The COOH-terminal tail of the GAT-2 GABA transporter contains a novel motif that plays a role in basolateral targeting

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Submitted 9 July 2003; accepted in final form 10 December 2003

Brown, Andrea, Theodore Muth, and Michael Caplan. The COOH-terminal tail of the GAT-2 GABA transporter contains a novel motif that plays a role in basolateral targeting. Am J Physiol Cell Physiol 286: C1071–C1077, 2004.—The ability of polarized epithelia to perform vectorial transport depends on the asymmetrical distribution of transmembrane proteins among their plasma membrane domains. The establishment and maintenance of these polar distributions relies on molecular signals embedded in the proteins themselves and the interpretation of these signals by cellular sorting machinery. Using Madin-Darby canine kidney (MDCK) cells as an in vitro model of polarized epithelia, our laboratory has previously shown that the COOH-terminal cytoplasmic 22 amino acids of the GAT-2 isoform of the γ-amino butyric acid (GABA) transporter are necessary for its basolateral distribution. We demonstrate that the COOH-terminal tail of the transporter can function as an autonomous basolateral distribution signal, independently of the rest of the transporter. We find that the three-amino acid PDZ domain-interacting motif at the COOH-terminus of GAT-2 is not necessary for its basolateral distribution. Instead, the more proximal seven amino acids are necessary both for targeting and for steady-state distribution. Because this sequence resembles no other known basolateral sorting information, we conclude that these seven amino acids contain a novel basolateral targeting and distribution motif. GABA transporter; traffic; sorting signal; targeting signal

EPITHELIAL CELLS DISTIBUTE their surface transmembrane proteins with polarity, restricting them to either the apical or the basolateral surfaces of their plasma membranes. This asymmetrical distribution is required for the directional and selective transport of fluid and solutes across epithelia, which in turn regulates the composition of body fluids. The generation and maintenance of this asymmetry require that membrane proteins contain encoded localization information and that cells possess mechanisms capable of interpreting this information. Much remains to be learned about the molecular nature of this information and about the cellular machinery that responds to it.

Whereas glycosyl phosphatidylinositol (GPI) and carbohydrate modification have been shown to serve as signals encoding the apical distribution of several proteins (4, 6, 12, 37), the information responsible for distributing proteins to the basolateral membrane appears to be embedded exclusively within the amino acid sequences of these proteins. Tyrosine- and dileucine-based motifs present in the cytoplasmic tails of a number of proteins specify the basolateral distributions of these proteins (21, 22, 24, 29). In the case of the Caenorhabditis elegans LET-23 receptor (5, 15), a PDZ-binding motif present at the extreme COOH-terminus of the protein mediates stable basolateral localization.

Previous experiments from our laboratory have examined the distribution information in the GAT-2 isoform of the γ-amino butyric acid (GABA) transporter, a polytopic membrane protein. Initial studies of the basolateral sorting signals of GAT-2 were conducted by deleting and exchanging portions of the basolateral GAT-2 and apical GAT-3 GABA transporters. These two transport proteins are 65% identical, and yet they exhibit dramatically different subcellular localizations. The COOH-terminal 32 amino acids of the GAT-3 transporter are necessary for its apical localization; without them, the transporter is present equally at the apical and basolateral membranes of Madin-Darby canine kidney (MDCK) cells. The COOH-terminal 22 amino acids of GAT-2 are necessary for its basolateral distribution, and their removal results in its random cell surface distribution. When the COOH-terminal 32 amino acids of the GAT-3 transporter are replaced with the COOH-terminal 22 amino acids of GAT-2, the transporter accumulates at the basolateral surface. This observation demonstrates that these 22 amino acids are necessary and sufficient to ensure the basolateral localization of the GAT-2 transporter at steady state in MDCK cells (26). In the present study, we endeavored to determine whether the basolateral signal in the COOH-terminal tail of GAT-2 can function autonomously in the absence of other GABA transporter sequences. We also determined which portions of the tail sequence comprise the basolateral distribution signal and asked whether this information accounts for the localization of the protein by specifying its initial biosynthetic targeting or by stabilizing its basolateral steady-state distribution.

