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Micropuncture gene delivery and intravital two-photon visualization of protein expression in rat kidney

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Tanner, George A., Ruben M. Sandoval, Bruce A. Molitoris, James R. Bamburg, and Sharon L. Ashworth. Micropuncture gene delivery and intravital two-photon visualization of protein expression in rat kidney. *Am J Physiol Renal Physiol* 289: F638–F643, 2005. First published May 10, 2005; doi:10.1152/ajprenal.00059.2005.—Understanding molecular mechanisms of pathophysiology and disease processes requires the development of new methods for studying proteins in animal tissues and organs. Here, we describe a method for adenoviral-mediated gene transfer into tubule or endothelial cells of the rat kidney. The left kidney of an anesthetized rat was exposed and the lumens of superficial proximal tubules or vascular welling points were microinfused, usually for 20 min. The microinfusion solution contained adenovirus with a cDNA construct of either *1*) *Xenopus laevis* actin depolymerizing factor/cofilin [XAC; wt-green fluorescent protein (GFP)], *2*) actin-GFP, or *3*) GFP. Sudan black-stained castor oil, injected into nearby tubules, allowed us to localize the microinfused structures for subsequent visualization. Two days later, the rat was anesthetized and the kidneys were fixed for tissue imaging or the left kidney was observed in vivo using two-photon microscopy. Expression of GFP and GFP-chimeric proteins was clearly seen in epithelial cells of the injected proximal tubules and the expressed proteins were localized similarly to their endogenously expressed counterparts. Only a minority of the cells in the virally exposed regions, however, expressed these proteins. Endothelial cells also expressed XAC-GFP after injection of the virus cDNA construct into vascular welling points. An advantage of the proximal tubule and vascular micropuncture approaches is that only minute amounts of virus are required to achieve protein expression in vivo. This micropuncture approach to gene transfer of the virus cDNA construct and intravital two-photon microscopy should be applicable to study of the behavior of any fluorescently tagged protein in the kidney and shows promise in studying renal physiology and pathophysiology.

actin; actin depolymerizing factor; adenovirus; green fluorescent protein; intravital microscopy

THE TRANSFER OF GENETIC MATERIAL into kidney cells in vivo is widely recognized as a potentially useful tool for studying renal pathophysiology and for gene therapy of renal diseases (4–6, 8). This approach can be used to express specific proteins in kidney cells. Our interest has been in examining the effects of ischemia on the kidney and in observing the behavior of specific protein molecules in response to an ischemic insult (1, 10). We hypothesized that expression of these molecules, labeled with a fluorescent tag, could permit study of their intracellular localization and behavior before, during, and after renal ischemia.

In unpublished studies (Ashworth S and Boyd-White J), we attempted to express protein chimeras with green fluorescent protein (GFP) by injection of an adenovirus vector containing the cDNA construct into the renal artery of the rat kidney. We saw modest levels of expression of the GFP-tagged proteins in these experiments but encountered several problems. First, the injection method involved a period of renal ischemia, which could have had consequences or altered the response to a second ischemic insult induced after protein expression. Second, the intra-arterial injection of a large dose of adenovirus caused renal vascular inflammation and possible systemic effects. Third, we only saw expression of GFP in the vasculature, not in kidney tubule cells.

In this study, we present a novel approach to accomplish gene transfer and visualization of a protein product in the rat kidney. We used micropuncture methods to infuse adenovirus containing *1*) *Xenopus laevis* actin depolymerizing factor/cofilin isoform (wild type) linked to GFP [XAC(wt)-GFP], *2*) actin-GFP, or *3*) GFP cDNA constructs (7) into the lumens of single proximal tubules or vascular welling points on the kidney cortex surface of anesthetized rats. The ADF/cofilins are actin-binding proteins that regulate actin filament dynamics and participate in proximal tubule microvillus disruption after an ischemic insult (1, 10). Two days after recovery from anesthesia and surgery, expression of GFP-tagged proteins was observed in proximal tubule and endothelial cells. In vivo imaging with a two-photon microscope (3) was used to visualize the GFP-tagged proteins. This approach should have wide applicability in studying the behavior of fluorescently tagged proteins in vivo.

