Localization of an organic anion transporter-GFP fusion construct (rROAT1-GFP) in intact proximal tubules

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Sweet, Douglas H., David S. Miller, and John B. Pritchard. Localization of an organic anion transporter-GFP fusion construct (rROAT1-GFP) in intact proximal tubules. Am. J. Physiol. 276 (Renal Physiol. 45): F864–F873, 1999.—The organic anion transporter, rROAT1, is a dicarboxylate/organic anion exchanger, a function associated with the basolateral membrane in rat proximal tubule. To directly establish the subcellular localization of rROAT1 in renal epithelia, we made a rROAT1-green fluorescent protein (GFP) fusion construct (rROAT1-GFP). Plasma membrane-associated fluorescence was observed in rROAT1-GFP-expressing Xenopus oocytes examined by confocal microscopy. Uptake of 3H-labeled p-aminodhippurate (PAH) increased 2.5-fold in rROAT1-GFP-expressing Xenopus oocytes, and this increase was abolished by 1 mM probenecid. Thus the construct was capable of specific organic anion transport. Cultured renal epithelial cell lines (MDCK and LLC-PK1) transfected with the vector pEGFP-C3 showed a diffuse, evenly distributed cytoplasmic signal. However, when transfected with pEGFP-C3/rROAT1 (vector coding for rROAT1-GFP), both cell lines showed predominantly plasma membrane fluorescence. The expression and distribution of rROAT1-GFP in intact renal proximal tubules was also investigated. Isolated killifish (Fundulus heteroclitus) renal tubules transfected with pEGFP-C3/rROAT1 showed marked basal and lateral membrane-associated fluorescence, but no detectable signal in the nucleus or the apical pole of tubule cells. Tubules transfected with pEGFP-C3 showed diffuse cytoplasmic fluorescence. Function of the rROAT1-GFP construct was demonstrated in transfected killifish tubules by fluorescent transport assay. These results demonstrate the basolateral subcellular localization of rROAT1 in polarized renal epithelium and validate a new technique for localizing cloned transporters within intact renal tubules.

A major function of the renal organic anion secretory system in proximal renal tubule is to limit the toxicity of anionic xenobiotics, xenobiotic metabolites, and waste products of metabolism. Studies with intact kidneys, tissue slices, isolated renal tubules, and membrane vesicles clearly demonstrate this organic anion transport is driven by basolateral and apical membrane transporters and that these transporters have separate and distinct physiological characteristics. Movement across the basolateral membrane is a tertiary active process that indirectly taps the energy of the sodium gradient (for review, see Ref. 16). Once within the cell, substrates may be bound or sequestered within vesicles (5, 13), followed by transport across the luminal membrane, apparently by anion exchange or facilitated diffusion (1, 9, 27).

Although the physiological role of the kidney in the elimination of charged xenobiotic compounds from the body has been investigated for decades, it was only recently that we began to understand such transport at the molecular level. Through the application of molecular biology techniques and the Xenopus oocyte expression assay system, cDNAs encoding some of these transporters have been cloned (21), allowing their transport properties to be examined in isolation, under controlled conditions. On the basis of such physiological analyses, transporter positions within the renal transport model, and thus their subcellular localization, could be assigned. However, there are examples in the recent literature in which subsequent analysis has yielded conflicting data as to subcellular distribution. When the kidney-specific organic anion transporter rOAT-K1 was expressed and characterized in LLC-PK1 cells, it was concluded that rOAT-K1 was a basolateral membrane transporter in proximal tubule (18). However, when a rOAT-K1 antisem was used to examine kidney basolateral and brush-border membrane preparations by Western blot, an immunoreactive band was detected in the brush border, but none was found in the basolateral membranes (10). Similarly, when the organic cation transporter, rOCT2, was characterized in Xenopus oocytes, it met the physiological criteria of a basolateral transporter in proximal tubule (14). Yet, when a pig OCT2 homolog was cloned from LLC-PK1 cells and subsequently expressed and characterized in human embryonic kidney 293 cells, it was concluded that pOCT2 had the predicted physiological profile of an apical membrane cation transporter (3). Additionally, the human homolog, hOCT2, was detected in the apical membranes of distal (not proximal) tubule cells in human kidney by in situ hybridization and immunocytochemistry (2).

