THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 21, Issue of May 25, pp. 18540–18550, 2001
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Printed in U.S.A.

Intra-endosomal pH-sensitive Recruitment of the Arf-nucleotide Exchange Factor ARNO and Arf6 from Cytoplasm to Proximal Tubule Endosomes*

Received for publication, December 21, 2000, and in revised form, January 30, 2001
Published, JBC Papers in Press, February 13, 2001, DOI 10.1074/jbc.M011577200

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Kidney proximal tubule epithelial cells have an extensive apical endocytotic apparatus that is critical for the reabsorption and degradation of proteins that traverse the glomerular filtration barrier and that is also involved in the extensive recycling of functionally important apical plasma membrane transporters. We show here that an Arf-nucleotide exchange factor, ARNO (ADP-ribosylation factor nucleotide site opener) as well as Arf6 and Arf1 small GTPases are located in the kidney proximal tubule receptor-mediated endocytosis pathway, and that ARNO and Arf6 recruitment from cytosol to endosomes is pH-dependent. In proximal tubules *in situ*, ARNO and Arf6 partially co-localized with the V-ATPase in apical endosomes in proximal tubules. Arf1 was localized both at the apical pole of proximal tubule epithelial cells, but also in the Golgi. By Western blot analysis ARNO, Arf6, and Arf1 were detected both in purified endosomes and in proximal tubule cytosol. A translocation assay showed that ATP-driven endosomal acidification triggered the recruitment of ARNO and Arf6 from proximal tubule cytosol to endosomal membranes. The translocation of both ARNO and Arf6 was reversed by V-type ATPase inhibitors and by uncouplers of endosomal intralumenal pH, and was correlated with the magnitude of intra-endosomal acidification. Our data suggest that V-type ATPase-dependent acidification stimulates the selective recruitment of ARNO and Arf6 to proximal tubule early endosomes. This mechanism may play an important role in the pH-dependent regulation of receptor-mediated endocytosis in proximal tubules *in situ*.

In eukaryotic cells, exo- and endocytotic pathways contribute to various important functions such as secretion and uptake of soluble proteins as well as recycling of membrane proteins. Receptor-mediated endocytosis (RME)[1] is used by eukaryotic cells to internalize and process various macromolecules such as signaling molecules, proteins, nutrients, and toxins. In kidney epithelial cells, vesicular trafficking plays a crucial role in many processes, including fluid, ion, and metabolite homeostasis (1). In particular, kidney proximal tubule epithelial cells are highly specialized for protein reabsorption and membrane protein recycling via the RME pathway.

Recently, important progress has been made in our understanding of the regulation of exo- and endocytotic pathways. Key roles are played by V-type ATPase (V-ATPase)-dependent intravesicular acidification as well as by the ADP-ribosylation factor (Arf) subfamily of small GTPases. Earlier studies on the localization and distribution of overexpressed Arf proteins in cultured cells demonstrated that Arf1 was exclusively associated with the Golgi complex (2, 3) where it is required for β-COP recruitment (4, 5), formation of Golgi-derived COP-coated vesicles (6), and for formation and maintenance of Golgi complex structure (7, 8). In contrast, Arf6 was localized to the plasma membrane of Chinese hamster ovary cells (2, 9). It was proposed that Arf6 is an important regulator of the RME trafficking pathway (10) and is also implicated in rearrangement of the actin cytoskeleton (11). Some of these effects may be mediated via activation of phospholipase D isoforms by Arf proteins (12, 13). *In situ,* all six members of the Arf family have been identified in rat and mouse tissues (14). Recently we also identified Arf isoforms in human, dog, and rat kidneys (15, 16).

Arf small GTPases have low intrinsic GTPase activity, yet nucleotide exchange is required for their function as a "molecular switch" during the GTP/GDP cycle and cytosol to target membrane shuttling. Regulatory factors including Arf-specific GTPase-activating proteins (Arf-GAP) and guanine nucleotide exchange factors (Arf-GEF) are, therefore, essential for Arf function. ARNO (ADP-ribosylation factor nucleotide site opener) is a member of a family of Arf-GEFs which also includes cytohesin-1 (17), GRP1 (18), and EFA6 (19). ARNO is located at the plasma membrane and in contrast to another

* This work was supported by Medical Research Council of Canada Grant MT-7875 (to V. M. and P. V.) and National Institutes of Health Grants DK42956 (to D. B.) and DK38452 (to D. A. A., V. M., J. E. C., and D. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: RME, receptor-mediated endocytosis; Arf, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; ARNO ADP-ribosylation factor nucleotide site opener; BHK, baby hamster kidney; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GTPγS, guanosine-5′-O-(3-thiotriphosphate); GDPβS, guanosine-5′-O-(2-thiodiphosphate); PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BBM, brush-border membrane; DCCD, dicyclohexylcarbodiimide ; TGN, trans-Golgi network; PSP, pH-sensitive protein.