To investigate these questions, we appended the entire COOH-terminal tail of the GAT-2 GABA transporter to PLAP-TMR, a membrane protein construct composed of the transmembrane domain (TMR) of the vesicular stomatitis virus G (VSVG) protein and the human placental alkaline phosphatase (PLAP) extracellular domain. Previous studies have established that the GPI anchor normally attached to PLAP is required for its apical membrane distribution in polarized epithelial cells (6). Without this anchor, the protein is secreted without polarity, implying that the extracellular domain of PLAP has no intrinsic sorting information (6). The transmembrane domain of the chimera, derived from the membrane-spanning domain of the VSVG protein, also has been shown to be devoid of intrinsic sorting information in polarized epithelia (17, 35). The construct combining these two domains (PLAP-TMR) should, therefore, contain no intrinsic sorting informa-
tion. The COOH-terminal tail of GAT-2 was appended to PLAP-TMR to generate PLAP-GAT-2. All of these constructs can be detected at the surfaces of intact cells with anti-PLAP antibodies. Immunofluorescence confocal microscopy and domain-specific cell surface immunoprecipitation of radioactively labeled PLAP-GAT-2 allowed us to evaluate the polar localization of the tail and tail deletion mutant constructs expressed by transfection in polarized MDCK cells.

We found that the tail has a motif that can function independently, in the absence of other transporter sequences, to mediate both basolateral targeting and distribution. This basolateral distribution motif is encoded in the seven amino acids proximal to the COOH-terminal PDZ-domain-interacting motif. Thus a PDZ interaction does not play a significant role in mediating the sorting of GAT-2 or in stabilizing its basolateral distribution.

MATERIALS AND METHODS

Plasmid construction. To create the PLAP-GAT-2 chimera in pc86, the PLAP ectodomain was cut out of the pcASPAR vector (30). This 1,500-bp fragment contains the entire amino-terminal extracellular domain of PLAP. The fragment was inserted into a modified pBluescript vector. Oligonucleotides were annealed to generate the transmembrane domain of the VSVG protein. These oligonucleotides were ligated into the pBluescript vector containing the ectodomain of PLAP. The PLAP ectodomain and VSVG transmembrane coding sequence were removed from pBluescript by digestion, and the PLAP-TMR construct was generated by ligating this 1,700-bp fragment into the pc86 expression vector. The PLAP-GAT-2 tail, NH2-RTLKGPLRER LRQLVCPAED LPQKSQPELT SPATPMTSLL COOH, was inserted into this construct by ligating together and inserting two pairs of oligonucleotides that together encode the entire COOH terminus. Oligonucleotide sequences and cloning strategy details are available upon request. Constructs were sequenced.

To delete the COOH-terminal three amino acids, we modified an oligonucleotide pair encoding the extreme COOH terminus through deletion of the bases coding for the extreme COOH terminus through a single-strand annealing reaction. Altered oligonucleotides were annealed and then ligated into the pBluescript vector containing the ectodomain of PLAP. The PLAP ectodomain and VSVG transmembrane coding sequence were removed from pBluescript by digestion, and the PLAP-TMR construct was generated by ligating this 1,700-bp fragment into the pc86 expression vector. The PLAP-GAT-2 tail, NH2-RTLKGPLRER LRQLVCPAED LPQKSQPELT SPATPMTSLL COOH, was inserted into this construct by ligating together and inserting two pairs of oligonucleotides that together encode the entire COOH terminus. Oligonucleotide sequences and cloning strategy details are available upon request. Constructs were sequenced.

To prepare the PLAP-GAT-2 Δ583–592 and PLAP-GAT-2 Δ592–602 chimeras, we again employed oligonucleotides encoding an alternate COOH-terminal sequence. Oligonucleotides designed for the PLAP-GAT-2 Δ583–592 construct were the same as above but had base pairs encoding amino acids 583–592 of the tail deleted. Oligonucleotides generated for the PLAP-GAT-2 Δ592–602 chimera had a sequence encoding amino acids 593–602 of the tail deleted and replaced with a stop codon. Constructs were sequenced to confirm the deletion.

To prepare the PLAP-GAT-2 Δ583–592 and PLAP-GAT-2 Δ592–602 chimeras, we again employed oligonucleotides encoding an alternate COOH-terminal sequence. Oligonucleotides designed for the PLAP-GAT-2 Δ583–592 construct were the same as above but had base pairs encoding amino acids 583–592 of the tail deleted. Oligonucleotides generated for the PLAP-GAT-2 Δ592–602 chimera had a sequence encoding amino acids 593–602 of the tail deleted and replaced with a stop codon. Constructs were sequenced to confirm the mutations. Oligonucleotide sequences are available upon request.