MATERIALS AND METHODS

Microinjection of adenovirus. Micropuncture experiments were done in a biosafety level 2-certified facility on 32 male Sprague-Dawley rats, weighing 210–430 g. The rats were fasted overnight, with free access to drinking water, and anesthetized intraperitoneally with 40 mg/kg pentobarbital sodium. The animal was placed on a heated animal board. With the use of sterile techniques, the left kidney was exposed via a subcostal flank incision and was supported in a micropuncture cup. Surgery and kidney micropuncture were done in a biological safety cabinet (model SG-603 SterilGARD III, Baker, Sanford, ME). The hood was fitted with a Leica MZ-6 microscope

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with ergotube head (W. Nuhsbaum, McHenry, IL). A fiberoptic light source was used to illuminate the kidney. The exposed kidney was kept moist by periodically dripping sterile 0.9% NaCl solution onto its surface.

The replication-incompetent adenovirus containing the cDNA for expressing XAC(wt)-GFP, actin-GFP, or GFP (used as a control) was suspended in DMEM at a concentration of 3–5 $\times$ 10^8 pfu/ml and was kept frozen at −80°C before use (7). The adenovirus constructs had been previously tested for efficient infection and transgene expression in LLC-PK cultured renal cells (2). We found that a higher virus titer was necessary for adenoviral infection and transgene expression via the vascular route, and in these experiments the viruses were concentrated to 3–5 $\times$ 10^{11} pfu/ml using the Viraprep Adenovirus Purification Kit (Virapur, Carlsbad, CA) and then suspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) just before use. The injection solutions were colored with lissamine green (30 mg/dl) or FD & C green dye no. 3 (100 mg/dl).

The virus suspension was aspirated into a sharpened micropipette (tip diameter 7–8 μm) that was filled with Sudan black-stained heavy mineral oil. We used constant bore, 0.55-mm (SD 0.02) inner diameter, capillary tubing to prepare these micropipettes. The solution volume injected was estimated from the length of the fluid column before and after injection and the relation 1-mm length = ~0.23 μl. The tip of the micropipette was filled with light mineral oil or a tiny amount of Sudan black-stained castor oil. The micropipette was mounted in a holder on a Leitz micromanipulator and was connected to a mercury leveling bulb which could be used to change the pressure in the micropipette and inject its contents in a controlled manner. The manipulator was clamped to a metal plate on the hood surface for better control of its movements.

The usual procedure for tubule injections was as follows. The kidney was observed at a magnification of ×96. A surface proximal tubule was selected, micropunctured, and the small droplet of castor oil in the micropipette tip (about 3 tubule diameters in length) was injected into the tubule lumen. The oil droplet flowed downstream and disappeared from view. The purpose of the oil droplet was to reduce dilution of the virus suspension by freshly formed glomerular filtrate. This was achieved by impeding flow through the narrow loop of Henle with the viscous oil; this causes proximal tubular pressure to rise and single nephron glomerular filtration rate to fall. In this "stopped-flow" condition, the injected virus suspension was concentrated by tubular water reabsorption, as was readily apparent from the increased dye intensity in the lumen of blocked tubules. The virus suspension was injected slowly, under visual observation, for an average of 17 min (SD 5), n = 33 tubules (range 5–22 min, median = 20 min). During the injection, colored injection solution usually appeared in a surface distal tubule after a delay of many minutes. The injected oil droplet occasionally appeared in a distal tubule segment, and the injection rate then had to be increased.

We also used a "free-flow" approach to tubule injections. In this case, all oil was expelled from the micropipette tip before puncturing the tubule and no oil was injected into the tubule lumen. The infusion rate was adjusted so as to prevent retrograde flow of the colored solution toward the glomerulus. In these experiments, microinfusion