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Arf-GEF activity located in the Golgi complex, it is not sensitive to brefeldin A (2, 20). Recently the insulin receptor-dependent translocation of ARNO from cytosol to plasma membrane of adipocytes *in vivo* was demonstrated (21) and its coimmunoprecipitation with the insulin receptor has been reported (22). ARNO is 4 times more efficient in activating Arf6 than Arf1 during exchange of GDP by GTP *in vitro* (23), indicating that its physiological function may be to regulate Arf6 activity.

Numerous mammalian intracellular organelles and carrier vesicles including clathrin-coated vesicles, endosomes, lysosomes, and the Golgi/TGN complex have an acidic lumen generated by a V-ATPase in conjunction with a parallel chloride conductance (24–26). Individual endocytotic vesicles from kidney proximal tubules showed considerable pH heterogeneity (27), suggesting that endocytotic trafficking is associated with a progressive acidification as internalized content passes from early endosomes to late endosomes and finally to lysosomes. Two central questions need to be answered. 1) What are the molecular mechanisms by which this differential acidification in the RME pathway is achieved? 2) What is the functional role of the intra-endosomal acidification process in regulation of the RME pathway? One potential regulatory mechanism involves a pH-dependent interaction of transport vesicles with Arf small GTPases and other cytosolic coat proteins. Previously, a pH-dependent interaction of Arf protein(s) with pancreatic microsomal vesicles (28, 29) as well as β-COP (30–32) with endosomal vesicles of BHK-21 cells *in vitro* was reported. However, the specific members of the Arf subfamily involved, as well as the molecular mechanism of pH-dependent recruitment were not established (33). We now report the expression and distribution of ARNO, Arf6, and Arf1 in kidney proximal tubule epithelial cells and describe their co-localization with apical acidifiable endosomal vesicles *in situ*. We show that V-ATPase-dependent intra-endosomal acidification stimulates the recruitment of ARNO and Arf6 from proximal tubule cytosol to endosomal membranes, implicating this process in endosomal function *in situ*.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Wheat germ agglutinin, aprotinin, pepstatin A, chymostatin, and phenylmethylsulfonyl fluoride, concanamycin A (folimycin), FCCP, and nigericin were purchased from Sigma. Acridine orange was from Molecular Probes (Eugene, OR). ATP, creatine phosphate, creatine phosphokinase, and bacterial collagenase A (from *Clostridium histolyticum*) were obtained from Roche Molecular Biochemicals (GmbH, Germany). Non-hydrolyzable analogs of the guanine nucleotides GTPγS and GDPβS were supplied by Calbiochem (La Jolla, CA). Percoll, low-molecular weight protein calibration kit, IF standards for two-dimensional PAGE and PhastGel silver staining kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Kaleidoscope prestained protein standards and all other reagents for SDS-PAGE and Western blotting were from Bio-Rad Laboratories. Immobilon-P transfer polyvinylidene difluoride membranes were obtained from the Millipore Corporation (Bedford, MO). ECL Western blotting detection kits, donkey horseradish peroxidase-conjugated anti-rabbit antibody, and sheep horseradish peroxidase-conjugated anti-mouse antibody were purchased from Amersham Pharmacia Biotech. Goat anti-rabbit (GAR-Alexa 488) and goat anti-mouse (GAM-Alexa 488) IgG conjugated to Alexa 488 were obtained from Molecular Probes (Eugene, OR). Goat anti-rabbit (GAR-Cy5) and goat anti-mouse (GAM-Cy5) IgG conjugated to Cy5 were obtained from Jackson Immunoresearch (West Grove, PA).

