2). In contrast, the amino acid transport system ATA2 remained unaltered.

A complementary approach was to use inhibitors of protein kinase C (PKC), as well as long-term downregulation by phorbol ester, to determine if direct blockade of phosphorylation by this enzyme would inhibit hypertonic activation of BGT1 transport activity. The inhibitors tested were Go6976 which targets the classical protein kinases C α and C β [32] and staurosporine which targets a broad spectrum of protein kinases in MDCK cells [33]. In addition, overnight (16 hr) incubation with phorbol 12-myristate 13-acetate (PMA) also

FIGURE 2: Inhibition of protein serine/threonine phosphatases by 5 h incubation in hypertonic medium containing okadaic acid (0–0.3 μM) stimulated transport activity of BGT1 but not amino acid transport system ATA2. Data are mean ± SD of 3–4 experiments. *Significantly different ($P < 0.05$) compared to isotonic controls.

FIGURE 3: MDCK cells were incubated overnight (16 h) in isotonic or hypertonic medium and in the absence (controls C) or presence of conditions that inhibit or downregulate protein kinase C activity. The latter impaired hypertonic upregulation of BGT1 transport but not ATA2 or TauT. *Significantly different ($P < 0.02$, $n = 3$) compared to hypertonic controls. Final concentrations were 10 μM for Go6976 (Go), 50 nM for phorbol 12-myristate 13-acetate (PMA), and 0.3 μM for staurosporine (Stau).

FIGURE 4: Na⁺-dependent GABA transport by HEK 293 cells after transfection with EGFP-tagged BGT1 or mutants followed by hypertonic stress (450 mOsm) for 6 h. Controls were transfected with empty EGFP vector. Data are mean $\pm$ SD of 6–8 separate experiments. *Significantly different ($P < 0.02$) compared to controls.

was used to downregulate PKC activity in MDCK cells [34]. As before, these drugs were applied at the same time as cells were switched to hypertonic medium and BGT1 transport activity was measured 16 h later. As expected after prolonged hypertonicity, BGT1 transport activity was increased 10-fold in hypertonic controls, but the upregulation was significantly reduced in the presence of each of the tested drugs (Figure 3). In contrast, there was no effect on the ATA2 and TauT transport systems, indicating specific action on BGT1.

These initial data do not yield any new information on the possible effect of hypertonic stress on protein phosphatase activity. Overall they provide indirect support for a potential role for phosphorylation in the upregulation of BGT1 transport during hypertonic stress. To determine if direct phosphorylation of BGT1 was required, we tested the behavior of four different EGFP-tagged mutants in which potential PKC phosphorylation sites in BGT1 were blocked by substitution with alanine. The mutants were expressed in MDCK cells, subjected to hypertonic stress (500 mOsm) overnight (16 h), followed by fixation in paraformaldehyde and processing for confocal microscopy. Three of the mutants, namely, T235A, S418A, and S564A were localized primarily in the plasma membrane identical to the distribution of native EGFP-BGT1. In contrast, the fourth mutant, T40A, remained intracellular. The same was true for the truncated mutant K560Δ which lacks plasma membrane targeting motifs. Substitution of T40 with glutamate (T40E) or aspartate (T40D) to mimic phosphorylation at this site fully restored the normal localization at the plasma membrane (data not shown).

The absence of T40A from the plasma membrane was confirmed by whole cell transport studies following transfection of the EGFP-tagged mutants in HEK293 cells that lack endogenous BGT1 [19]. Transport activity was determined after 6 h exposure to hypertonic medium (450 mOsm). Compared to controls transfected with empty vector, Na⁺/GABA cotransport was increased 15–20-fold in cells transfected with native BGT1. However, in marked contrast, transport activity

in cells expressing T40A was not different from the controls (Figure 4), confirming that T40A was not located at the plasma membrane. As expected, cells expressing the truncated mutant K560Δ also demonstrated a low level of Na⁺/GABA cotransport that was not different from the controls and T40A. In contrast, Na⁺/GABA cotransport in cells expressing T40E was not significantly different from cells expressing native BGT1 (Figure 4), confirming a normal plasma membrane location. T40D could not be tested because of a low expression level in HEK293 cells. Taken together these data in two different cell lines suggest that phosphorylation at T40 may be required for successful trafficking of BGT1 protein to the plasma membrane.