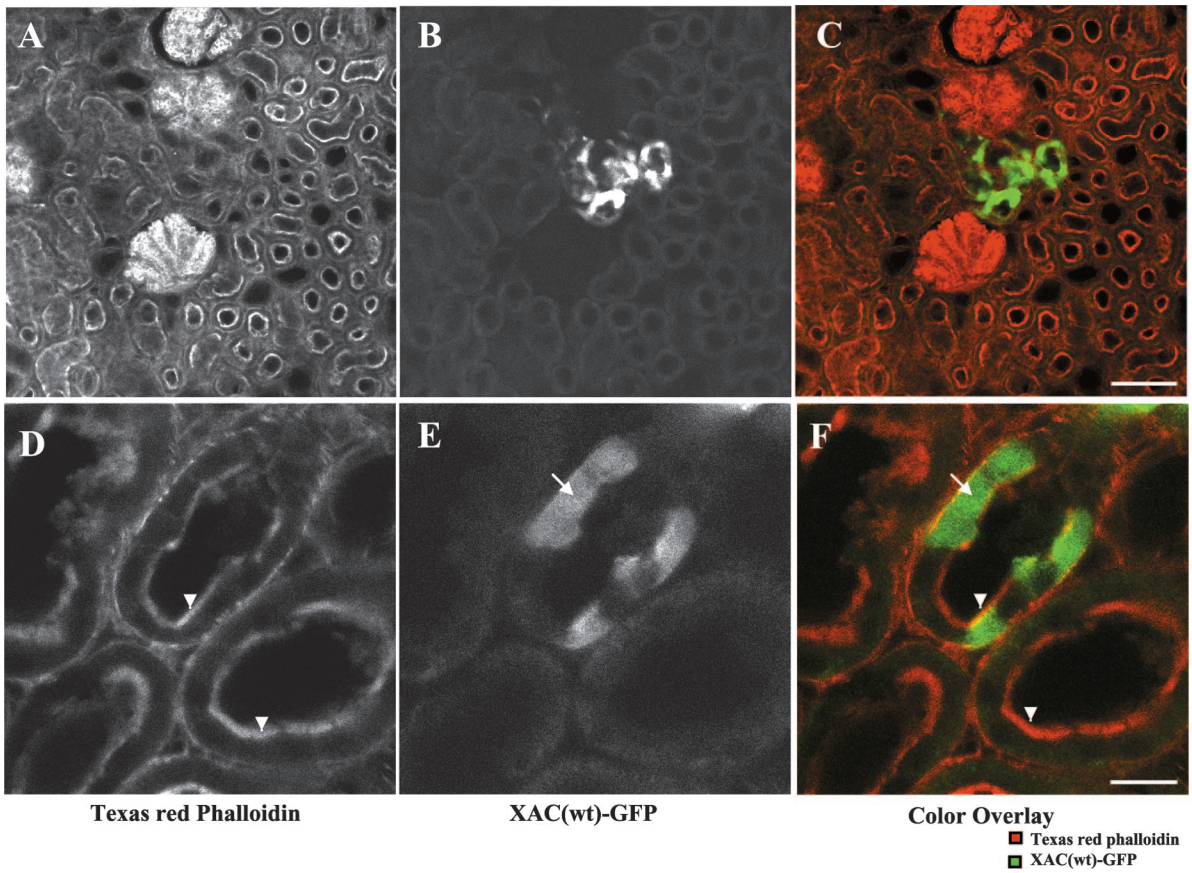

Fig. 1. Sections of a rat kidney cortex showing *Xenopus laevis* actin-depolymerizing factor/cofilin [XAC;(wt)]-green fluorescent protein (GFP)-expressing proximal tubule cells 48 h after microinjection. The low-power images in *A–C* show that labeling of proximal tubule cells is restricted to the micropunctured nephron. Typically only a minority of the cells in the infused tubules expressed XAC(wt)-GFP (*E*). The XAC(wt)-GFP appeared to be evenly distributed throughout the cytoplasm (arrow, *E*, *F*). Texas red-phalloidin staining of the actin cytoskeleton (*D*) appeared to be unaffected by XAC(wt)-GFP expression in the infected cells, compared with uninfected cells (arrowheads, *D*, *F*). Scale bars = 60 μm (*A–C*); 10 μm (*D–F*).