Production and purification of recombinant rec-Arf1 and rec-Arf6 proteins as well as production and characterization of the rabbit polyclonal anti-Arf1 (SYL1) and monoclonal anti-Arf6 (SYL6) antibodies have been previously described (15). The polyclonal anti-ARNO antibodies (Lap18) were raised against recombinant ARNO. The chicken polyclonal anti-V-ATPase (subunit E, 31 kDa) antibodies were generated against a KLH-coupled C terminus peptide (C-GANANRKFLD) and affinity purified using Pierce "Sulfolink" column (Rockford, IL). They were generously provided by Dr. Sylvie Breton, MGH Program in Membrane Biology, and have been characterized previously (34). Rabbit polyclonal anti-megalin (a proximal tubule apical membrane receptor) antibodies were obtained from Dr. R. T. McCluskey, Department of Pathology, Massachusetts General Hospital (35). Mouse monoclonal anti-TGN38 (Clone 2) (trans-Golgi network marker) antibodies were obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-Rab11 antibodies (recycling endosome marker) were purchased from Zymed Laboratories Inc. (San Francisco, CA). Mouse monoclonal anti-β-COP (Clone M3A5) and anti-GP58 (Clone 58K-9) (marker of the Golgi apparatus) were supplied by Sigma. Rabbit polyclonal anti-Rab5 antibodies (early endosome marker) were obtained from StressGen Corp. (Victoria, BC, Canada).

Expression, Distribution, and Co-localization of Endogenous ARNO and Arf Isoforms in Kidney Proximal Tubules in Situ—Immunofluorescence experiments were performed on cryostat sections of rat kidney using an antigen retrieval technique (36). Kidneys from Harlan Sprague-Dawley rats were fixed in paraformaldehyde-lysine periodate by intravascular perfusion. Kidney slices were further fixed overnight at 4 °C, before being stored in phosphate-buffered saline (PBS). Kidney slices were cryo-protected in 30% sucrose/PBS, mounted for cryosectioning in Tissue-Tek embedding medium and quick-frozen in liquid nitrogen. Sections were cut at a thickness of 5 μm on a Reichert-Frigocut cryostat and collected on Fisher Superfrost Plus microscope slides. Antigen retrieval was performed by treatment of cryostat sections with 1% SDS for 4 min as previously described (36). After washing in PBS buffer (2 times, 5 min) sections were blocked with 1% bovine serum albumin in PBS for 10 min. Primary polyclonal anti-ARNO (1:20 dilution), anti-V-ATPase (1:200 dilution), anti-megalin (1:1,000 dilution), anti-Arf1 (1:20 dilution), anti-Rab11 (1:10 dilution), anti-Rab5 (1:100 dilution), and monoclonal anti-Arf6 (1:10 dilution) were then applied and incubated overnight at 4 °C. Secondary GAR-Alexa 488 or GAM-Alexa 488 were diluted in DAKO medium (1:100) and incubated for 60 min at room temperature. After washing in PBS, slides were counterstained with Evans Blue. In co-localization experiments, primary mouse monoclonal anti-Arf6, rabbit polyclonal anti-ARNO, and chicken polyclonal anti-V-ATPase antibodies were used as above. Primary antibodies were then detected using goat anti-mouse, goat anti-rabbit, or donkey anti-chicken IgG conjugated to Alexa 488 or Cy5 as appropriate.

Endocytosis of FITC-dextran by Kidney Proximal Tubules in Vivo—FITC-dextran (25 mg in 1 ml of 0.9% NaCl) was injected into the jugular vein of an anesthetized adult rat. After 10 min kidneys were perfused with PBS followed by paraformaldehyde-lysine periodate and sucrose solution. After removing the kidneys, they were immersed in paraformaldehyde-lysine periodate for 2 h for additional fixation, and FITC-dextran was visualized in 5-μm cryostat sections as previously described (37).

Confocal and Conventional Immunofluorescence Microscopy—Incubated sections were mounted in a 2:1 mixture of Vectashield mounting medium (Vector Labs, Burlingham, CA) in 1.5 M Tris solution (pH 8.9). Epifluorescence analysis was performed on a Nikon Eclipse E800 epifluorescence microscope connected to a Macintosh G4 computer. Images were captured using a Hamamatsu Orca CCD camera and IPLab Spectrum (Version 3.1a) image processing software (Scanalytics Inc., Fairfax, VA). Confocal analysis was performed on a Bio-Rad Radiance 2000 confocal microscope using LaserSharp 2000 software. All images were transferred into Adobe Photoshop 5.0, paginated using Adobe Illustrator 9.0, and printed on a Epson Stylus Photo750 color printer.

Preparation of Dog Kidney Proximal Tubules in Suspension—Cortical tubules (>85% proximal) were prepared from slices of dog renal cortical tissue. Briefly, both kidneys were removed from anesthetized animals and immediately immersed in ice-cold modified Krebs-Henseleit saline containing: 120 mM NaCl, 3.2 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 50 mM mannitol. The renal cortex was sliced with a Stadie Riggs microtome and tubules were separated and isolated by collagenase digestion as previously described (38, 39). The final suspension of cortical tubules containing around 60 mg wet weight per ml was kept at 4 °C in Krebs-Henseleit saline fully gassed with 5% CO_2, 95% O_2 until use.

