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PDZ-mediated interactions retain the epithelial GABA transporter on the basolateral surface of polarized epithelial cells

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The PDZ target motifs located in the C-terminal end of many receptors and ion channels mediate protein–protein interactions by binding to specific PDZ-containing proteins. These interactions are involved in the localization of surface proteins on specialized membrane domains of neuronal and epithelial cells. However, the molecular mechanism responsible for this PDZ protein-dependent polarized localization is still unclear. This study first demonstrated that the epithelial γ-aminobutyric acid (GABA) transporter (BGT-1) contains a PDZ target motif that mediates the interaction with the PDZ protein LIN-7 in Madin–Darby canine kidney (MDCK) cells, and then investigated the role of this interaction in the basolateral localization of the transporter. It was found that although the transporters from which the PDZ target motif was deleted were still targeted to the basolateral surface, they were not retained but internalized in an endosomal recycling compartment. Furthermore, an interfering BGT peptide determined the intracellular relocation of the native transporter. These data indicate that interactions with PDZ proteins determine the polarized surface localization of target proteins by means of retention and not targeting mechanisms. PDZ proteins may, therefore, act as a sort of membrane protein sorting machinery which, by recognizing retention signals (the PDZ target sequences), prevents protein internalization.
Keywords: cell polarity/endocytosis/neurotransmitter transporters/PDZ-containing proteins/protein sorting

Introduction

In order to exert their vectorial functions, polarized cells require the selective localization of distinct populations of receptors, ion channels and carriers on their site of functional residence. Many of these surface proteins have been shown to contain conserved sequences in their C-terminal end, which are target motifs recognized by the modular protein-binding sites found in membrane-associated proteins (called PDZ domains because they were recognized initially as sequence repeats in PSD-95, Dlg and ZO-1 proteins) (Saras and Heldin, 1996). PDZ target motifs are characterized by a C-terminal hydrophobic residue and a free carboxylate group at the –2 position (T/S-X-V/L) in relation to the C-terminus. The specificity of the binding seems to be conferred by residues at the –2 to –4 positions of the target proteins (Pawson and Scott, 1997), and these interactions have been found to be involved in the supramolecular organization of receptors and signal transduction pathways (Sheng, 1996; Craven and Bredt, 1998).

Various groups have shown recently that PDZ complexes play a role in sorting their interacting proteins to specialized membrane domains of neuronal and epithelial cells (Simske *et al*., 1996; Kim, 1997; Craven and Bredt, 1998; Rongo *et al*., 1998), both of which have two compositionally and functionally distinct plasma membrane domains: axonal and somatodendritic in the former, and apical and basolateral in the latter. In order to polarize their surfaces, these cells require the continuous sorting of newly synthesized and internalized plasma membrane proteins. This sorting can occur by means of direct targeting and/or retention mechanisms.

Sorting by direct targeting takes place at the level of the *trans*-Golgi network (TGN) and endosomal compartments, in which the sequences (sorting signals) contained in the proteins and specifying their final destination are recognized (Matter and Mellman, 1994). So far, targeting signals have been investigated mainly in epithelial cells: basolateral targeting signals reside in cytoplasmic domains, and can be divided into those that are co-linear with clathrin-coated pit signals and those that are not (Matter *et al*., 1994; Keller and Simons, 1997); apical signals appear to be luminal and, in some cases, have been shown to rely on lipid (glycosyl-phosphatidylinositol anchor) modifications (Lisanti *et al*., 1988; Rodriguez-Boulan, 1992) or asparagine-linked carbohydrates (Scheiffele *et al*., 1995; Gut *et al*., 1998).

Retention mechanisms operate on the final surface domain, and have the function of preventing protein internalization. A similar mechanism is used by hepatocytes, in which all proteins are first delivered to the basolateral membrane (where resident basolateral proteins are retained), and the apical membrane proteins are internalized and then redirected to the apical surface (Bartles *et al*., 1987). The signals responsible for the surface retention of transmembrane proteins have been little investigated, but one example is Na, K-ATPase, whose retention in the correct plasma membrane domain seems to be determined by interactions with the membrane cytoskeleton (Mays *et al*., 1995).

It has been shown that many apical and basolateral proteins localize on the axonal and somatodendritic surfaces of neurons, thus indicating that both epithelial and neuronal cells make use of similar sorting mechanisms to polarize their surfaces (Dotti and Simons, 1990; Dotti *et al.*, 1991): a representative example is the GLR-1 glutamate receptor, which is postsynaptic in neurons and localizes basolaterally when ectopically expressed in *Caenorhabditis elegans* epithelial cells (Rongo *et al.*, 1998). Interestingly, receptor localization in both cell types is mediated by LIN-10 which, together with LIN-2 and LIN-7, forms an evolutionarily conserved tripartite protein complex that is involved in fundamental aspects of protein sorting in epithelial and neuronal cells (Bredt, 1998; Butz *et al.*, 1998; Kaech *et al.*, 1998; Rongo *et al.*, 1998).

In order to investigate whether this complex mediates sorting by means of a targeting or retention mechanism, we analysed the role of the LIN-7-mediated interactions in the basolateral localization of an epithelial γ-aminobutyric acid (GABA) transporter (BGT-1, hereinafter BGT) in Madin–Darby canine kidney (MDCK) cells, a well characterized polarized epithelial cell line. BGT belongs to the Na, Cl-dependent neurotransmitter transporter gene family, and is expressed in renal medulla and on the basolateral surface of hypertonically cultured MDCK cells (Yamauchi *et al.*, 1991), in which it accumulates the small betaine osmolite. We have shown previously that BGT, which is exogenously overexpressed in MDCK cells, contains basolateral sorting signals in its cytosolic C-terminal domain (Perego *et al.*, 1997); we show here the presence and function of a typical (T-X-L) PDZ target motif. The T-X-L residues, as well as those that confer PDZ domain binding specificity, are identical in BGT and *C.elegans* LET-23 receptor tyrosine kinase. The PDZ target motif of LET-23 mediates binding to LIN-7 (Kaech *et al.*, 1998), whose expression is required in order to localize the receptor to the basolateral membrane of worm epithelial cells (Simske *et al.*, 1996). Mouse homologues of *C.elegans* LIN-7 have been identified and named LIN-7A (or Veli 2) and LIN-7 B (or Veli 3) (Butz *et al.*, 1998; Kaech *at al.*, 1998). The only previously identified partner of LIN-7 was the *C.elegans* LET-23, but we demonstrate here that the epithelial GABA transporter is a mammalian target of LIN-7 in MDCK cells. The interaction of BGT with LIN-7 is mediated by the last five residues in the C-terminal end of BGT, and is required for the exclusively basolateral localization of the transporter. When this interaction is prevented by means of the removal of the PDZ target motif from the transporter or the injection of an interfering peptide in MDCK cells, the transporter reaches the basolateral surface but instead of being retained, it is internalized in an endosomal recycling compartment partially overlapping that containing the transferrin receptor. These data indicate a novel role for PDZ proteins as membrane protein sorting machinery that promotes the selective retention of membrane proteins in the plasma membrane by sequestering them from pathways of internalization. The cells may therefore regulate surface protein density by modulating protein interactions with PDZ proteins.