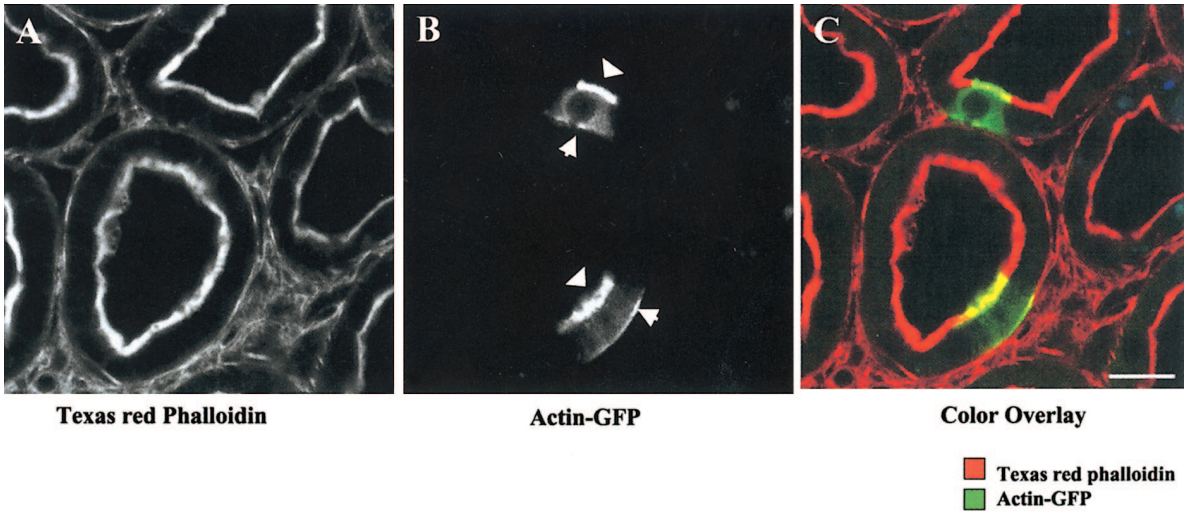

Fig. 2. Cortical kidney sections demonstrating rat proximal tubule cells expressing actin-GFP. Actin-GFP fluorescence intensity is increased in the apical brush-border microvilli (arrowheads) and in the basal aspects of the cell (arrows) compared with the actin-GFP signal in the cytoplasm (*B*). Expressed actin-GFP colocalizes with Texas red phalloidin-stained actin filaments (*A*) in the microvilli and at the basal membrane as shown in the image overlay (*C*). Scale bar = 10 μm (*A–C*).

rates averaged 107 nl/min (SD 64; range 26–194 nl/min) and lasted 18 min (SD 4; range 11–21 min; $n = 11$ tubules).

Vascular injections of colored virus suspension were done by microinjection into welling points, the place where a superficial efferent arteriole breaks up into the peritubular capillaries on the kidney surface. The infusion rates ranged from 180 to 700 nl/min and lasted 9–18 min (4 welling points).

We usually injected one or two tubules or blood vessels per animal. To aid subsequent identification of the injected tubules or blood vessels, a nearby proximal tubule was micropunctured, its lumen was completely filled with Sudan black-stained castor oil, and a map of the puncture sites was drawn. The flank incision was closed by apposing the muscle layers with sterile sutures and by using metal clips on the skin. The rat was allowed to recover from anesthesia and housed in an individual cage. No mortality or morbidity was observed.

Observations on the kidneys 2 days after adenovirus microinjection. Two days later, the rat was studied after an overnight fast. The animal was anesthetized with Inactin (130 mg/kg body wt) intraperitoneally. For observations with the two-photon microscope, the animal was placed on the microscope stage and kept warm with a circulating water heating blanket. The left kidney, which had been exposed by a small flank incision, was under the animal in a dish of isotonic saline (3). The nephron that had been filled with Sudan black-stained castor oil was located, and nearby GFP-expressing tubules were identified. Images were collected using a Bio-Rad two-photon microscope, with a titanium-sapphire laser set at a wavelength of 860 nm (3). To visualize proximal tubule cells in vivo more clearly, we injected a bolus of Texas red-conjugated folate (100–200 μg dissolved in isotonic saline) into a tail vein before collecting images in two rats (9). These rats had been on a folate-deficient diet for 6–7 days. Folate accumulates in the brush border and is endocytosed by proximal tubule cells (9). In two experiments, we injected a bolus of 1.6 mg Texas red-labeled neutral dextran 3,000 (Molecular Probes, Eugene, OR) to outline the tubular lumens.

In 18 experiments, the kidneys in the anesthetized rats were fixed for tissue imaging using retrograde aortic perfusion. The fixation solution contained 3% paraformaldehyde, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 4 mM Na_2HPO_4, and 2 mM picric acid (pH