Purification of Endosomes and Cytosol from Dog Kidney Proximal Tubules—Endosomes and cytosol from dog kidney proximal tubules in suspension were also prepared as previously described (39). Briefly, the suspension of cortical tubules was homogenized in the presence of protease inhibitors: 0.1 μM aprotinin, 1 μM pepstatin A, 10 μM chymostatin, and 100 μM phenylmethylsulfonyl fluoride. The suspension was then centrifuged at 7,700 × *g* for 15 min and the supernatant was recentrifuged at 20,000 × *g* for 30 min. The supernatant from the second centrifugation was recentrifuged at 150,000 × *g* for 1 h and was kept on ice until use. The pellet containing a mixture of brush-border membrane (BBM) vesicles and early endosomes was collected and ho-

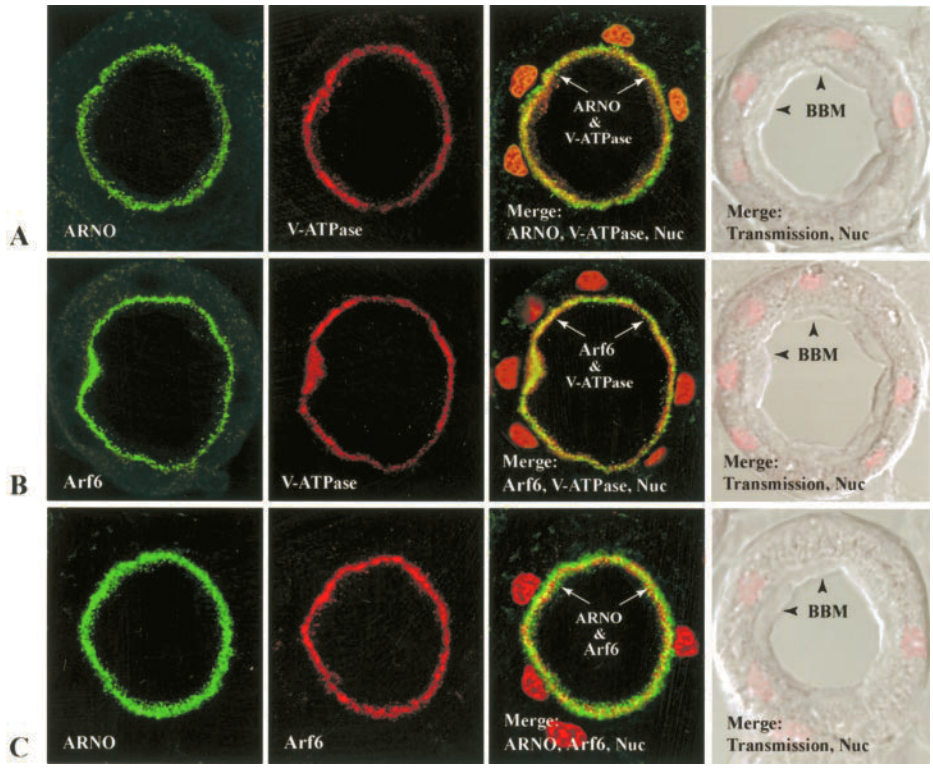

FIG. 1. **Co-localization of ARNO and Arf6 with V-ATPase in acidifiable endosomes *in situ*.** Confocal microscopy analysis of partial co-localization of ARNO with V-ATPase (*A*), Arf6 with V-ATPase (*B*), and ARNO with Arf6 (*C*) in kidney proximal tubule apical endosomes *in situ*. In *panels A* and *B*, primary anti-ARNO and anti-Arf6 antibodies were detected with GAR-Alexa 488 and GAM-Alexa 488, respectively (*green*). Primary anti-V-ATPase antibodies were detected with DAC-Cy5 (*red*). In *panel C*, primary anti-ARNO antibodies were detected with GAR-Alexa 488 (*green*) while anti-Arf6 was detected with GAM-Cy5 (*red*). Nuclear (*Nuc*) staining was performed with propidium iodide. The *right panel* in each row was obtained using the transmitted light mode of the confocal microscope, and shows the localization of the BBM.