Finally, the intracellular location of T40A was examined in light of the possibility that it was simply trapped in the endoplasmic reticulum, because the alanine substitution caused misfolding of the expressed protein [35]. This appears unlikely since substitution of T40 with glutamate (T40E) or aspartate (T40D) did not impair normal trafficking to the plasma membrane. Live-cell epifluorescence imaging was used after cotransfection of MDCK cells with EGFP-tagged BGT1 or mutants and mCherry-tagged markers for endoplasmic reticulum, endosomes, or Golgi. The exposure to hypertonic stress (500 mOsm) was limited to 4 h, because we showed previously in live MDCK cells [19] that this time interval allows for easily observable localization of EGFP-BGT1 in both cytoplasm and plasma membrane. This was confirmed in the present study. While some EGFP-BGT1 remained in the cytoplasm, there was significant localization at the plasma membrane (green fluorescence, Figure 5(a)) and there was no colocalization with the red fluorescence of calnexin (endoplasmic reticulum), RhoB-GTPase (endosomes), or SiT (Golgi). As expected, the K560Δ mutant was not located in the plasma membrane, but its distribution almost completely overlapped calnexin, as indicated by the yellow color generated by the merging of green and red fluorescence (Figure 5(b)). There was no colocalization with RhoB-GTPase or SiT confirming that K560Δ remained in the endoplasmic reticulum due to loss of plasma membrane trafficking motifs and presumably was destined for degradation in the cytosol [35]. The T40A mutant also showed no plasma membrane localization and no colocalization with calnexin. However, there was significant colocalization with RhoB-GTPase and some colocalization with SiT (yellow color, Figure 5(c)). This strongly suggests that T40A is properly folded and assembled and exits the endoplasmic reticulum [35]. It is trafficked to the Golgi and endosomal compartments but cannot be inserted into (or retained in) the plasma membrane.

The intracellular location of T40A was examined also by immunohistochemistry of fixed MDCK cells labeled with an antibody to furin, an endoprotease in the trans-Golgi network. Prior to fixation, the cells were transfected with EGFP-tagged BGT1 or T40A overnight and subjected to hypertonic stress (500 mOsm) for 5 h. Confocal microscopy revealed colocalization of EGFP-tagged T40A with furin which was located using a secondary antibody tagged with Cy5 (Figures 6(b) and 6(c)). As in Figure 5, there was significant localization of native EGFP-BGT1 in the plasma

FIGURE 5: Epifluorescent images of live MDCK cells expressing EGFP-tagged BGT1 (a), K560 Δ (b), or T40A (c). Cells were cotransfected with mCherry-tagged markers (red fluorescence) for endoplasmic reticulum (calnexin), endosomes (RhoB-GTPase), and Golgi (sialyltransferase, SiT). Images were captured after hypertonic stress for 4 h.

membrane (Figure 6(a)) even after this relatively short period of hypertonic stress.

It might be argued that the localization of T40A protein with the endosomal compartment (Figure 5(c)) reflects rapid removal from the plasma membrane, because phosphorylation at T40 is required for stable plasma membrane insertion and attachment. However, it was reported previously that endocytic removal of BGT1 from the plasma membrane required PKC-mediated phosphorylation at T612 [23]. Consequently, our finding that inhibitors of PKC impaired the

normal upregulation of BGT1 transport activity in whole cells during overnight hypertonic stress (Figure 3) does not support this mechanism. Instead it supports the notion that normal trafficking of BGT1 to the plasma membrane requires normal PKC activity and phosphorylation at T40, in addition to C-terminal trafficking motifs. Pertinent additional data are the observations that hypertonic stress of hepatocytes activates isoforms of PKC which in turn can activate cation channels [36, 37]. This is the first report that a residue near the N terminus may play an important role in intracellular

FIGURE 6: Confocal images of EGFP-tagged native BGT1 or T40A mutant expressed in MDCK cells following hypertonic stress for 5 h. At the end of this period, the cells were fixed and immunostained for furin (red fluorescence), a marker for the trans-Golgi network. BGT1 showed significant localization in the plasma membrane (panel (a)). In contrast, T40A remained intracellular and colocalized with furin (arrows, panels (b) and (c)). Cell outline is shown in white.

trafficking of BGT1 protein. It remains to be determined if BGT1 is phosphorylated at T40 during hypertonic stress and if this is mediated by PKC. This will be addressed in future studies.

In summary, the control of BGT1 transport activity in kidney cells occurs at several levels starting with regulation of gene expression of the TonEBP transcription factor [38] and culminating with posttranslational regulation of abundance of BGT1 protein in the plasma membrane by insertion and retrieval [19, 23, 39]. Apart from residues 565–572 (28) and T612 [23], the function of specific regions within BGT1 has barely been explored, and an additional layer of complexity may emerge as the protein structure-function relationships are better understood. The BetP protein in certain bacteria is an example of such complexity. It is a Na^+ -coupled betaine transporter which, in addition to its transport function, is capable of both osmosensing and osmoregulation [40].

This is the first report suggesting that posttranslational modification may be involved in BGT1 regulation. It is possible that similar modifications also upregulate the transport proteins responsible for accumulation of other osmolytes, such as *myo*-inositol (SMIT) and taurine (TauT), but there have been no reports so far. Unlike BGT1, the system A amino acid transporter (ATA2) is upregulated much more rapidly by hypertonic stress, so that amino acids can be accumulated as an interim source of osmolytes [41]. Similar to BGT1, the density of ATA2 functioning on the cell surface is reduced by endocytotic sequestration and proteasomal degradation [39, 42].

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