Cell culture. Wild-type MDCK cells were maintained in α-MEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS (Sigma, St. Louis, MO), 50 U/ml penicillin and streptomycin (GIBCO BRL), and 2 mM glutamine (GIBCO BRL). The incubator was kept at 5% CO2, 37°C, and 100% humidity. Stable cell lines were selected and maintained under the same conditions with the addition of 0.9 g/l geneticin (GIBCO BRL) to the medium.

Preparation of stable cell lines. Transfection of MDCK cells for the establishment of stable cell lines was performed with PerFect lipid number 2 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After transfection, cells were split 1:10 into 100-mm dishes containing medium supplemented with 0.9 g/l geneticin. Cells were incubated for 8–10 days in this selection medium, and clones were isolated by cloning rings (Belco Glass, Vineland, NJ). Cells harvested from the same individual colonies were seeded into both 24- and 96-well plates to facilitate the first round of screening.

This first round of screening was performed by using a colorimetric PLAP enzymatic assay (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in the 96-well plates when the clones reached confluence. Positive colonies were then evaluated by immunofluorescence microscopy after plating on eight-chamber glass slides (Becton Dickinson Labware, Franklin Lakes, NJ). Immunofluorescent detection of the PLAP chimeras was carried out as described previously (1). Cells were incubated with 1:1:100 dilution of Fitzgerald rabbit anti-PLAP antibody (Fitzgerald Industries, Concord, MA) followed by a 1:100 dilution of rhodamine-conjugated goat anti-rabbit secondary antibody (Sigma). Slides were mounted with Aquamount (Lerner Laboratories, Pittsburgh, PA) and examined with a Zeiss Axioskop upright fluorescence microscope (Carl Zeiss, Thornwood, NY).

Immunofluorescence confocal microscopy. Approximately 5 × 105 cells were plated on 2.4 cm polycarbonate Transwell filter 0.45-µm inserts (Corning Costar, Cambridge, MA). These cells were allowed to grow for 5–10 days under standard conditions. Cells were washed three times in PBS+, and monolayers were then incubated for 1 h on ice in a 1:100 dilution of Fitzgerald rabbit anti-PLAP antibody in PBS+/1% BSA present in both the apical and basolateral media compartments. Cells were then washed three times for 5 min in unmodified PBS+/1% BSA and fixed with 4% paraformaldehyde in PBS++. After fixation, cells were prepared for secondary antibody incubation and stained with a 1:100 dilution of fluorescein- or rhodamine-conjugated goat anti-rabbit secondary antibody (Sigma) as described previously (1). Filters were mounted with Aquamount. Immunofluorescence images were obtained with a Zeiss LSM 410 scanning confocal microscope (Carl Zeiss). Brightness and contrast were set so that all the pixels were within the linear range and all images were the product of eightfold line averaging. We then produced x-z sections with the use of a 0.2-µm motor step.

Cell surface immunoprecipitation. Approximately 5 × 105 cells were plated per 2.4 cm polycarbonate Transwell filter 0.45-µm insert (Corning Costar). These cells were allowed to grow for 5–10 days under standard conditions. For targeting experiments, cells were labeled for 1 h at 37°C with 500–700 µl of cysteine/methionine-free medium supplemented to 0.2 µCi/ml with [35S]cysteine/methionine (Amersham Biosciences, Piscataway, NJ) at the basolateral side of the filter and 500–700 µl of cysteine/methionine-free medium at the apical side. For steady-state labeling experiments, the labeling medium contained cold cysteine and methionine at 0.01 mM (10% of the level in growth medium). This medium was supplemented to 0.1 µCi/ml with [35S]cysteine/methionine, and the cells were labeled overnight. After labeling, the plates holding the filter inserts were placed on wet ice and washed twice with ice-cold PBS++. Filters were incubated at either the apical or basolateral side with 500–700 µl of ice-cold PBS++ supplemented with 1% BSA to block nonspecific antibody binding. A 1:200 dilution of Fitzgerald rabbit anti-PLAP antibody was added to the either the apical or basolateral medium compartment. Filters were incubated on ice with antibody for 2 h, washed three times with ice-cold PBS++, cut out of the holder, and lysed in ice-cold TBS (150 mM NaCl, 5.0 mM EDTA, 50 mM Tris, pH 7.4) with 1% Triton X-100 for 30 min. Cells were scraped off the filters and transferred into microcentrifuge tubes. Lysates were spun at maximum speed for 10 min to pellet cell debris, and the cleared supernatant was transferred to another microfuge tube. Forty microliters of a 1:50 (vol/vol) slurry of protein A-Sepharose beads (Pierce Biotechnology, Rockford, IL) were added to each supernatant, and the samples were rotated overnight at 4°C. Samples were washed three times in 1 ml of TBS plus 1% Triton X-100, and proteins were eluted from the beads with SDS-PAGE sample buffer before being run on 10% SDS-PAGE gels. Gels were stained for 1 h in 10% acetic acid, 20% methanol and then washed for at least 2 h in several changes of distilled water before being dried under a vacuum. Radioactivity was detected by phosphorimaging (Amersham) and quantitated using
ImageQuant software (Amersham). Each cell line was assayed in triplicate.