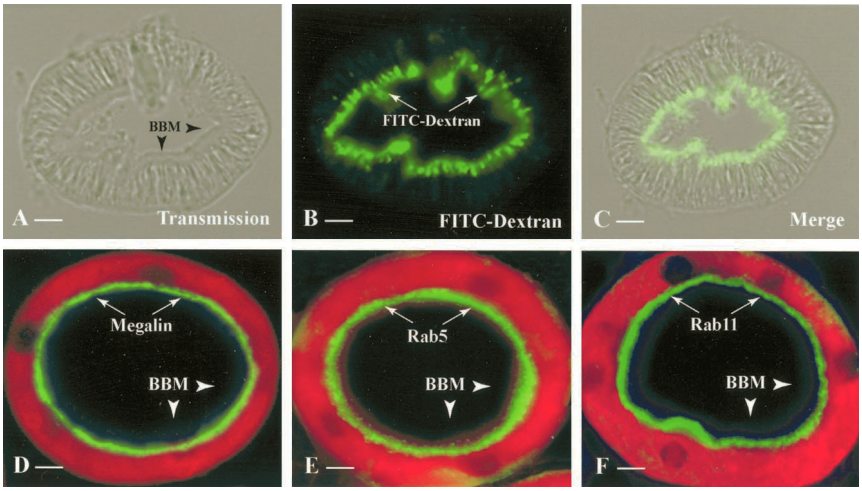

FIG. 2. **The apical domain of proximal tubules corresponds to the active RME pathway of epithelial cells and contains numerous endocytotic vesicles.** FITC-dextran (non-proteinaceous marker for fluid phase endocytosis) is taken by proximal tubules and concentrated in endosomal vesicles immediately below the apical brush-border membrane *in vivo* (*A-C*). Megalin (apical receptor for broad range of luminal proteins)(*D*), Rab5 (early endosome marker)(*E*), and Rab11 (recycling endosome marker)(*F*) showed a similar pattern of apical localization, immediately below the apical brush-border membrane. Tissue sections (*D-F*) were counter-stained with 1% Evan's Blue (red fluorescence). *Bar* = 5 μm.

mogenized by aspiration through a 25 5/8-gauge steel needle and re-centrifuged at 1,900 × *g* for 15 min. Endosome-enriched BBM vesicles were pelleted by centrifugation at 31,000 × *g* for 30 min, resuspended in 150 mM KCl, 5 mM Tris-Hepes (pH 7.4) (1 mg of protein/ml), and used to purify the early endosomal fraction. To separate early endosomes (E) from BBM vesicles (BBMV) a wheat germ agglutinin negative selection technique was employed as previously described (39). Protein concentration of proximal tubules, early endosomal, and 150,000 × *g* cytosol fractions was measured after solubilization of membranes in 0.1% SDS with Pierce bicinchroninic acid protein assay kit (Rockford, IL) using albumin as standard (40). Freshly prepared endosomes were resuspended to yield 5–10 mg of protein/ml and stored on ice until use. Estimation of the purity of the endosomal preparation (39) as well as characterization of their acidification machinery (41) was also previously described in detail. In our experiments, we used only freshly prepared early endosomal and cytosolic fractions to measure endosomal acidification and ARNO/Arf translocation. Freezing in liquid nitrogen with consequent thawing drastically diminished the acidification capacity of endosomes and interfered with the translocation assay (data not shown).

Purification of Golgi Stacks from Kidney Tissue—Isolation of Golgi stacks from kidney cortex was performed using a discontinuous sucrose gradient as previously described (42).

Endosomal Acidification Assay—Acidification of endosomes was measured simultaneously with translocation of ARNO/Arf under the same conditions, and using freshly prepared endosomal and cytosolic fractions. Endosomal acidification was measured at 37 °C by acridine orange fluorescence quenching as previously described (41). Briefly, 100 μg of endosomal protein was added to 2 ml of acidification/translocation buffer (1.5 mg/ml cytosol supplemented with 150 mM KCl, 50 mM Tris-Hepes (pH 7.4), 5 mM $MgSO_4$, 1 mM ATP, 5 mM creatine phosphate, 10 units/ml creatine phosphokinase, 5 μM acridine orange) to initiate proton transport. Fluorescence measurements were performed using a Deltascan Model RFM-2001 spectrofluorimeter (Photon Technology International, South Brunswick, NJ) with excitation at 450 nm (slit width 1 nm) and emission at 525 nm (slit width 2 nm). Fluorescence was recorded using the Felix^{TM} software. Folimycin (0.1 μM) or DCCD (100 μM) were used to inhibit the V-ATPase while FCCP (1 μM), nigericin (1 μM), or NH_4Cl (10 mM) were used to dissipate intra-endosomal proton gradients.