Results

The last five amino acids of BGT mediate the interaction with the PDZ protein LIN-7

In order to verify whether the last five amino acids of BGT contain a functional PDZ target motif that mediates protein–protein interaction, we tested the ability of the C-terminal end of BGT to bind LIN-7, a single PDZ protein that has been shown to recognize a very similar PDZ target motif in the *C.elegans* LET-23 tyrosine kinase receptor (Kaech *et al.*, 1998). Figure 1B shows the *in vitro* interaction between a peptide consisting of the last 17 amino acids of BGT conjugated to Sepharose beads and a bacterial lysate expressing the recombinant GST–LIN-7 fusion protein. The peptide containing the PDZ motif of BGT (17mer) efficiently retained the worm LIN-7 and mouse LIN-7A fusion proteins, whereas the peptides in which the motif was removed (14mer) or mutagenized (19mer) (Figure 1A) did not show any interaction with the BGT peptide. Binding was also prevented by an excess of free 17mer peptide in the incubation reaction, and no specific binding was detected when an unrelated peptide was used.

To investigate the putative interaction of BGT and LIN-7 *in vivo*, we used an antibody raised against mLIN-7A (A.Hajnal, in preparation). This antibody recognizes a single band of the expected mol. wt (26 kDa) in MDCK lysate (Figure 1C), and immunofluorescence experiments have shown that it has a basolateral staining pattern similar to that of BGT (data not shown). When the BGT C-terminal peptides conjugated to Sepharose beads were incubated with MDCK lysate, the 17mer (but not the 14mer and 19mer) synthetic peptide bound LIN-7 (Figure 1C), thus indicating that the epithelial GABA transporter is a partner of LIN-7 in MDCK cells.

To confirm the *in vivo* interaction of LIN-7 and BGT, a co-immunoprecipitation experiment was performed in MDCK cells expressing wild-type BGT (Figure 1D). When the specific BGT antibody was used in the immunoprecipitation assay, a protein of 26 kDa (recognized by the antibody raised against mLIN-7A) was co-immunoprecipitated. However, although LIN-7 protein is expressed abundantly in MDCK cells (Figure 1D, lysate), only a small amount was co-immunoprecipitated. One possible explanation for this is that the LIN-7–BGT interaction is weak and the conditions necessary for extracting both proteins do not maintain the complex, as has been reported in the case of other LIN proteins (Cohen *et al.*, 1998). Moreover, LIN-7 probably binds multiple proteins via its PDZ domain, and so it may be that only a small fraction is complexed with BGT in MDCK cells.

The PDZ target sequence of BGT is not required to localize the transporter on the basolateral surface of MDCK cells

Having demonstrated that the PDZ target motif of BGT mediates the physical interaction with LIN-7 in MDCK cells, we investigated the role played by this interaction in the basolateral localization of BGT. To this end, two alternative approaches to preventing the BGT–LIN-7 interaction were adopted: (i) the removal of the PDZ target motif from BGT; and (ii) the injection of an excess of the 17mer BGT peptide in MDCK cells overexpressing