Therefore, to investigate the issue of subcellular localization from a different perspective, we combined the technology of green fluorescent protein (GFP) with the unique assay system of isolated teleost renal proximal tubules. Each of these components offers several advantages for this type of study. Since GFP fluorescence has been documented in a wide variety of organisms, either it does not require any cofactors, substrates, or additional gene products from the source organism (Aequorea victoria), or the factors it requires are ubiquitous (20, 26). GFP is ideal for use in comparative studies, because it is stable when expressed in various organisms and its fluorescence is species inde-
expression of rROAT1-GFP in intact proximal tubules

Dependent. There is also evidence that GFP provides greater sensitivity and resolution than staining with fluorescently labeled antibodies and that it is more resistant to photobleaching (25). Perhaps one of the greatest attributes of GFP is that detection can be performed in living cells and tissues, e.g., cultured cells and isolated renal tubules. Teleost fish represent a particularly good tissue source of proximal tubules, because their nephrons contain a very high proportion of proximal segment. Teleost proximal tubules are easily obtained, remain viable for long periods of time, and broken ends rapidly reseal, maintaining a fluid-filled lumen that only communicates with the medium through the tubular epithelium (15). Additionally, the mechanisms of transport that exist in teleost renal tubules appear to be the same as those in mammalian proximal tubules (15).

For the present study, we made a fusion construct of rat renal organic anion transporter 1 (rROAT1, Ref. 24) and GFP, which allows direct visual observation of the subcellular localization of the fusion protein (rROAT1-GFP). Function of rROAT1-GFP was confirmed by Xenopus oocyte expression assay. The fusion construct was transfected into cultured renal cell lines, and a plasma membrane localization of the construct was transfected into cultured renal cell lines, and a plasma membrane localization of the fusion protein (rROAT1-GFP) under the control of the human cytomegalovirus promoter. The nucleotide sequence of the rROAT1-GFP fusion coding region was removed from pEGFP-C3/rROAT1 by restriction enzyme digestion with SnaBI and BamHI. The fragment was gel purified and ligated into pSPORT1 (GIBCO-BRL; Life Technologies, Gaithersburg, MD) cut with SnaBI and BamHI I. The resulting plasmid, pSPORT1/rROAT1-GFP was linearized and used as template for in vitro cRNA synthesis.

The organic cation transporter 2 (ROCT2)-GFP fusion construct was made as follows: pSPORT1/OCT2, which contains the full-length roct2 cDNA (22), was cut with the restriction enzymes BamHI and KpnI; the fragment containing the roct2 cDNA was gel-isolated and ligated into pEGFP-C3 cut with BamHI I and KpnI I, resulting in the plasmid pEGFP-C3/roct2, which contains an in-frame fusion of roct2 to the carboxy terminus of GFP (ROCT2-GFP). For Xenopus oocyte expression studies, the exact same strategy that was used to make pSPORT1/rROAT1-GFP was employed, resulting in the plasmid pSPORT1/roct2-GFP.

The plasmid pCMV/myc/mito/GFP was purchased from Invitrogen (Carlsbad, CA), and the ER-GFP plasmid was a gift from Dr. Richard McKay. Xenopus oocyte expression assay. Oocyte isolation procedures and uptake assay were performed as described previously (24). Briefly, adult female Xenopus laevis (Xenopus One, Ann Arbor, MI) were anesthetized by hypothermia and decapitated. Stage V and stage VI oocytes were manually dissected free of the ovary, and the follicles were removed by treatment with collagenase A. Oocytes were maintained at 18°C in Barth's medium containing 0.05 mg/ml gentamicin sulfate, 2.5 mM sodium pyruvate, and 5% heat-inactivated horse serum. Overnight recovery was allowed before injection. An in vitro transcription kit (mMessage mMachine; Ambion, Austin, TX) was used to synthesize capped cRNA from plasmid DNA linearized with BamH I. The cRNA products were quantitated in a spectrophotometer and diluted prior to injection to allow delivery of 20–60 ng of cRNA/oocyte in 17 nl with a 10-s injection.

Six days after injection, the oocytes were divided into experimental groups (containing 10 oocytes each) and incubated at 18–22°C for 60 min in oocyte Ringer 2 (OR-2) containing 50 µM 3H-labeled p-aminohippurate ([3H]PAH, 4 µCi/ml) in the absence or presence of 1 mM probenecid. After uptake, oocytes were rapidly rinsed three times with ice-cold OR-2 and placed into individual scintillation vials containing 0.5 ml 1 M NaOH, incubated at 65°C for 20 min, and neutralized with 0.5 ml 1 M HCl. Finally, 4.7 ml of Econotube (ICN Biomedical, Cleveland, OH) was added, and oocyte radioactivity measured in disintegrations per minute in a Packard 1600TR liquid scintillation counter with external quench correction.