In Vitro ARNO, Arf6, and Arf1 Translocation Assay—The ARNO and Arf translocation assays were performed in the same buffer used for the acidification measurements (see above), except that 5 μM acridine orange was replaced with 100 μM phenylmethylsulfonyl fluoride. Also, for the study of GTP/GDP cycle-dependent Arf translocation, the ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, 10 units/ml creatine phosphokinase) was replaced by GTPγS (200 μM) and/or

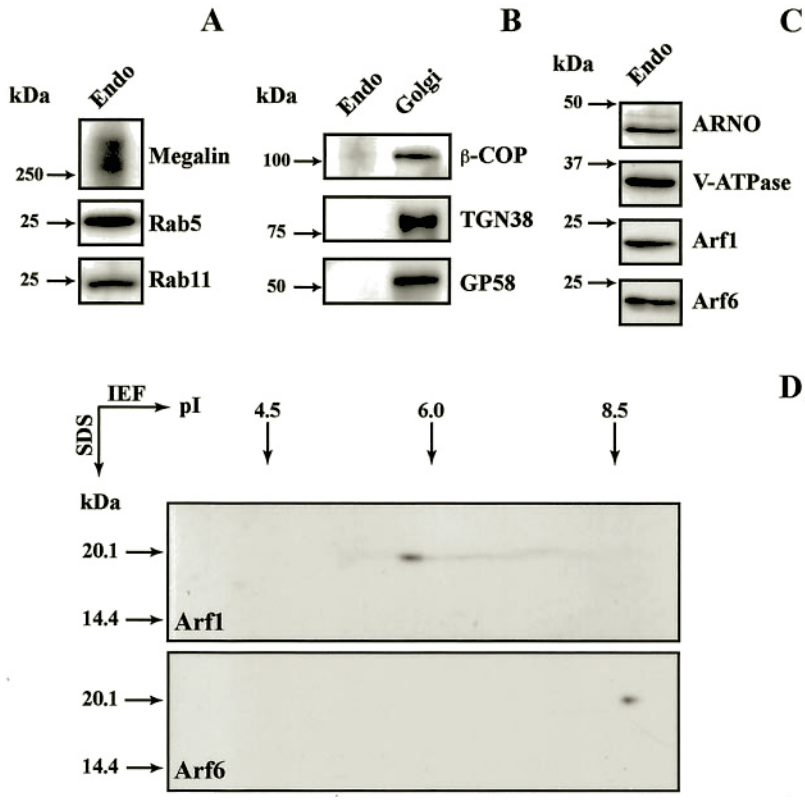

FIG. 3. **Isolated kidney proximal tubule endosomes are not contaminated with Golgi and/or TGN membranes and contain ARNO, Arf6, Arf1, and V-ATPase.** Purified endosomes are enriched with megalin receptor and endosomal markers Rab5, Rab11 (*A*), do not contain the Golgi/TGN markers GP58, TGN38 (*B*), and contain ARNO, V-ATPase, Arf6, and Arf1 (*C*). Detection and mapping of Arf6 and Arf1 in the cytoplasm of kidney proximal tubules by two-dimensional gel electrophoresis and Western blot analysis (*D*). The polyclonal anti-Arf1 antibody recognized a single protein (estimated molecular mass ≈ 20 kDa and pI ≈ 6.0) while monoclonal anti-Arf6 antibody recognized a single protein (estimated molecular mass ≈ 20 kDa and pI ≈ 8.6) in the cytoplasm. There was no cross-reactivity of these antibodies with other Arf isoforms in kidney proximal tubules cytosol.

GDPβS (200 μM) as indicated in the figure legends. Freshly prepared endosomes (100 μg) were mixed with 500 μl of freshly prepared cytosol (1 mg/ml protein) and incubated for 10 min at 37 °C. Translocation was stopped by putting the tubes on ice for 2 min, and endosomes were recovered by centrifugation at 16,000 × *g*, 4 °C for 60 min. The endosomal pellet was rinsed with 200 μl of rinsing buffer (200 mM sucrose, 25 mM Hepes-KOH, 50 mM KCl, 1 mM $MgCl_2$, pH 7, 4). After determination of protein concentration, endosomes were resuspended in 150 mM KCl, 5 mM Tris-Hepes (pH 7.4), and in SDS-PAGE sample buffer to give 1 mg/ml protein.