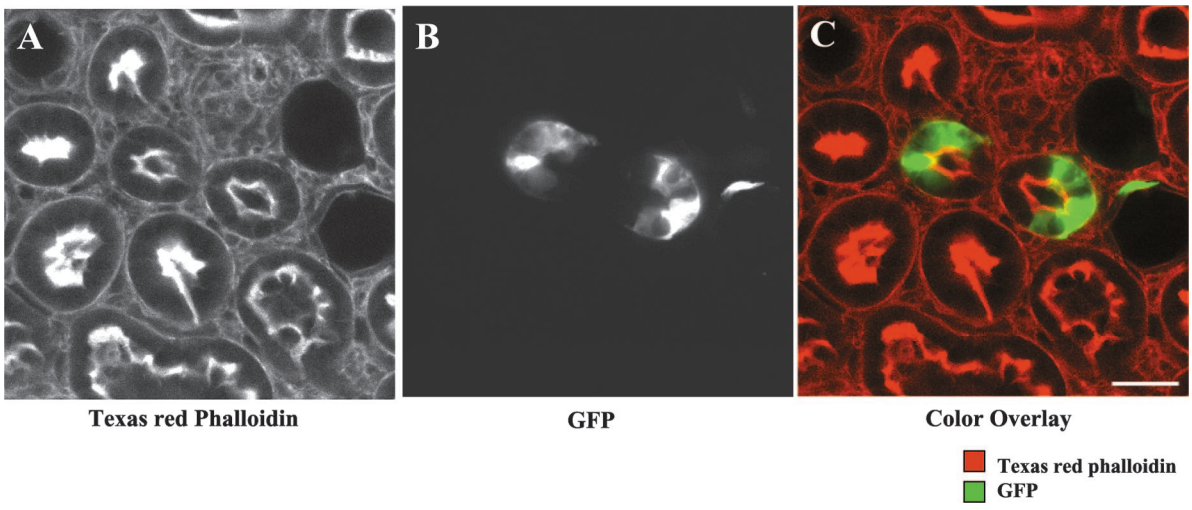

Fig. 3. Cortical sections from a normal kidney expressing GFP. Adenovirus for GFP expression (no XAC or actin) was microinjected into single tubules. The GFP signal was observed throughout the cytoplasm (*B*) with no localized accumulation within the proximal tubule cells. The Texas red-stained actin cytoskeleton (*A*) in the GFP-expressing cells was comparable to the actin cytoskeleton in the non-GFP-expressing cells. Overlay of images (*C*). Scale bar = 20 μm (*A–C*).

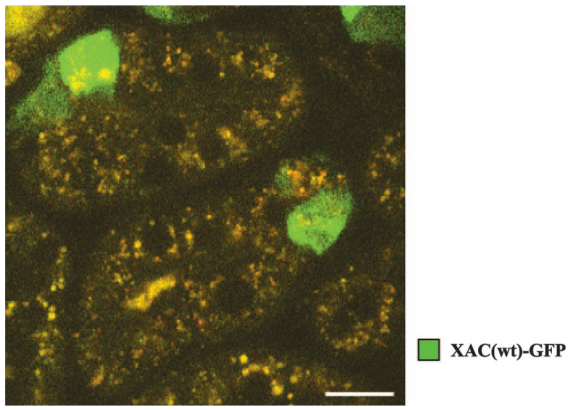

Fig. 4. Proximal tubule cells expressing XAC(wt)-GFP in vivo. Observations were made using the two-photon microscope. The XAC(wt)-GFP signal is diffusely distributed in the cytoplasm of proximal tubule cells. The autofluorescence signal enabled visualization of the proximal tubule. Scale bar = 10 μm.

adjusted to 7.4 with NaOH) and was delivered at a perfusion pressure of 150–170 mmHg for 20 min. The kidney was kept in the fixative solution at 4°C for several days, and then a block of kidney tissue containing the micropunctured area was cut with a scalpel. The kidney block was sectioned with a vibrotome and 100-μm-thick sections were stained for actin using Texas red-phalloidin diluted 1:200 in blocking buffer (2% bovine serum albumin and 0.1% Triton X-100 diluted 1:200 in phosphate-buffered saline). The sections were imaged using confocal and two-photon microscopes.

Experiments were conducted in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals,* and protocols were approved by the Indiana University School of Medicine Animal Care and Use Committee.

RESULTS

Two days after microinjection of adenovirus cDNA constructs for XAC(wt)-GFP, actin-GFP, or GFP into the lumen of single proximal tubules, GFP was expressed in tubule cells in 23 of 28 experiments. Failure to express the proteins or very weak expression appeared to be associated with low virus titer, short durations of injection (<10 min), or free-flow, as opposed to stopped-flow, injections. Figure 1 shows a typical example of expression of XAC(wt)-GFP in the renal cortex. Expression of GFP appeared to be restricted to the injected tubule, as shown in the low-power images (Fig. 1, *A-C*), but only a minority of its cells expressed the GFP signal, as shown in the higher magnification images (Fig. 1, *C-F*). The XAC(wt)-GFP complex appeared to be distributed throughout the cytoplasm as was previously observed for the distribution of endogenous cofilin (2). Texas red-phalloidin staining of the actin cytoskeleton appeared to be unaffected by XAC(wt)-GFP expression in the infected cells, compared with uninfected cells (Fig. 1).