RESULTS

The GAT-2 tail is an independent targeting signal. To determine whether the basolateral targeting information present in the COOH-terminal tail of GAT-2 can function autonomously, we appended the GAT-2 COOH-terminal tail to a transmembrane protein that is not homologous to the GABA transporters and that has no intrinsic polarized distribution information (Fig. 1). We used PLAP-TMR, a fusion between the extracellular domain of human PLAP and a transmembrane domain from the VSVG protein, for this purpose. To determine the cell surface distribution of PLAP-TMR in MDCK cells, we grew stable cell lines expressing this construct to confluence on 0.4-μm polycarbonate filter supports. The surface distribution of the PLAP-TMR construct was examined by surface labeling of live cells with an anti-PLAP antibody added to both the apical and basolateral medium compartments. Cells were chilled to 4°C to prevent internalization or redistribution of surface-bound antibody. After incubation with anti-PLAP antibody, cells were fixed and incubated with rhodamine-conjugated secondary antibodies. The stained cells were analyzed by confocal microscopy (Fig. 2). The fine punctate pattern visible in the en face images (Fig. 2) demonstrates the presence of apically distributed protein, and cell outlines detected at a deeper plane of focus (Fig. 2) indicate the presence of basolateral localization as well. The ASNC tail perturbed its basolateral distribution (PLAP-GAT-2 Δ592–602), whereas deleting the 10 residues internal to those (PLAP-GAT-2 Δ583–592) or the final 3 residues of the tail (PLAP-GAT-2 ΔSNC) had a much less dramatic effect.

To obtain a quantitative biochemical measurement of the distribution of PLAP-TMR, we performed cell surface immunoprecipitation. Cells were metabolically labeled to steady state and also illustrated that deleting the final 10 amino acids of the GAT-2 tail perturbed its basolateral distribution (PLAP-GAT-2 Δ592–602), whereas deleting the 10 residues internal to those (PLAP-GAT-2 Δ583–592) or the final 3 residues of the tail (PLAP-GAT-2 ΔSNC) had a much less dramatic effect.

To determine whether the basolateral targeting information inherent in the COOH-terminal 48 amino acids of GAT-2 (Δ592–602) was predominantly basolaterally localized at steady state and also illustrate that deleting the final 10 amino acids of the GAT-2 tail perturbed its basolateral distribution (PLAP-GAT-2 Δ592–602), whereas deleting the 10 residues internal to those (PLAP-GAT-2 Δ583–592) or the final 3 residues of the tail (PLAP-GAT-2 ΔSNC) had a much less dramatic effect.

Fig. 1. GABA transporter GAT-2 and the PLAP-GAT-2 constructs. Constructs were prepared to examine the targeting information inherent in the COOH-terminal 48 amino acids of GAT-2 (Δ). This COOH-terminal tail was combined with the extracellular domain of placental alkaline phosphatase (PLAP) and the transmembrane domain of vesicular stomatitis virus protein G (VSVG-TMR), which provide an epitope tag and a membrane anchor, respectively. The PLAP-TMR chimera incorporates no intrinsic sorting information. The PLAP-GAT-2 full-length (FL) chimera was prepared by appending the entire GAT-2 tail to PLAP-TMR. PLAP-GAT-2 ΔSNC was created from the FL construct by deleting the final 3 amino acids. The PLAP-GAT-2 Δ583–592 chimera was made by deleting the amino acids highlighted in solid brackets, and the Δ592–602 chimera was made by deleting the amino acids highlighted in shaded brackets, from the FL construct.