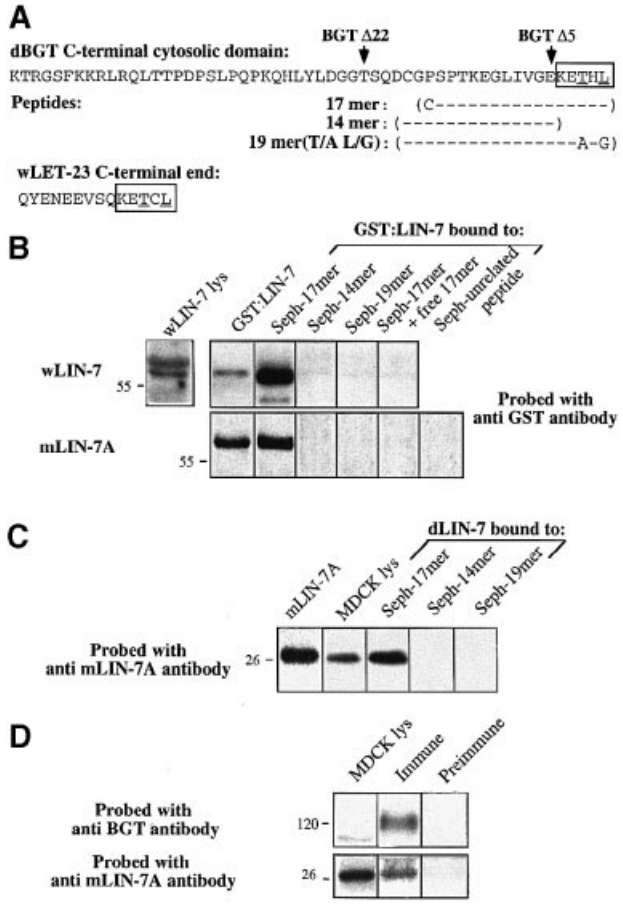

Fig. 1. The PDZ target motif of the epithelial GABA transporter mediates the interaction with LIN-7. (**A**) Sequence of the C-terminal cytosolic domains of the dog BGT (dBGT) and *C.elegans* LET-23 (wLET-23) receptor. The two arrows indicate the last amino acid contained in the BGT Δ22 and Δ5 transporters, the binding region to LIN-7 is boxed (compare with that in wLET-23) and the most conserved residues among PDZ motifs are underlined. The dashed lines indicate the amino acids contained in the BGT synthetic peptides used in the interaction assays; the cysteine extra sequence added at the N-terminus of the 17mer and the mutagenized residues in the 19mer are also indicated. (**B**) Binding of worm LIN-7 or mouse LIN-7A (GST:LIN-7) to BGT peptides immobilized to CNBr-activated Sepharose 4B. The bound GST–LIN-7 fusion proteins were detected using the monoclonal antibody directed against GST. Ten percent of the total wLIN-7 fusion protein used in the binding experiments is shown in the first lane. GST–LIN-7 purified on glutathione–Sepharose 4B from bacterial lysates was probed in the same blot as the control. The 55 kDa mol. wt standard is indicated. (**C**) Binding of dog LIN-7 (dLIN-7) to immobilized BGT peptides; 10% of the cell lysate used in the binding assay is shown in lane 2. The bound LIN-7 was detected by immunostaining the blot with the antibody raised against mLIN-7A. This antibody recognizes the mouse LIN-7A digested from the GST fusion protein (lane 1) and a single band of the same mol. wt in the MDCK cell lysate (lane 2). (**D**) Co-immunoprecipitation of BGT and LIN-7 in MDCK cells. MDCK cells expressing BGT were lysed and immunoprecipitated with an antiserum to BGT or a pre-immune serum. The immunoprecipitates were immunoblotted with anti-BGT and anti-mLIN-7A antibodies, as indicated. Two percent of the cell lysate used in the immunoprecipitation assay is shown in lane 1. The BGT antibody (but not the pre-immune serum) was able to co-immunoprecipitate LIN-7 from the MDCK cell lysate.

wild-type BGT. In the case of the first approach, the sorting behaviour of truncated BGT (BGT Δ5 and Δ22; Figure 1A) stably expressed in MDCK cells, was analysed functionally and immunohistochemically. [^{3}H]GABA uptake and immunofluorescence experiments (Figure 2A and B) showed that the truncated transporters maintained the same sorting behaviour as the original BGT. In the experiments shown in Figure 2A, MDCK cells grown on permeable Transwell filters were incubated with [^{3}H]GABA added to the apical or basolateral medium compartments. The GABA influxes through the basolateral surface were higher (75–85%) than those through the apical surface, regardless of whether they expressed wild-type or truncated transporters. The basolateral localization of the transporters was supported further by the results of double immunofluorescence experiments using antibodies directed against BGT (BGT-KLH, red staining) and the α-subunit of Na, K-ATPase used as a basolateral marker (mAb 6H, green staining) (Figure 2B). The yellow colour obtained by merging horizontal confocal sections revealed the co-localization of both wild-type and truncated transporters with the basolateral marker (upper panels); however, unlike the wild-type, the truncated transporters were also distributed in the perinuclear subapical region. Spot-like staining reminiscent of intracellular vesicular structures, which was particularly enriched in the perinuclear cell regions, could be clearly seen when the cells were grown at a lower level of confluence on glass coverslips (Figure 2B, lower panels). The intracellular localization of the truncated transporter was found to be independent of the level of transporter expression (data not shown). The removal of the PDZ motif therefore affected the regular transport of the carrier protein to or from the basolateral cell surface, but not its targeting.

The removal of the PDZ target sequence does not affect the rate of protein maturation and degradation

In order to analyse the step at which the mutant transporters accumulate in these intracellular structures, we followed the passage of wild-type and truncated transporters along their biosynthetic and degradative pathways by means of pulse–chase experiments coupled to glycosidase digestions and immunohistochemical experiments. Transporters metabolically labelled for 3 h with [^{35}S]methionine and [^{35}S]cysteine migrated in SDS–PAGE as two bands of 60 and 120 kDa, which glycosidase digestions revealed to correspond to the immature (endoplasmic reticulum; ER) and the fully glycosylated dimeric mature transporter, respectively. The rate of maturation and degradation was measured by following the conversion from the lower (immature BGT) to the higher (mature BGT) band, and the disappearance of the latter. These experiments revealed that maturation and degradation of both wild-type and truncated transporters occurred at the same time points of the chase experiments (Figure 3A), thus arguing against intracellular accumulation of truncated transporters during these processes. Furthermore, confocal analysis revealed no co-localization of the truncated transporters with markers of the biosynthetic (ER, Golgi apparatus) and degradative pathways (late endosomes, lysosomes) (Figure 3B). Similar rates of transport along the biosynthetic pathway were also observed in experiments using MDCK cells injected with the cDNAs of wild-type and Δ5 transporters (Figure 3C). The wild-type and truncated carrier proteins were both transported slowly along the ER and Golgi compartment before accumulating on the plasma membrane. These experiments also revealed