SDS-PAGE and Western Blot Analysis—Electrophoresis was performed using 12% SDS-Tris glycine-polyacrylamide gels (SDS-PAGE) according to Laemmli (43). Endosomal membranes collected after the *in vitro* translocation assay were applied to the gel (20 μg of protein per lane) and submitted to electrophoresis using a conventional Mini-Protean II electrophoresis cell (Bio-Rad). A graphite electroblotter system MilliBlot™ (Millipore Corp., Bedford, MA) was used to transfer proteins from the gels to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Nonspecific binding sites were blocked by exposing the membrane to 5% nonfat dry milk for 1 h. Membranes were incubated for 1 h with an antibody against Arf6 (1:1,000 dilution) or Arf1 (1:1,000 dilution) diluted in TBS/Tween/albumin buffer (15 mM NaCl, 0.1% (v/v) Tween 20, 3% (w/v) bovine serum albumin, 5 mM Tris-HCl, pH 7.0). After washing four times in TBS/Tween buffer, the membranes were exposed to horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse antibodies (dilution 1:3,000) in TBS/Tween/milk buffer for 1 h. Membranes were then washed four times in TBS/Tween buffer and proteins revealed using the ECL technique. Quantification of Arf6 and/or Arf1 was made using highly purified rec-Arf1 and rec-Arf6 calibration curves running simultaneously. Quantitative densitometric analysis was performed using NIH Image 1.62 software.

Two-dimensional PAGE Analysis of Soluble Cytosolic Proteins of Dog Kidney Proximal Tubules—Mapping of Arf proteins in the cytoplasm from dog kidney proximal tubules was performed by two-dimensional gel electrophoresis (two-dimensional PAGE) and Western blot analysis. A combination of isoelectric focusing (IEF) (3/10 ampholyte, Bio-Rad) and 12% SDS-PAGE was used to resolve 25 μg of protein from the 150,000 × *g* cytosolic fraction of dog kidney proximal tubules. Electrophoresis was carried out using a Mini-Protean II 2D (Bio-Rad) electrophoresis system. Electrotransfer of proteins from the two-dimensional gels to Immobilon-P polyvinylidene difluoride membranes and immunoblotting with anti-Arf1 (polyclonal SYL1, 1:1,000 dilution) and anti-Arf6 (monoclonal SYL6, 1:1,000 dilution) antibodies were performed as described above.

Statistical Analysis—All experiments were performed at least three times and data were analyzed using appropriate ANOVA analyses (SuperANOVA software, Abacus).

RESULTS

Endogenous ARNO and Arf6 Are Co-localized with V-ATPase in Apical, Acidifiable Endosomes in Proximal Tubules—By confocal immunofluorescence, endogenous ARNO, an Arf-GEF, was detected in the apical pole of proximal tubules in association with numerous vesicles immediately below the apical brush-border membrane and co-localized with V-ATPase in acidifiable endosomes (Fig. 1*A*). Small GTPase Arf6 was also detected in the apical pole of proximal tubules in association with numerous vesicles and co-localized with V-ATPase in acidifiable endosomes (Fig. 1*B*). Moreover, confocal immunofluorescence analysis revealed that Arf6 co-localized with its exchange factor ARNO in apical proximal tubule endosomes *in situ* (Fig. 1*C*).

The Apical Domain of Proximal Tubules contains Numerous Endocytotic Vesicles and Corresponds to the Active RME Pathway of These Cells—As shown in Fig. 2 (*panels A-C*), internalized FITC-dextran was concentrated in endosomal vesicles immediately below the apical brush-border membrane of kidney proximal tubules 10 min after FITC-dextran injection into the jugular vein of rats *in vivo*. The apical receptor protein megalin (Fig. 2*D*) showed a similar pattern of apical localization, immediately below the apical brush-border membrane. Antibodies against Rab5 (an early endosome marker) and Rab11 (a recycling endosome marker), also label subapical compartments in proximal tubule epithelial cells (Fig. 2, *E* and *F*). In contrast, endogenous Arf1 was detected in the perinuclear region, probably corresponding to the Golgi complex, similar to previous findings in cultured LLC-PK_1 cells (44). However, Arf1 was also found associated with apical microvilli and with vesicles at the base of brush-border membrane (not shown). These results are in agreement with our previous electron microscopic data