Tissue culture The MDCK (established cell line derived from renal distal tubules of a 3- to 4-wk old male pig) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell lines were negative for mycoplasma upon receipt from ATCC. The MDCK line was retested before publication and found to be negative for mycoplasma. Both cell lines were maintained in Eagle's modified essential medium supplemented with 10% fetal bovine serum in a humidified incubator at 37°C with 5% CO2. Cultures were split 1:20 approximately every 3–4 days.

Killifish (Fundulus heteroclitus) were collected near the Duke University Marine Laboratory (Beaufort, NC) and maintained in tanks with recirculating artificial sea water at the National Institute of Environmental Health Sciences. After decapitation, renal tubular masses were isolated, adherent hematopoietic tissue was removed, and individual killifish proximal tubules were dissected. After transfection, the tubules were maintained in a confocal chamber containing 2 ml of medium 199 at 12°C.

MATERIALS AND METHODS

Plasmid construction. The coding region of the rROAT1 gene was amplified from a full-length cDNA done (23) using PCR and the primers

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\text{R1/GFPccw:} \quad 5'\text{CAGTCGGATCCCGTACAGCTGACCTGACC-3'}
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\text{R1/GFPccw:} \quad 5'\text{GCGTCGAGCTCAGCTGACCTGACC-3'}
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Cycle parameters were as follows: denature at 94°C for 4 min; followed by 10 cycles of 94°C denature for 1 min, 35°C anneal for 30 s, and 72°C extension for 2 min; followed by 30 cycles of 94°C denature for 1 min, 55°C anneal for 30 s, 72°C extension for 2 min; with a final 10-min extension at 72°C. The PCR product was digested with the restriction enzyme Kpnl, gel isolated, and ligated into the vector pEGFP-C3 (Clontech Laboratories, Palo Alto, CA) cut with Kpnl I. The resulting expression vector, pEGFP-C3/rROAT1, contains an in-frame fusion of rROAT1 to the carboxy terminus of green fluorescent protein (rROAT1-GFP) under the control of the human cytomegalovirus promoter. The nucleotide sequence of rROAT1-GFP was confirmed by fluorescent sequencing on a DNA sequencer (model 377; Applied Biosystems, Foster City, CA).

For Xenopus oocyte expression studies, the DNA fragment containing the rROAT1-GFP fusion coding region was removed from pEGFP-C3/rROAT1 by restriction enzyme digestion with SnaBI I and BamHI I. The fragment was gel purified and ligated into pSPORT1 (GIBCO-BRL; Life Technologies, Gaithersburg, MD) cut with SnaBI I and BamHI I. The resulting plasmid, pSPORT1/rROAT1-GFP was linearized and used as template for in vitro cRNA synthesis.

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Transfection. One day prior to transfection, $1.5 \times 10^5$ cells were plated into poly-l-lysine-treated glass-bottomed confocal chambers (4.9 cm²). Cells were transfected with 8 µg plasmid DNA for 1 h at 37°C using SuperFect Reagent (2 µl SuperFect/µg DNA; Qiagen, Chatsworth, CA). Transfected cells were washed with PBS, given fresh medium, and maintained at 37°C with 5% CO₂ for the remainder of the experiment. Cells were examined by confocal fluorescence microscopy 24 and 48 h after transfection.

Groups of killifish tubules were transfected with 8 µg plasmid DNA for 1 h at 12°C using SuperFect Reagent (2 µl SuperFect/µg DNA). The tubules were subsequently rinsed with fresh medium 199 and placed in a confocal chamber containing 2 ml of medium 199 and maintained at 12°C for the remainder of the experiment. Tubules were examined by confocal fluorescence microscopy 24 and 48 h after transfection. Additionally, rROAT1-GFP transport function was demonstrated in transfected tubules by fluorescein (FL) transport assay. Two days after transfection with either pEGFP-C3 or pEGFP-C3/rROAT1, tubules were exposed to 1 µM FL in their culture medium. After 30 min, confocal images were acquired.