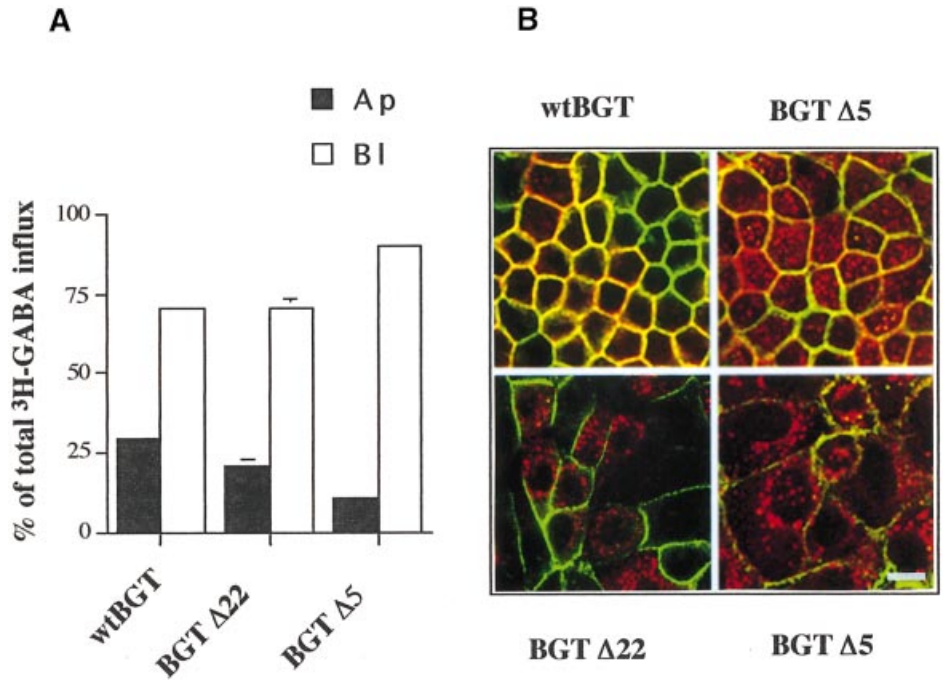

Fig. 2. The PDZ target motif of BGT is required for the exclusive basolateral localization of the transporter. (**A**) Functional analysis of the surface distribution of the wild-type and truncated BGT revealed by [^{3}H]GABA influx measurements. Similar levels of total activity (5 pmol/min/sample) were measured in the wild-type and truncated transporters. The values are expressed as the percentage of total cell surface transporter activity, and represent the means ± SE of at least three independent experiments performed in duplicate. Ap, apical surface; Bl, basolateral surface. (**B**) Immunofluorescence analysis. After ice-cold methanol fixation, the cells grown to confluent density on Transwell filters (upper panels), or to subconfluent density on glass coverslips (lower panels), were double stained with the polyclonal BGT-KLH (red) and the monoclonal 6H anti-α1 Na, K-ATPase (green) antibodies. Merged confocal horizontal sections (taken in a subapical region of the cells in the right upper panel) show co-localization of the transporters with the basolateral marker α1 Na, K-ATPase, as revealed by the yellow staining. Whereas the wild-type transporter has an exclusively basolateral localization, the truncated transporter also accumulates in large intracellular perinuclear structures, located in the subapical region of confluent cells. Bar, 7 μm.

the simultaneous appearance of BGT Δ5 in the intracellular structures and on the cell surface (24 h; Figure 3C).

The PDZ target motif of BGT is required to retain the transporter on the basolateral surface

The nature and function of the intracellular structures containing truncated transporters was assessed immunohistochemically and biochemically. In the former assay, internalized wheat germ agglutinin (WGA)-labelled surface glycoproteins were found to accumulate in the intracellular structures containing truncated transporters (Figure 4A), thus indicating that these structures are located along the endocytic pathway. Similar labelled structures were observed in cells not expressing BGT Δ5, which suggests that they are not generated by the overexpression of the truncated transporter (see asterisk in Figure 4A). A biotin assay for endocytosis made it possible to demonstrate that the mutated transporters are internalized, and to measure the kinetics of this internalization. Basolateral surface transporters labelled with a cleavable biotin analogue (NHS-SS-biotin) at 0°C were internalized at 37°C, and the extracellular biotin was removed by cleaving the disulfide linkage with glutathione, a membrane-impermeant thiol-reducing agent. The amount of labelled transporter resistant to glutathione reduction was taken as a measure of its internalization. As shown in Figure 4B, both the wild-type and truncated surface transporters were sensitive to reduction directly after labelling (0 chase + GTH), but only the wild-type BGT remained sensitive after a chase at 37°C. Maximum insensitivity to glutathione reduction was measured after 30 min of chase (Figure 4B), which indicates that most of the labelled truncated transporters are internalized during this time. Interestingly, a large number of the biotinylated truncated transporters apparently regain sensitivity to glutathione reduction after 1 h of chase. Given the unaffected protein stability, and that only 20–25% of biotin is removed in the absence of glutathione treatment over the period of time analysed (data not shown), this result is consistent with the recycling of the transporter to the basolateral plasma membrane. In an attempt to characterize this compartment further, we followed the internalization of Texas Red-labelled transferrin for 30 min at 37°C in MDCK cells expressing BGT Δ5. Confocal microscopy analysis showed partial co-localization of the truncated transporter with transferrin, which was particularly clear in the perinuclear region, but absent at the cell periphery (Figure 5). Co-localization of the transporter with the internalized transferrin was also revealed in the subapical region of cells grown to confluence on Transwell filters (data not shown), thus indicating that the intracellular compartment in which the transporter is accumulated largely corresponds to the recycling endosomal compartment.

We also prevented the interaction of BGT and LIN-7 by injecting synthetic peptides into the cytoplasm of MDCK cells overexpressing the wild-type transporter (Figure 6). The BGT 17mer peptide relocated the wild-type transporter in large perinuclear structures (>80% of the injected cells expressing BGT showed intracellular