Expression of actin-GFP (Fig. 2) was induced by microinjecting an adenovirus suspension containing a cDNA construct of actin-GFP. GFP fluorescence was distributed throughout the cytoplasm but was significantly increased in the dense F-actin bundles found in the apical microvilli of the brush border and also at the base of the cell.

Figure 3 shows results obtained from an experiment in which an adenovirus-GFP construct was injected 2 days earlier. Because this construct did not contain XAC or actin, these experiments served as a control for the XAC or actin experiments. Although the GFP was present throughout the cell cytoplasm, protein expression levels varied from cell to cell.

With two-photon microscopy, we next imaged XAC(wt)-GFP in vivo. We often had difficulty visualizing non-GFP-expressing proximal tubule cells in the absence of a fluorescent marker. In Fig. 4, the brightness level of autofluorescence was increased digitally to see the outlines of non-GFP-expressing proximal tubule cells. Two GFP-expressing proximal tubule cells are clearly visible. Intravenous injection of a bolus of Texas red-labeled dextran 3,000, a freely filtered molecule, allowed us to visualize the lumen of the microinjected nephron (Fig. 5). The presence of an open lumen with dextran in it indicated that the glomerulus of the nephron that had been microinjected 2 days earlier continued to filter the plasma. Another approach was to inject intravenously a Texas red-folate conjugate (Fig. 6). Filtered folate accumulated in the

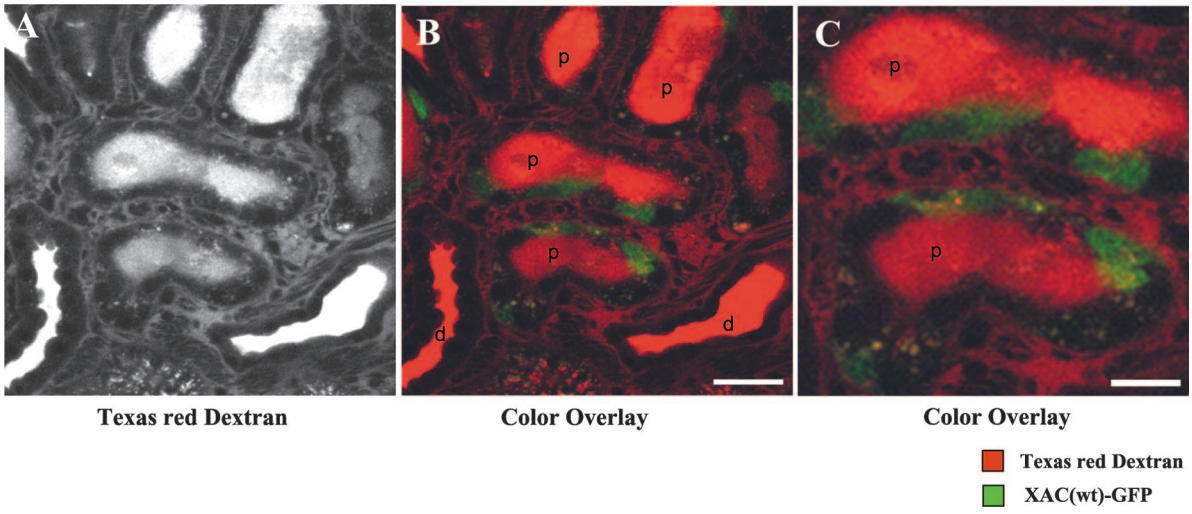

Fig. 5. Filtered Texas red-labeled dextran is concentrated in the tubule lumen, due to water reabsorption, and outlines the lumen (*A*). The proximal tubules contain XAC(wt)-GFP-expressing and -nonexpressing cells (*B*, *C*). Overlay images (*B*, *C*) with p = proximal tubule, d = distal tubule. Scale bar = 20 μm (*A*, *B*); 10 μm (*C*).