Confocal fluorescence microscopy. Cultured cells and tubules were imaged using a Zeiss model 410 inverted laser-scanning confocal microscope fitted with a 340 water-immersion objective (NA 1.2). Fluorescent images were collected by illuminating samples with an Ar-Kr laser at 488 nm. A 510-nm dichroic filter was positioned in the light path and a 515-nm long-pass emission filter was positioned in front of the detector. Confocal images (512 × 512 × 8 bits) were acquired as single 8- or 16-s scans, saved to an optical disk or high-capacity floppy disk (Jaz, Iomega), and analyzed on a Power Macintosh 9600 computer using NIH Image 1.61 software. For the FL transport studies, cellular and luminal fluorescence intensities were measured from the stored confocal images as described previously (11, 12). Briefly, for each tubule, two to three adjacent cellular and luminal areas were selected. After background subtraction, the average pixel intensity for each area was calculated, and the values used for each tubule were the means for all selected areas. Measurements were made from 9–12 tubules in each group, and mean values were determined.

Statistics. Uptake data are presented as means ± SE. Differences in mean values were considered to be significant when $P < 0.05$, as determined by unpaired Student’s t-test.

Chemicals. [3H]PAH (3.7 Ci/mmol) was obtained from Dupont-NEN (Boston, MA). FL and the fluorescent Golgi-specific marker 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminooxy)hexanoyl)shingosine (NBD C₆-ceramide) were purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from commercial sources and were of the highest grade available.

RESULTS

Xenopus oocytes. Oocytes expressing rROAT1-GFP exhibited a low but detectable level of plasma membrane-associated fluorescence, and water-injected oocytes showed no such signal (Fig. 1). Transport function of the rROAT1-GFP fusion protein was confirmed by Xenopus oocyte expression assay (Fig. 2). Uptake of 50 µM [3H]PAH by oocytes 6 days after injection with rROAT1-GFP cRNA was significantly higher than in...
water-injected oocytes (2.5-fold increase, \( P < 0.01 \)), or in oocytes injected with rOCT2-GFP cRNA. Treatment with 1 mM probenecid reduced uptake to the level in water-injected oocytes, demonstrating that rROAT1-GFP mediated specific PAH transport.

Renal cell lines. LLC-PK₁ cells were transfected with pEGFP-C3 (expresses cytoplasmic GFP), pCMV/myc/mito/GFP (expresses mitochondrial-targeted GFP), ER-GFP (expresses endoplasmic reticulum-targeted GFP), or pEGFP-C3/rROAT1 (expresses rROAT1-GFP). Confluent monolayers were examined 24 and 48 h after transfection by confocal microscopy. Cells transfected with pEGFP-C3 had a diffuse, evenly distributed signal over the entire cell including both cytoplasm and nucleus.

Fig. 3. Confocal images of transfected LLC-PK₁ cells in culture. Transfection was carried out as described in MATERIALS AND METHODS, and confluent monolayers were examined by confocal microscopy. A: pEGFP-C3 transfection. Cells show a diffuse signal that fills the cytoplasm and nucleus. B: pCMV/myc/mito/GFP transfection. Cells show a punctate staining in the cytoplasm (mitochondria) with no nuclear or plasma membrane-localized fluorescence. C: pEGFP-C3/rROAT1 transfection. Note the strong plasma membrane-localized fluorescence and exclusion of signal from the nucleus.