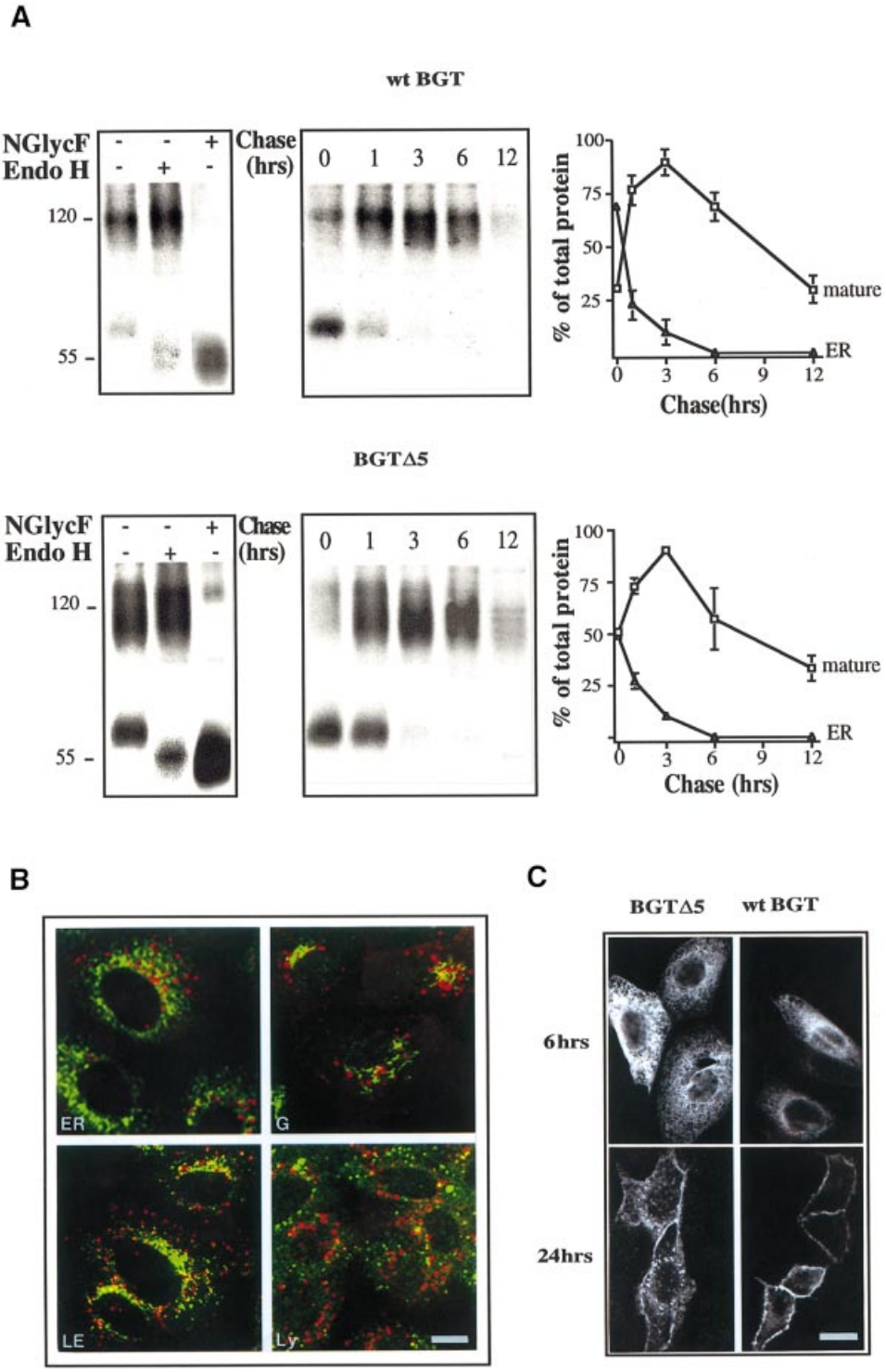

Fig. 3. Removal of the PDZ target motif of BGT does not affect the rate of maturation and degradation of the transporter. (**A**) Pulse–chase and glycosidase digestion experiments. Wild-type and truncated transporters metabolically labelled for 3 h were chased at 37°C and analysed on 10% SDS–PAGE after immunoprecipitation with the BGT-KLH antibody. Where indicated, after labelling, the transporters were deglycosylated with endoglycosidase H (Endo H) or *N*-glycosidase F (NGlycF). The positions of the molecular weight standards are indicated in kDa. Expressed as the percentage of total protein [immature (60 kDa) + mature (120 kDa) transporters at t_0 of chase], the quantitation reflects the average of at least duplicate experiments, one of which is represented by the gels shown in the figure. (**B**) Double immunofluorescence staining of MDCK cells expressing BGT Δ5. The red staining identifies the transporter, and the green the ER, the Golgi apparatus (G), the late endosomes (LE) and the lysosomes (Ly). No co-localization of the truncated transporter with the analysed markers was revealed by merging the confocal horizontal sections. Bar, 5 μm. (**C**) Immunofluorescence staining of MDCK cells injected with wild-type or BGT Δ5 cDNAs. Six hours after the injection, newly synthesized transporters were found in the ER, but it was only after 24 h that they were accumulated on the cell surface. The BGT Δ5, together with its surface appearance, also localized in the intracellular perinuclear structures. Bar, 10 μm.

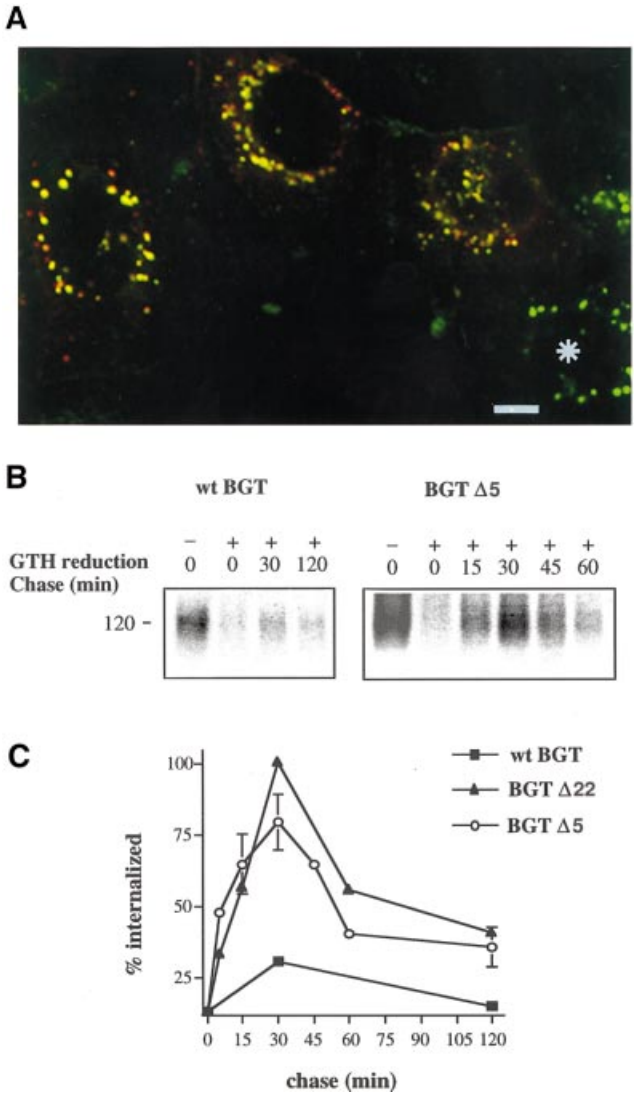

Fig. 4. Transporters without the PDZ target motif are not stable on the cell surface and accumulate in an endocytic compartment. (**A**) Truncated transporters co-localize with internalized FITC-labelled glycoproteins. MDCK cells stably expressing BGT Δ5 were labelled with FITC–WGA at 0°C, and chased at 37°C for 30 min prior to fixation and staining with the BGT-KLH antibody. The merged confocal horizontal section shows co-localization (yellow staining) of BGT Δ5 (red) with the internalized labelled glycoproteins (green). The asterisk indicates a cell not expressing the truncated transporter. Bar, 4 μm. (**B**) The PDZ target sequence prevents BGT internalization. MDCK cells expressing wild-type or truncated transporters grown to confluence on Transwell filters were metabolically labelled. The basolateral surface transporters were biotinylated and, where indicated (+), reduced with glutathione prior to immunoprecipitation with BGT-KLH. The quantitation (performed by scanning the developed fluorograph) reflects the average of at least two (wild-type and Δ22) or five (Δ5) independent experiments, one of which is represented in the figure. The values obtained were corrected for the loss of biotin measured in the absence of glutathione in the control samples. Truncated but not wild-type transporters internalize and then recycle to the basolateral surface.

staining of the transporter), whereas an unrelated 17mer peptide, as well as the 19mer and 14mer peptides (data not shown), did not affect its surface localization (virtually no intracellular staining was observed in the injected cells). These experiments further demonstrate that native BGT interacts with LIN-7 through its PDZ target motif in MDCK cells, and that this interaction is required to retain the transporter on the surface.