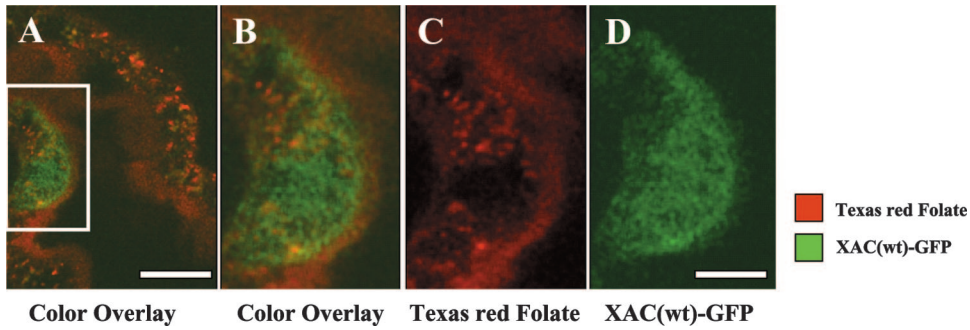

Fig. 6. Texas red-labeled folate fluoresces in the brush border and is incorporated into endocytic vesicles. This probe permits better visualization of proximal tubule cells in vivo. *B*: composite of *C* and *D*. *C*: Texas red-folate conjugate. *D*: XAC(wt)-GFP. *B*, *C*, and *D*: enlargements of the rectangular area in *A*. Scale bars = 10 μm (*A*); 5 μm (*B–D*).

brush-border region and was incorporated into endocytic vesicles in proximal tubule cells, greatly improving visualization of these cells. Qualitatively, the uptake of folate did not appear to differ in cells which did or did not express the XAC(wt)-GFP.

Infusion of a high titer adenovirus-XAC(wt)-GFP construct into vascular welling points on the kidney cortex surface led to expression of XAC(wt)-GFP in peritubular capillary endothelial cells (Fig. 7*A*). With the increased adenovirus titer, GFP signal was sometimes observed in adjacent proximal tubule cells (Fig. 7*B*), demonstrating that tubular cells can also be infected and labeled by the vascular route. This limited GFP expression may be a result of extravasation of the virus, possibly due to pressure effects on the endothelium, and labels far fewer epithelial cells than tubule lumen microinjection.

DISCUSSION

Delivery of adenovirus constructs directly into kidney proximal tubules and microvasculature resulted in expression of GFP-labeled proteins in kidney tubule and endothelial cells. Several conclusions can be made from the present experiments. First, infusion of the adenovirus over 10–20 min appears to be necessary for adequate cellular uptake, as protein expression was not achieved when the virus suspension was infused into tubule lumens for <10 min. This is why most infusions lasted 20 min. Second, tubule infusions in which a stopped-flow condition existed gave more consistent expression than free-flow infusions into tubules. We do not know whether this results from greater concentration of the virus suspension under stopped-flow conditions, raised intratubular pressure, or persistent tubule blockade (12, 13). In many of the stopped-flow tubule injections, we did see colored dye solution in a distal tubule segment or the sudden appearance of oil in a distal segment, but usually only after many minutes of injection. This suggests that the tubules were not completely or permanently blocked. Third, successful gene expression of XAC(wt)-GFP in vascular endothelial cells can be achieved by injection of a high titer adenovirus suspension, but not with the much lower titers used for tubular injections.

The present study raises a number of issues, some of which will need to be answered by further experiments.

First, we do not know why only a minority of cells in the injected tubules visibly expressed the GFP-tagged proteins, and why these particular cells, and not others, did so. It is difficult to estimate the exact percentage of tubule cells that expressed the GFP signal, because tubule cross sections that belong to the injected nephron might not be labeled and hence would not be recognized. Furthermore, in some experiments we could not find the injected nephron on *day 2*, possibly due to lack of GFP expression. In 31 tubule cross sections that contained at least one GFP-expressing cell, we estimated that an average of 43% of the cells (SD 29) expressed the GFP; the true frequency of GFP expression is likely much lower. We also noted in cell culture that not all cells in the monolayer became infected with the adenovirus and expressed the transgene protein (2). This may be due to the adenovirus titer or to the length of infection time. In one additional rat (unpublished observations), we saw robust expression of actin-GFP 16 days after microinjection of the adenovirus construct. Thus gene expression persists in vivo and its extent may be time dependent.