Fig. 2. Localization of PLAP-TMR and PLAP-GAT-2 constructs in Madin-Darby canine kidney (MDCK) cells. Clonal MDCK cell lines expressing the PLAP-TMR (A), PLAP-GAT-2 FL (B), PLAP-GAT-2 Δ583–592 (C), PLAP-GAT-2 Δ592–602 (D), or PLAP-GAT-2 ΔSNC (E) were grown to confluence on filters, and anti-PLAP surface immunofluorescence was performed. These en face and x-z cross-sectional confocal micrographs show that PLAP-TMR was both apically and basolaterally localized at steady state and that addition of the COOH-terminal tail of GAT-2 resulted in a construct that exhibits a predominantly basolateral distribution. The images also confirm the results of cell surface immunoprecipitation, demonstrating that PLAP-GAT-2 Δ583–592 and ΔSNC were primarily basolateral in MDCK cells but that PLAP-GAT-2 Δ592–602 was randomly distributed. These confocal micrographs show that the COOH-terminal tail of PLAP-GAT-2 was sufficient to cause basolateral localization of the normally randomly distributed PLAP-TMR construct at steady state and also illustrate that deleting the final 10 amino acids of the GAT-2 tail perturbed its basolateral distribution (PLAP-GAT-2 Δ592–602), whereas deleting the 10 residues internal to those (PLAP-GAT-2 Δ583–592) or the final 3 residues of the tail (PLAP-GAT-2 ΔSNC) had a much less dramatic effect.
compartment. Unbound antibody was removed by washing, after which cells were solubilized in 1% Triton X-100. Antibody-bound PLAP-TMR was then precipitated through the addition of protein A-Sepharose beads to the detergent lysate. Analysis of the immunoprecipitated, radiolabeled PLAP-TMR protein revealed an apical-to-basolateral ratio of 0.99 ± 0.21 (Fig. 3). These experiments demonstrate that the populations of PLAP-TMR at the apical and basolateral membranes are approximately equal and, thus, that the chimera has no intrinsic localization information. This property makes this construct a useful tool for studying sorting and targeting information in sequences appended to its cytoplasmic COOH terminus.

The sequence of the entire GAT-2 tail was appended to PLAP-TMR to make PLAP-GAT-2 FL (Fig. 1). Stable MDCK cell lines expressing this construct were generated, and the distribution of PLAP-GAT-2 FL was evaluated initially by immunofluorescence microscopy. Cells were grown to confluence on 0.4-μm polycarbonate filter supports, and anti-PLAP antibody staining and confocal microscopy were carried out as described above. The en face view (Fig. 2B) indicated that the distribution of this construct was predominantly basolateral. This finding was confirmed through analysis of the x-z sections, which show almost exclusively lateral staining. Further confirmation was provided by domain-specific steady-state immunoprecipitation experiments. These experiments yielded an apical-to-basolateral ratio of 0.21 ± 0.03 (Fig. 3). These results indicate that the COOH-terminal tail of GAT-2 contains a basolateral localization motif that can function independently and without contributions from the rest of the transporter.

The last 10 amino acids of the GAT-2 tail are necessary for basolateral steady-state localization. We next wanted to define the portion of the GAT-2 tail necessary for basolateral sorting. Earlier experiments employing the entire transporter demonstrated that critical basolateral distribution information is contained in the final 22 amino acids (26). Alanine mutagenesis experiments indicated that a dileucine motif at amino acids 592 and 593 does not play any detectable role in generating basolateral distribution of the entire GAT-2 transporter protein (26). The polar distributions of several membrane proteins, including CFTR (25), syndecan (9), and the C. elegans LET-23 epidermal growth factor receptor (36), are at least partly dependent on PDZ-binding motifs displayed at their extreme COOH termini. The final three amino acids of the GAT-2 tail—serine, asparagine, and cysteine (SNC)—resemble a type I PDZ-binding motif. To investigate the potential contribution of this putative PDZ domain-binding motif to the basolateral sorting information in the GAT-2 tail, we made a truncated PLAP-GAT-2 construct in which these COOH-terminal three amino acids were removed: PLAP-GAT-2 ΔSNC (Fig. 1). To assay the distribution of this construct in MDCK cells, we created stable cell lines and performed cell surface immunoprecipitation and immunofluorescence confocal microscopy as described for the PLAP-TMR and PLAP-GAT-2 FL constructs. The en face immunofluorescence image (Fig. 2B) indicates that the construct exhibits a basolateral distribution. This assessment is borne out by the lateral staining pattern revealed on the x-z sections (Fig. 2B). Domain-specific steady-state surface immunoprecipitation experiments also showed that elimination of these three amino acids causes no statistically significant increase in the apical-to-basolateral ratio of the chimera (Fig. 3), indicating that these amino acids play no role in restricting the chimera to the basolateral membrane.