Discussion

Role of PDZ-mediated interactions in localizing plasma membrane proteins in specialized surface domains

Our data demonstrate that the epithelial GABA transporter contains a functional PDZ target motif that is required for its association with the canine homologue of LIN-7A. This interaction is not necessary for targeting the transporter to the basolateral surface of MDCK cells, but is essential for retaining it on that surface. Polarized transporter distribution is therefore a consequence of targeting and retention mechanisms existing in distinct compartments, and which rely on distinct motifs (Matter and Mellman, 1994). We show that only the retentional mechanism is dependent on the PDZ motif, and so one or more other motifs recognized at the level of the TGN and/or endosomes must determine the polarized distribution of BGT.

It has been shown previously that the proper localization of other surface proteins depends on PDZ-mediated interactions, but the molecular mechanisms responsible for this localization are still unknown. It has been reported that the postsynaptic localization of the GLR-1 glutamate receptor and the basolateral localization of the LET-23 tyrosine kinase receptor in *C.elegans* require the expression of their interacting PDZ proteins LIN-10 and LIN-7 (Simske *et al.*, 1996; Rongo *et al.*, 1998), and that the localization of the Shaker potassium channel at the neuromuscular junction is controlled by Disk-Large (Dlg), a *Drosophila* homologue of PSD-95 (Zito *et al.*, 1997). Our data indicate that PDZ interactions make use of retention and not targeting mechanisms to operate the selective localization of the target receptors and ion channels, thus suggesting a general mechanism to localize surface proteins in polarized cells. PDZ target motifs are therefore retention signals recognized by PDZ proteins that function as membrane protein sorting machinery. The binding of PDZ proteins to the retention signal of the target protein determines a plasma membrane localization, whereas a lack of binding determines the removal of the protein from the cell surface (internalization).

Role of PDZ-mediated interactions in the regulation of surface protein density

Our results also suggest that PDZ-mediated interactions play a role in the regulation of target protein surface density.

Surface proteins are internalized via distinct endocytic processes, the most studied of which uses clathrin and adaptor protein complex to sequester receptors into coated pits at the level of the plasma membrane. This internalization is signal mediated and usually involves critical aromatic (usually tyrosine) residues positioned in a context of one or more amino acids with large hydrophobic side chains (Mellman, 1996). A second class of coated pit localization signals involves di-leucine residues, or leucine plus another small hydrophobic amino acid. Two potential internalization signals are present in the cytosolic tail of BGT, one containing a tyrosine residue (position –27 in relation to the C-terminus) and the other

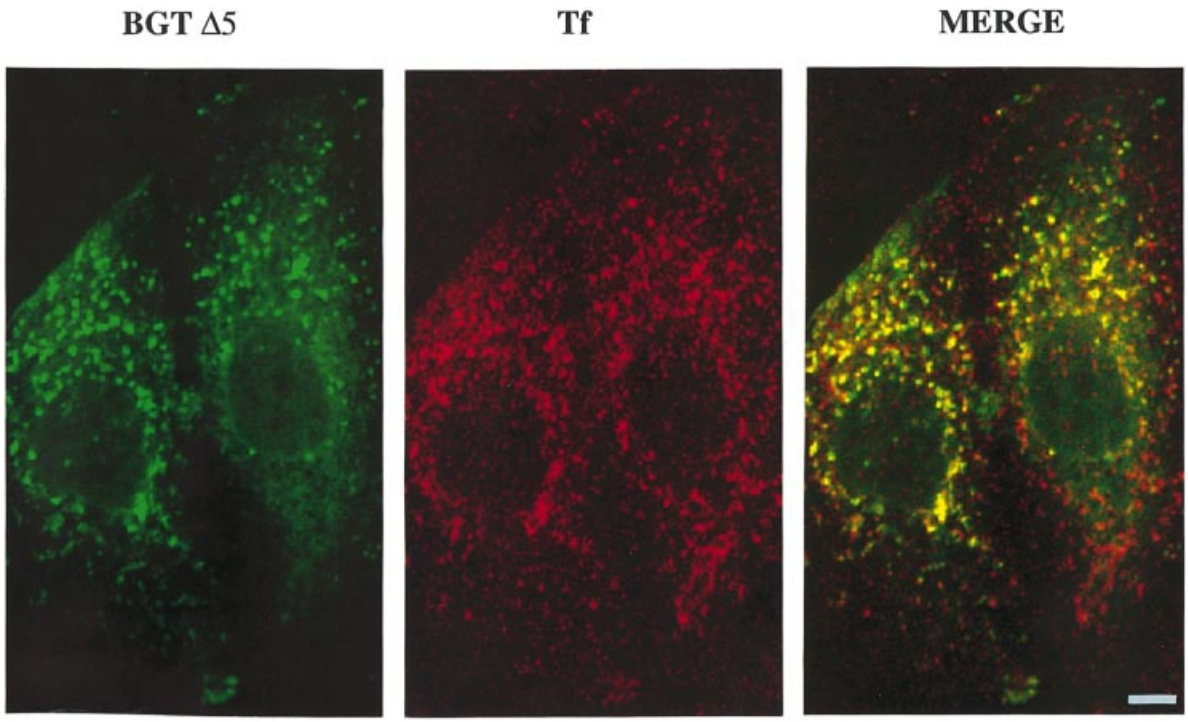

Fig. 5. Partial co-localization of BGT Δ5 with the transferrin-containing perinuclear recycling compartment. Texas Red–transferrin uptake experiments were performed in pre-confluent MDCK cells expressing BGT Δ5 for 30 min at 37°C before fixation and immunostaining. Confocal analysis revealed partial co-localization of the truncated transporter with perinuclear Texas Red–transferrin-labelled vesicles. No apparent co-localization was found at the cell periphery. Bar, 3 μm.