Second, whether the procedure described injures the tubule or affects tubule function needs further study. Our observations on fixed tissue suggest that the microinjection procedure was usually innocuous and did not cause cell injury, but occasionally we saw debris in the tubule lumen. Potentially, the virus

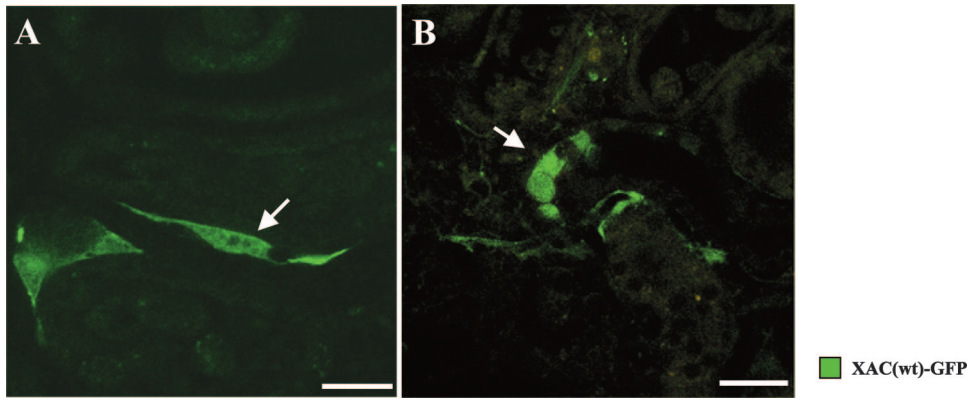

Fig. 7. Expression of XAC(wt)-GFP 2 days after infusion of a high titer adenovirus suspension into a vascular welling point in the anesthetized rat. *A*: capillary endothelial cell (arrow) is clearly labeled with XAC(wt)-GFP. *B*: XAC(wt)-GFP signal was also observed in proximal tubule cells (arrow), demonstrating that tubular cells can be labeled by the vascular route. Scale bars = 10 μm (*A*); 20 μm (*B*).

proteins could initiate an immune response or the expressed protein, if at high enough levels, might be toxic. We did not see any evidence of an immune response (e.g., edema or white cells), but no staining for leukocytes was done.

The intratubular microinfusion rates in the present study (average 107 nl/min) were high, exceeding a normal single nephron glomerular filtration (~30 nl/min in a rat) several-fold. The high intratubular infusion rate minimized dilution of the injected virus suspension by glomerular filtrate, but at the same time would have increased intratubular pressure to values close to, but not above, the stopped-flow pressure. We saw no evidence (e.g., dye uptake by the cells) that these high pressures acutely damaged the tubule cells. It has been common practice in micropuncture studies to elevate, for short periods of time, tubular pressures (e.g., during stopped-flow pressure measurements or in split oil-droplet experiments), with no detrimental effects reported. There was no apparent relationship between the rate of infusion and the expression of the GFP protein.

The vascular microinfusion rates were similar to those used by Spitzer and Windhager (11). These authors noted that adequate capillary perfusion rates could be achieved only at infusion rates of 400–800 nl/min, which is higher than the normal blood flow in a single efferent arteriole (~200 nl/min). The capillary network perfused clearly exceeds the number of peritubular capillaries originating directly from a single efferent arteriole.

It is possible to study the functional properties of the injected structures and their individual cells using imaging techniques. Limited observations suggest that the proximal tubule cells in the microinjected nephrons continue to endocytose folate and small molecular mass dextran.

Finally, we do not know whether cellular expression of the injected molecules will alter the response to an insult such as ischemia.

In this report, we highlighted observations made on fixed kidneys 2 days after microinjection (Figs. 1–3). The main advantages of the fixed tissue are that it provides an opportunity for staining and allows a clearer picture of cell morphology. Observations on fixed tissue sections, however, provide only a snapshot at one point of time. As a result of our ability to express specific protein molecules in vivo, we will now be able to make sequential observations on molecule behavior using live-animal imaging and the two-photon microscope.

The methods described in this paper allow the study of expression, cellular localization, and behavior of fluorescently tagged proteins in the living kidney. Both tubule cells and capillary endothelial cells can be made to express GFP-tagged proteins and on rare occasions we also saw expression in cortical collecting duct cells. A clear advantage of this approach is that only minute quantities of virus are injected into the lumen of a single tubule or microscopic blood vessel, so that the whole kidney or animal is not labeled or infected. By varying the injection site and virus dose, it might be possible, in future experiments, to label specifically glomerular cells, peritubular capillary endothelial cells, proximal tubules, or distal tubules. This approach should be generally useful in studying the in vivo behavior of a variety of molecules in numerous pathophysiological conditions.

GRANTS

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