Fig. 4. Cross-sectional image analysis of rROAT1-GFP-expressing LLC-PK₁ cells in culture. Transfection was carried out as described in MATERIALS AND METHODS, and confluent monolayers were examined by confocal microscopy. Microscope was focused at bottom of the chamber, and 8-s scans were collected 0.5 µm apart (i.e., up through the cell). Individual images were then compiled to render a three-dimensional reconstruction of the tissue. A: fluorescence image (single plane) of pEGFP-C3/rROAT1-transfected cells. Diagonal line designates the axis used for a cross section through the image stack. B: cross-sectional projection corresponding to line in A. Note strong labeling of lateral plasma membranes and absence of signal in nucleus.
nucleus (Fig. 3A). No membrane-localized fluorescence could be detected. Cells expressing the mitochondrial-targeted GFP had a distinctive, punctate fluorescence found exclusively in the cytoplasm (Fig. 3B). In contrast, cells transfected with pEGFP-C3/rROAT1 exhibited strong plasma membrane fluorescence, but no nuclear fluorescence (Figs. 3C and 4). Additionally, a perinuclear region of high fluorescence, believed to be in the area of the Golgi complex, was detected (see below). When rROAT1-GFP-expressing cells were examined in cross section, the extensive labeling of the lateral membranes was evident, as was the absence of a nuclear signal (Fig. 4B). However, whether there was labeling of the basal and/or apical membranes was not readily discerned. A weak reticulate pattern of cytoplasmic fluorescence was also observed that was similar to the pattern seen in ER-GFP-transfected cells (Fig. 5). The plasmid constructs were also transfected into MDCK cells. As was observed in the LLC-PK1 cells, pEGFP-C3-transfected MDCK cells exhibited an in-
tense, evenly distributed cytoplasmic fluorescence that penetrated the nucleus, but was not associated with the plasma membrane (not shown). Again, rROAT1-GFP was highly localized to the plasma membrane, but was excluded from the nucleus (Fig. 6B). The perinuclear region of high fluorescence was seen, as well (Fig. 6B). Examination of a cross-sectional view through an image stack again showed the localization of rROAT1-GFP to the lateral, and perhaps basal, plasma membranes, as well as its exclusion from the nucleus (Fig. 7B). Targeting of rROAT1-GFP to the apical membrane could not be ruled out by these images.

To confirm that the region of highly localized perinuclear fluorescence was indeed Golgi, the fluorescent Golgi-specific dye NBD C6-ceramide was used (Fig. 8). While observing a field of cells containing both pEGFP-C3/rROAT1-transfected and nontransfected cells, NBD C6-ceramide was added to the culture. The subsequent appearance of NBD C6-ceramide-induced Golgi fluorescence correlated with the perinuclear fluorescence seen in the rROAT1-GFP-expressing cells (Fig. 8), suggesting that the localized perinuclear GFP fluorescence represented some step in the Golgi processing of rROAT1-GFP.

Renal proximal tubules. Although targeting of rROAT1-GFP to the lateral plasma membrane of renal cells in culture was clear, evidence of insertion into the basal plasma membrane was not as convincing, and insertion into the apical membrane could not be ruled out. Therefore, we attempted to determine whether intact renal tubules could be transfected and, if so, whether an unambiguous localization could be obtained. Dissected renal tubules from killifish were transfected with the pEGFP-C3 and pEGFP-C3/rROAT1 plasmids and examined by confocal microscopy at 24 and 48 h after transfection. Untransfected control tubules had a low level of autofluorescence (Fig. 9A). As with the cultured cells, pEGFP-C3-transfected tubules showed a strong, evenly distributed cytoplasmic and nuclear signal (Fig. 9B). In contrast, tubules transfected with pEGFP-C3/rROAT1 showed specific localization to the basal region of tubule cells (Fig. 9, C and D). No signal was observed in the nucleus or above the nucleus at the apical pole of the cells. Cross sections...
made through an image stack of a rROAT1-GFP-expressing tubule clearly demonstrated the basal localization (Fig. 10).

Active transport of FL by cytoplasmic GFP-expressing tubules, 2 days after transfection, demonstrated the long-term viability of killifish tubules in culture (Figs. 11 and 12). Function of the expressed rROAT1-GFP fusion protein in transfected killifish tubules was demonstrated by their increased capacity to accumulate the organic anion FL, over that of pEGFP-C3-transfected tubules (Fig. 11). At steady state, the luminal concentration of FL in tubules from the pEGFP-C3/rROAT1-GFP-transfected group exceeded that observed in the pEGFP-C3-transfected control group (Fig. 11). The average fluorescence intensities of both the cells (2-fold increase, \( P < 0.05 \)) and lumina (3-fold increase, \( P < 0.0001 \)) of pEGFP-C3/rROAT1-transfected tubules were significantly higher than in pEGFP-C3-transfected tubules, indicating increased transport capacity in the rROAT1-GFP-expressing tubules (Fig. 12).