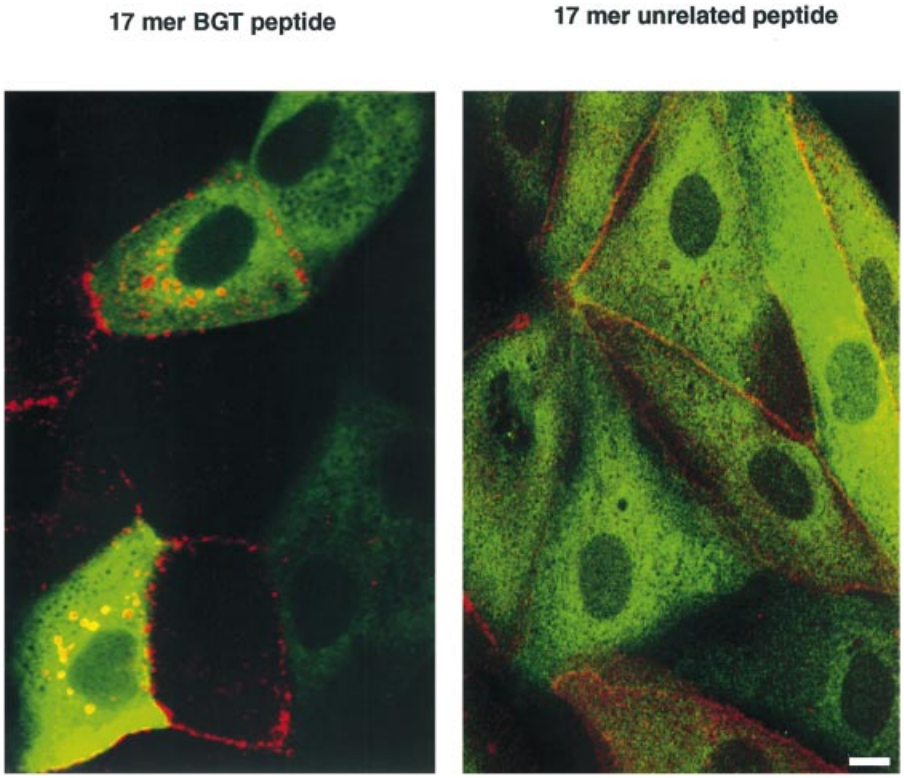

Fig. 6. The LIN-7-mediated interaction is responsible for the cell surface stability of wild-type BGT in MDCK cells. The cells were microinjected with the 17mer peptide or 17mer unrelated peptide and, 30 min later, processed for immunofluorescence. Merged confocal horizontal sections show the injected cells (green) and those expressing the transporter (red). The 17mer peptide (but not the unrelated peptide) interfered with the BGT–LIN-7 interaction, thus determining the intracellular relocation of the wild-type transporter. Bar, 4 μm.

belonging to the di-leucine-type (positions –9 and –10) (see Figure 1). The removal of the di-leucine type (BGT Δ22) or the tyrosine-containing sequence from the BGT Δ5 construct (data not shown) did not affect the internalization of the truncated transporters, thus indicating that each signal is sufficient by itself or that neither is required for transporter internalization. These results did not make it possible to distinguish whether BGT internalization depends on these or other signals masked by the PDZ interaction, or whether it occurs by default when this interaction is prevented.

After internalization, the molecules can be recycled selectively to the cell surface or routed to the degradative pathway, processes that take place within the early endosomes located in the cell periphery (also called sorting endosomes). Recycling molecules can be redirected rapidly to the plasma membrane or enter the recycling endosomal compartment located in the cell perinuclear region. These distinct recycling pathways have different kinetics, with recycling from the sorting endosomes being faster than that from the perinuclear compartment (Gruenberg and Maxfield, 1995; Mellman, 1996). The transferrin receptor and ligand are markers of both the fast and the slow recycling pathways. The recycling endosomes of non-polarized cells have been shown to correspond to MDCK cell apical recycling endosomes, which receive markers that are internalized from both the apical and basolateral surfaces (Odorizzi *et al.*, 1996). We found that the truncated transporter at least partially co-localizes with labelled transferrin in the perinuclear subapical region of polarized MDCK cells, whereas very little if any co-localization was seen at the cell periphery; this suggests that the intracellular compartment in which the truncated transporters accumulate may correspond to the apical recycling endosomes. On the other hand, the loading/unloading kinetics of the recycling compartment containing the truncated transporters (~30 min) are slower than those measured for the transferrin receptor (<10 min). These data together suggest that the truncated transporters might reach and possibly leave the apical recycling compartment via routes other than transferrin. Further experiments are required in order to identify the internalization mechanisms involved and further characterize the recycling compartment in which truncated transporters accumulate.

Our experiments showing that the 17mer peptide competes with BGT for the interaction with LIN-7 are in line with data obtained in other systems indicating the dynamic regulation of PDZ-mediated interactions (Cohen *et al.*, 1996; Hall *et al.*, 1998; Xiao *et al.*, 1998). In a recently proposed model for explaining the regulation of the α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA; GLuR2)-mediated synaptic transmission triggered by the binding of *N*-ethylmaleimide-sensitive fusion protein (NSF) to the AMPA receptor, the authors suggest that NSF binding might cause the disruption of the PDZ complex between the AMPA receptor and the synaptic PDZ protein GRIP, thus leading to the recycling of the receptor between the postsynaptic membrane and an intracellular pool (Nishimune *et al.*, 1998; Osten *et al.*, 1998). Our data demonstrate that preventing (or interfering with) the formation of the PDZ complex leads to surface protein internalization and recycling, and therefore suggest the mechanism by which PDZ complexes may regulate the surface density of key molecules involved in synaptic transmission.

Materials and methods

Constructs

BGT Δ22. The last 22 C-terminal amino acids were removed from the cDNA encoding myc-BGT (Perego *et al.*, 1997). A fragment containing an in-frame stop codon was originated by the insertion of a T in position 1895 of the BGT in a PCR using BGT as a template. The PCR product was digested with *Kpn–Cla*I and ligated into similarly digested pCB6-mycBGT.

BGT Δ5. The last five C-terminal amino acids were removed from the cDNA encoding myc-BGT by means of cassette replacement. The *Kpn–Cla*I fragment of myc-BGT was removed and replaced with a cassette in which an in-frame stop codon was originated by replacing an A with a T in position 1947 of the BGT.

The absence of unwanted substitutions in the mutant clones was checked by sequencing. The sequences of all of the primers are available on request.

Cell culture and transfection

The MDCK (strain II) cells were cultured and transfected as described previously (Perego *et al.*, 1997). The expression of recombinant proteins was assayed initially by GABA uptake and then by immunofluorescence (Perego *et al.*, 1997). At least two independent clones were analysed for each recombinant cell line. For the polarity studies, transfected MDCK cells were grown to confluence for >5 days on Transwell filter inserts with 0.4 μm pores (Costar).