Because the apical-to-basolateral distribution ratio of the PLAP-GAT-2 ΔSNC construct was not significantly different from that of the full-length chimera, we concluded that there must be basolateral sorting information in the tail that is not dependent on the final three amino acids. To define where this information might reside, we made two additional deletion constructs within the context of the PLAP-GAT-2 FL chimera (Fig. 1). These new protein constructs contain complementary halves of the COOH-terminal 22 amino acids of the GAT-2 tail. This 22-residue sequence was shown to be necessary and sufficient to ensure the basolateral localization of the full-length transporter (26). We deleted the most membrane distal 10 amino acids of the tail, including the SNC motif, to create PLAP-GAT-2 Δ592–602. We deleted the 10 amino acids just proximal to these distal 10 to create PLAP-GAT-2 Δ583–592 (Fig. 1). Stable MDCK cell lines expressing these chimeras were prepared. Confocal immunofluorescence microscopy and domain-specific steady-state cell surface immunoprecipitation were carried out as described previously.

The results of these assays were markedly different for the two constructs. En face confocal micrographs of MDCK cells expressing PLAP-GAT-2 Δ592–602 revealed a principally apical distribution of the construct (Fig. 2D). This was confirmed by the predominance of apical staining in the x-z section (Fig. 2D). Domain-specific steady-state immunoprecipitation assays yielded an apical-to-basolateral ratio of 1.8 ± 0.3 (Fig. 3), demonstrating that the final 10 amino acids of the tail are vital for its basolateral sorting activity. Deletion of the more proximal 10 amino acids had little effect on the distribution of the chimera. En face confocal micrographs of the PLAP-GAT-2 Δ583–592-expressing cell lines (Fig. 2E) are consistent with a basolateral distribution. Similarly, x-z sections (Fig. 2E)
show principally basolateral staining. Domain-specific steady-state immunoprecipitation assays revealed an apical-to-basolateral ratio for this chimera of 0.4 ± 0.02 (Fig. 3), confirming that the chimera is primarily present at the basolateral membrane. In summary, the steady-state analysis of all the PLAP-GAT-2 chimeras argues that the final 10 amino acids of the tail contain necessary basolateral sorting information.

The last 10 amino acids of the GAT-2 tail are necessary for targeting. For a membrane protein to achieve a polar distribution, it must either be delivered directly to the appropriate plasma membrane domain or be preferentially maintained there after random surface delivery. We wanted to determine whether the localization information present in the GAT-2 COOH terminus is capable of mediating delivery from the biosynthetic pathway directly to the basolateral surface. The polarity of the initial cell surface delivery was assayed after a pulse-labeling period of 1 h (8). The protocol is essentially the same as that described above for domain-specific cell surface immunoprecipitation at steady state, but a shorter labeling period allowed us to examine a cohort of newly synthesized and delivered protein. The distribution of newly delivered PLAP-GAT-2 FL and PLAP-TMR was not significantly different from their distribution at steady state: PLAP-GAT-2 was delivered in an apical-to-basolateral ratio of 0.21 ± 0.05, whereas for PLAP-TMR, this ratio was 0.8 ± 0.36 (Fig. 4). We found that disruption of the final three amino acids had little or no effect on the apical-to-basolateral ratio of newly delivered chimeric protein (Fig. 4), indicating that this sequence does not play an important role in the initial targeting of the protein.

Deletion of the distal 10 amino acids perturbed the polarity of the cell surface delivery of the construct, just as it disrupted the basolateral distribution of the protein at steady state. This chimera was initially delivered in an apical-to-basolateral ratio of 3.5 ± 0.24 (Fig. 4), demonstrating that these 10 amino acids contain information necessary for the initial polar delivery of the protein as well as for ensuring its steady-state distribution.