**DISCUSSION**

The cells of the renal proximal tubule are highly polarized with respect to structure and function, and the transport properties of the basolateral and apical membranes reflect this polarity (16). For the "classic" organic anion transport system, the entry step is mediated by organic anion/dicarboxylate exchange. Recently, organic anion transporters were cloned from rat (rROAT1; rOAT1), mouse (mNKT, also known as mROAT1), and winter flounder (fROAT) (7, 19, 24, 28). When expressed in Xenopus oocytes, rROAT1 possessed all of the known functional properties of the basolateral organic anion/dicarboxylate exchanger (24). Here we confirmed directly the precise subcellular localization of rROAT1 in renal epithelia through observation of a rROAT1-GFP fusion protein. rROAT1-GFP was found to be inserted into the plasma membrane of Xenopus oocytes (Fig. 1) and shown to possess transport function in those oocytes by expression assay (Fig. 2). Although rROAT1-GFP transport function was detected, its activity was clearly less than the native gene. Determining whether this is an indication that the GFP construct is not as efficient at transport, or that the mRNA is less stable or not as efficiently transcribed, will require additional studies.

Initially, standard transfection techniques were used to examine the expression of rROAT1-GFP in renal epithelial cell lines (LLC-PK1 and MDCK cells), and rROAT1-GFP localization to the plasma membrane was observed (Figs. 3, 4, 6, and 7). Analysis of cross-sectional image projections from stacks of confocal slices provided direct evidence of rROAT1 targeting to the lateral membranes (Figs. 4 and 7). However, localization to the basal membrane and exclusion from the
apical membrane were not as certain. Also, a reticulate cytoplasmic signal was observed in rROAT1-GFP-expressing cells that resembled the pattern observed with an endoplasmic reticulum-specific GFP construct (Fig. 5). We believe that this could reflect the trafficking of rROAT1-GFP protein as it is processed in the endoplasmic reticulum and transported through the Golgi for packaging and subsequent insertion into the plasma membrane. The Golgi-associated fluorescence detected in the rROAT1-GFP-expressing cells, which was absent from cytoplasmic or mitochondrial GFP-expressing cells, is consistent with this explanation (Fig. 8).

Since the polarity of rROAT1-GFP expression in the cell culture-based system was inconclusive, we investi-
gated whether the GFP constructs could be successfully transfected into intact renal tubules. Transfected renal proximal tubules from a teleost fish (killifish) showed cytoplasmic GFP distribution similar to that in renal cell lines in culture (Fig. 9B). In the intact renal tubules, rROAT1-GFP was clearly expressed in a polarized manner, with fluorescence localized to the basal and lateral membranes (Figs. 9, C and D, and 10). There appeared to be some internal fluorescence in the cells of rROAT1-GFP-transfected tubules. If this represented diffuse cytoplasmic fluorescence, then at some point it would have been visible in the region above the nucleus. This was not the case. Therefore, this eliminated the possibility of a free, diffuse cytoplasmic signal. It is well established that the basal and lateral membranes of proximal tubule cells are not flat sheets, but rather they have large infoldings that extend into the interior of the cell (4). Labeling of these infoldings with rROAT1-GFP would certainly make it appear as if the construct were localized to the basal cytoplasm. Thus the labeling patterns are consistent with basolateral localization.

The FL uptake studies were indicative of 1) the long-term viability of teleost proximal tubules in culture and 2) enhanced basolateral transport in rROAT1-GFP-transfected tubules (Figs. 11 and 12). The increased cellular fluorescence, relative to cytoplasmic GFP-expressing cells, is consistent with greater uptake across the basolateral membrane. The stronger luminal fluorescence is surely a consequence of the greater free cytoplasmic pool of FL available for secretion in rROAT1-GFP tubules, due to the increased uptake. These data confirm rROAT1-GFP retains transport function in an intact tubule and also suggest a basolateral localization.

The ability to use GFP technology in intact teleost tubules represents a significant technical advance. By using intact renal tubules the polarity and function of the native epithelium is preserved. Similar, but less conclusive, results were obtained with mammalian (rabbit) proximal tubules (not shown). However, the teleost system provides several distinct advantages. The teleost tubules are maintained at 12°C vs. 37°C for rabbit, and it has been documented that GFP fluorescence is stronger at lower temperatures (6). Teleost tubules can also be easily maintained in culture for up to 5 days without any special treatment or equipment (8), whereas the rabbit tubules were losing their integrity within 48 h. Perhaps most significant is the ability to make functional measurements of secretory transport with the teleost tubules.

Thus, through the combination of a comparative model (isolated killifish proximal tubules) and GFP technology, we have confirmed the basal and lateral plasma membrane localization of rROAT1 (Figs. 9 and 10). These are the first data demonstrating the usefulness of a powerful new technique with which to establish a correlation between the predicted position of a cloned transporter within the transport model, based on physiology, and its actual subcellular distribution within an intact, functional renal tubule.

REFERENCES