Antibodies and immunocytochemistry

The wild-type and truncated transporters were immunolocalized with the anti-peptide rabbit polyclonal antibody (BGT-KLH) or the monoclonal antibody (mAb) 9 E10 raised against the c-myc epitope (Oncogene Science). The α1 isoform of the Na, K-ATPase was localized using mAb 6H. The production and characterization of BGT-KLH and 6H antibodies have been described previously (Pietrini *et al.*, 1992; Perego *et al.*, 1997). The following antibodies were also used: the mAb against protein disulfide isomerase (PDI) as an ER marker (StressGen); the polyclonal antibody against Giantin as a Golgi marker (a gift from Dr M.Renz) (Seelig *et al.*, 1994); the polyclonal antibody against the bovine mannose-6-phosphate receptor as a late endosomal marker (a gift from Dr B.Hoflack) (Griffiths *et al.*, 1990); the polyclonal antibody against human cathepsin D as a lysosomal marker (a gift from Dr C.Isidoro) (Isidoro *et al.*, 1997); and the affinity-purified polyclonal antiserum raised against amino acids 86–207 of mLIN-7A as a means of localizing the endogenous protein in MDCK cells (a detailed characterization of this antibody will be published elsewhere; A.Hajnal, personal communication). Fluorescein isothiocyante (FITC)-conjugated anti-mouse IgG and Texas Red-conjugated anti-rabbit IgG from Jackson Immunoresearch (West Grove, PA) were used as secondary reagents. The confocal images were obtained using a Bio-Rad MRC-1024 confocal microscope. The micrographs were taken using either a Focus Imagecorder Plus (Focus Graphics Inc.) on Kodak film, or a Professional Color Point 2-dye sublimation printer (Seiko).

Biochemical binding assays

The expression of wLIN-7 and mLIN-7A fused to GST and inserted into the pGEX-1 vector (Kaech *et al.*, 1998) was induced in *Escherichia coli* DH5α. The fusion proteins were purified on glutathione–Sepharose 4B (Pharmacia) following the manufacturer's protocol. Approximately 2 μg of fusion proteins contained in 0.3 ml of bacterial lysate were incubated with ~20 μg of peptides immobilized on CNBr-activated Sepharose 4B (Pharmacia). In competition experiments, 60 μg of free peptides were pre-incubated with the bacterial lysate for 2 h at room temperature before overnight incubation with the Sepharose-bound peptides. Four washes at 4°C were carried out using 1.5 ml of phosphate-buffered saline (PBS) containing 0.5% Triton X-100 (50% of the detergent concentration of the bacterial lysate). The proteins bound to the Sepharose beads were solubilized, loaded onto 10% SDS–PAGE and blotted. The bound GST–LIN-7 fusion proteins were detected using the monoclonal antibody directed against GST (Santa Cruz Comp). For binding of the canine LIN-7 to immobilized BGT peptides, the MDCK cell lysate containing ~3×10^6 cells was incubated with 20 μg of

immobilized peptides, and the bound LIN-7 was detected by immunostaining the blot with the mLIN-7A antibody.

Immunoprecipitation

MDCK cells expressing wild-type BGT were washed twice with cold PBS and solubilized in lysis buffer [25 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM dithiothreitol (DTT), 5 mM EDTA, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and a cocktail of protease inhibitors]. After centrifugation at 14 000 r.p.m. for 15 min, the lysates were incubated overnight with BGT-KLH antibody (1:100) or preimmune serum at 4°C; 50 µl of protein A–Sepharose was added, and the immunocomplexes bound to beads were recovered after 2 h, washed four times with lysis buffer, separated by SDS–PAGE and analysed by immunoblotting with anti-BGT or mLIN-7A antibodies.

Pulse–chase and glycosidase digestion experiments

Confluent cells grown on 24 mm Transwell filters were starved for 30 min in Dulbecco's modified Eagle's medium (DMEM) without cysteine and methionine, and metabolically labelled for 3 h with 0.1 mCi/ml of Trans^{35}S-label (ICN Pharmaceuticals, Costa Mesa, CA) in starving medium (Lisanti *et al.*, 1989). The filters were washed at 4°C and chased for different times in culture medium at 37°C. The cells were lysed, and the transporters immunoprecipitated by incubation with BGT-KLH antibody (1:200), followed by a second incubation with protein A conjugated to Sepharose beads (Pierce) (Pietrini *et al.*, 1994). The glycosidase-digested transporters were eluted from the protein A–beads by boiling in 0.5% SDS, and then diluted 4-fold in digestion buffer [1% octyl glucoside and a cocktail of protease inhibitors in 100 mM Na-citrate buffer pH 5.7 for endoglycosidase H (Endo H) or in 100 mM Na-phosphate buffer pH 7.4 for *N*-glycosidase F (NGlycF)]. The transporters were deglycosylated by means of the addition of 8 mU of Endo H or 1.6 U of NGlycF in a total volume of 40 µl. The digests were then incubated overnight at 37°C. The transporters were solubilized and heated in SDS solubilization buffer. The reduced proteins were analysed on 10% SDS–PAGE.

Endocytosis assays

Immunohistochemical assay. Subconfluent cells grown on coverslips were incubated on ice with FITC–WGA in PBS + 0.1 mM Ca^{2+} + 1 mM Mg^{2+} + 1% bovine serum albumin (BSA) for 1 h. The cells were washed to remove unbound labelled lectin, chased at 37°C for different times, and then fixed in 4% paraformaldehyde.

Biotin assay. Confluent cells grown on 24 mm Transwell filters were starved and metabolically labelled overnight in starving medium supplemented with a 1/10 vol. of culture media (Lisanti *et al.*, 1989). The basolateral surface proteins were biotinylated with a cleavable biotin analogue, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3 dithiopropionate (NHS-SS-biotin; Pierce Chemical Co.), and reduced with glutathione as described by Graeve *et al.* (1989). The cell extracts were immunoprecipitated as described above, but the transporters were released from protein A–Sepharose beads by means of boiling in 10 µl of 10% SDS and then reprecipitated with streptavidin beads (Pierce) before being analysed in 10% SDS–PAGE. The biotinylated proteins were quantitated by scanning the developed fluorographs.

Internalization of Texas Red-conjugated transferrin. BGT Δ5-expressing MDCK cells grown to subconfluence on coverslips were incubated for 30 min at 37°C with 50 µg/ml Texas Red-labelled transferrin (Molecular Probes) in a serum-free medium containing 1% BSA. The coverslips were then rinsed and fixed in 4% paraformaldehyde, and indirect immunofluorescence was used to detect the transporter.

Microinjection experiments

The microinjection experiments were performed in MDCK cells grown on coverslips to a subconfluent density. In each experiment, 100–150 cells were selected and solutions containing 0.1 mg/ml of cDNAs or 4 mg/ml of synthetic peptides and 1 mg/ml of FITC–dextran were injected into the cell nuclei or cytoplasm, using an injection system (Eppendorf) and commercial glass microcapillaries with an outlet diameter of 0.5 ± 0.2 µm (Femtotips; Eppendorf).

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