**DISCUSSION**

By studying chimeric constructs composed of complementary sequence domains derived from the highly homologous GABA neurotransmitter transporter isoforms GAT-2 and GAT-3, Muth et al. (26) demonstrated that the COOH-terminal 22 amino acids of GAT-2 contain a sorting motif necessary for basolateral localization of this protein. Furthermore, when exchanged for the final 36 amino acids of GAT-3, this sequence is sufficient to redirect this normally apical transporter to the basolateral membrane (26). To determine whether the signal in the tail is independent of the rest of the transporter sequence, we appened the final 22 amino acids of GAT-2 to PLAP-TMR, an unrelated transmembrane protein that exhibits no inherent localization information when expressed by transfection in MDCK cells (Fig. 1). When the COOH-terminal tail of GAT-2 was appended to produce PLAP-GAT-2 FL, the construct was basolaterally distributed, with an apical-to-basolateral ratio of ~1:4 (Figs. 2–4). These data demonstrate that the GAT-2 basolateral signal operates autonomously and does not depend on additional GAT-2 transporter sequences to be functional. The goal of the present study was to determine which residues in the tail play an important role in specifying the basolateral distribution of the protein.

By making deletion mutants of the GAT-2 tail in the context of the PLAP-TMR chimera (Fig. 1) and assaying their polarity in MDCK cells by immunofluorescence confocal microscopy and domain-specific cell surface immunoprecipitation, we were able to define a sequence domain required for basolateral localization. Two of the deletion constructs, PLAP-GAT-2 ∆583–592 and PLAP-GAT-2 ∆592–602, each contained approximately half of the 22-amino acid signal that was initially shown to be necessary and sufficient for basolateral localization of the full-length transporter (Fig. 1). A third mutant construct deleted a PDZ-binding motif at the extreme COOH terminus (Fig. 1). For each of these constructs, the distribution at steady state was similar to the distribution upon delivery to the cell surface, indicating that the sorting signal in the tail encodes biosynthetic sorting information (Figs. 2–4). Deletion of amino acids 583–592, the 10 amino acids proximal to the most COOH-terminal 10 amino acids, did not disturb basolateral distribution of the PLAP-GAT-2 chimera (Figs. 2 and 3). Deletion of a putative PDZ-binding motif at the extreme COOH terminus of the tail to create the PLAP-GAT-2 ∆SNC chimera (Fig. 1) also had no effect on the basolateral distribution of the protein, either upon delivery (Fig. 4) or at steady state (Figs. 2 and 3). However, deletion of these three amino acids along with the seven residues NH₂-terminal to them had a profound effect on the sorting of the construct, resulting in an apical-to-basolateral ratio of ∼2:5:1, compared with the 1:4 apical-to-basolateral ratio characteristic of the full-length chimera (Figs. 2–4). This led us to conclude that amino acids 593–599 are required for the basolateral distribution of the GAT-2 protein.

Given the role that PDZ domain-containing proteins have been shown to play in establishing or maintaining the polar distribution of some membrane proteins, including Kir 2.3 (19) and the LET-23 epithelial growth factor receptor (15, 32), it is perhaps surprising that the deletion of the putative PDZ-interacting motif played no role in the polar delivery or steady-state distribution of the PLAP-GAT-2 chimera. In fact,
we have identified a PDZ domain-containing protein that is a binding partner for GAT-2. The protein identified as GIPC interacting partner COOH terminus (GIPC) interacts with the GAT-2 COOH terminus as well as with cytoskeletal motor proteins (7), transmembrane proteins including α6α/α6β-integrins (11, 34), and various soluble proteins (10). The role of the GIPC interaction in regulating the function or distribution of GAT-2 remains to be established. It may be that the GAT-2 PDZ-binding motif is important for extending the lifetime of the protein at the plasma membrane. The ability of GIPC to interact with integrins, proteins known to cluster molecules at the plasma membrane (for review, see Ref. 3), is also consistent with this role. Another GABA transporter family member, the basolateral betaine transporter BGT (27), clearly depends on its PDZ domain-interacting motif to ensure its stable residence at the plasma membrane. The PDZ domain-interacting motif, perhaps through its interaction with GIPC and, in turn, with cytoskeletal motor proteins, may also be important for moving GAT-2 through the secretory pathway. The PDZ interaction motif does not, however, constitute a vital part of the anisotropic distribution information embedded in the GAT-2 tail.

The portion of the GAT-2 tail required to specify basolateral distribution appears to reside within the seven-amino acid sequence immediately NH2-terminal to the PDZ interaction motif: LRLTELE. Two classes of motifs have been characterized as important for basolateral localization. Tyrosine-based motifs, such as those found in the LDL receptor and the β-subunit of the H+/K+-ATPase, can mediate basolateral localization in MDCK cells (21, 29). The interpretation of these motifs is thought to depend on interactions with adaptor complexes (for review, see Ref. 13). Dileucine motifs in the cytoplasmic tails of the macrophage Fc receptor (23) and epithelial adhesion molecule E-cadherin (24) direct basolateral sorting. Similarly, other dihydrophobic amino acid motifs in several proteins, including the furin receptor (31) and major histocompatibility complex class I chain-related protein MICA (33), are necessary for basolateral localization in various cell systems. Dileucine motifs have also been shown to interact with adaptor complexes (16).

Non-tyrosine-based and nondihydrophobic basolateral sorting information also has been described (14, 18). Residues 749–788 of the cytoplasmic tail of a neural cell adhesion molecule, N-CAM-140, contain necessary basolateral sorting information that involves neither tyrosine nor dihydrophobic residues. Similarly, a 23-amino acid juxtamembrane sequence (residues Lys652 to Ala674) in the cytoplasmic portion of human epidermal growth factor receptor is necessary and sufficient to ensure the basolateral distribution of this protein. Neither of the two dileucine motifs of this protein plays a role in establishing its basolateral distribution. The HRXXV motif in the COOH-terminal tail of the polymeric immunoglobulin receptor is required for its initial basolateral delivery from the trans-Golgi network in MDCK cells (2, 28), although its subsequent transcytosis to the apical membrane involves other sequences (20). The basolateral sorting information in the GAT-2 tail also does not involve tyrosine or dileucine motifs and is not similar in any obvious way to the other novel sequences discussed above. Thus it constitutes a new class of basolateral sorting motif and is interpreted by as-yet unidentified mechanisms within the cell.

Deleting the LRLTELE motif results in an apical-to-basolateral ratio that is 10–20 times higher than that of the full-length construct, producing an apical distribution ratio of 1.8 ± 0.27 at steady state and 3.5 ± 0.24 upon initial delivery. This not only demonstrates that the COOH-terminal 10 amino acids are vital for the basolateral sorting activity of the GAT-2 tail but also implies that some cryptic apical sorting information may be uncovered by this deletion. Deleting the final 24 amino acids of the full-length GAT-2 transporter also leads to an apically biased distribution, associated with an apical-to-basolateral ratio of ~4 (26). It is possible, therefore, that apical localization information is present in the 26 residues of the GAT-2 COOH-terminal tail that resides between the membrane and the extreme COOH-terminal 22 amino acids deleted by Muth et al. (26). The characteristics and physiological role of this putative second silent signal remain to be determined. Whatever cryptic apical targeting information may be contained in the GAT-2 tail is entirely subordinate to the basolateral message embodied in the LRLTELE sequence under normal circumstances. This basolateral targeting sequence does not correspond to any known basolateral targeting signal, suggesting that it belongs to a novel class of basolateral targeting motif. Because it plays its role in the delivery of the protein to the appropriate plasma membrane domain rather than simply mediating its retention at the basolateral surface of the plasma membrane, this motif might be involved in sorting the protein into appropriately targeted membrane vesicles during its biosynthetic passage through the trans-Golgi network. It might also recruit basolateral targeting machinery to the vesicles into which it is sorted. Future work is required to determine the nature of the cellular components that actually interpret this novel motif and guide the COOH-terminal tail of the GABA transporter to the basolateral domain of the epithelial plasma membrane.

ACKNOWLEDGMENTS

We thank Vanathy Rajendran for expert technical assistance and the members of the Caplan laboratory for helpful discussion and encouragement throughout these studies. We also thank Drs. Peter Aronson, Gary Rudnick, and James Anderson for insightful criticisms and very helpful suggestions.

GRANTS

This work was supported by National Institutes of Health Grants GM-42136 and DK-17433.

REFERENCES

19. Le Gall AH, Powell SK, Yeaman CA, and Rodriguez-Boulan E.