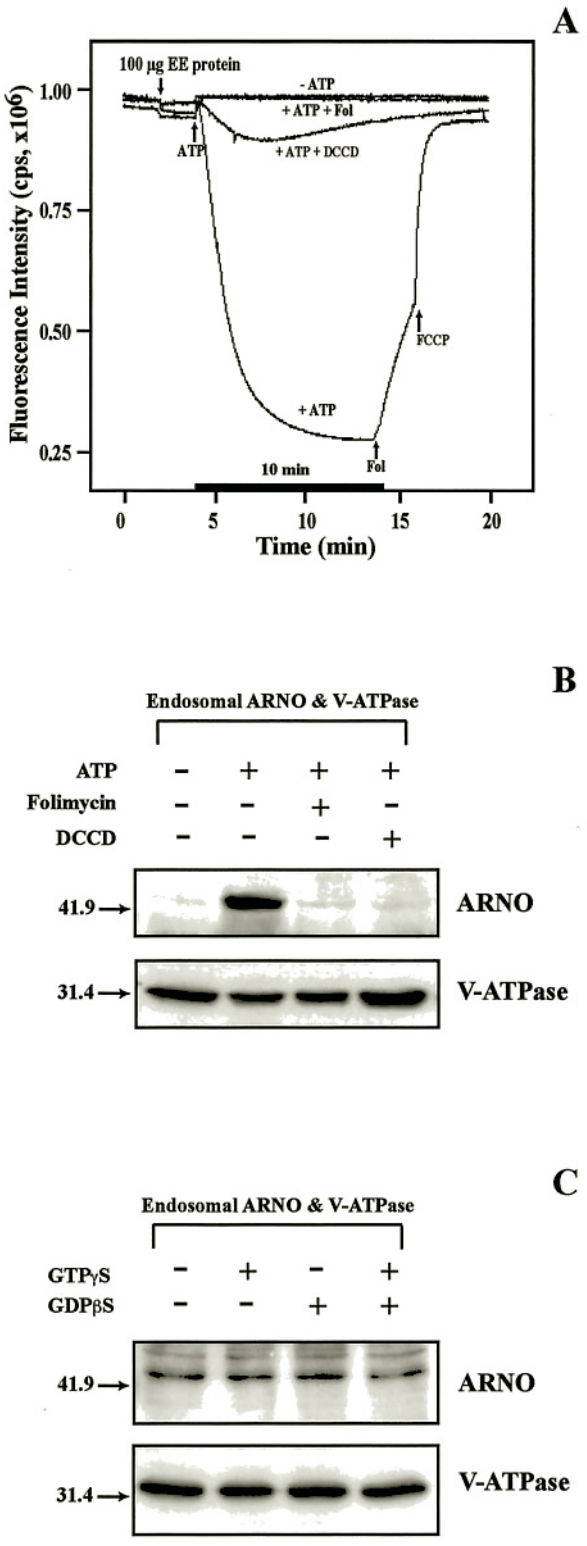

FIG. 4. **V-ATPase-dependent translocation of ARNO from cytosol to endosomal membranes *in vitro*.** Acidification of endosomes in the presence of ATP is completely abolished by folimycin and is greatly diminished by DCCD (*A*). Acridine orange fluorescence quenching was used to measure the acidification capacity of freshly prepared endosomes *in vitro*. The translocation of ARNO from cytosol to endosomes is induced in the presence of ATP and this translocation is abolished by inhibitors of the V-ATPase folimycin and DCCD (*B*). Recruitment of ARNO to endosomal membranes is not affected by GTPγS and/or GDPβS (*C*). The amount of subunit E of V-ATPase on endosomal membranes does not change during the experiment and serves as a protein loading control. Molecular sizes (kDa) of protein markers are indicated. Each *in vitro* translocation experiment was performed at least three times.

(16) showing that both Arf6 and Arf1 are detectable in the endocytotic pathway of proximal tubule epithelial cells *in situ*. Thus, ARNO, Arf6, and V-ATPase (Fig. 1) *in situ* are co-localized in apical acidifiable endosomes corresponding to the active RME pathway of these cells *in vivo*.

Isolated Kidney Proximal Tubule Endosomes Are Not Contaminated with Golgi and/or trans-Golgi Network (TGN) Membranes and Contain ARNO, Arf6, Arf1, and V-ATPase—We used a previously developed method to purify endosomes from proximal tubules (39). These endosomes contain megalin, an apical receptor protein, as well as the endosomal markers Rab5 and Rab11 (Fig. 3*A*), consistent with the apical localization of these proteins in tissue sections. The purified endosomes were not contaminated with Golgi (GP58) and/or trans-Golgi network (TGN38) membranes (Fig. 3*B*) and had a lower amount of β-COP than the Golgi. Thus, the presence of Arf6 and particularly Arf1 in the endosomal preparation together with variable amounts of ARNO and V-ATPase is consistent with their co-localization in endosomal vesicles *in situ* (Fig. 3*C*). Our present and previous kidney fractionation experiments (15, 33) also demonstrate that both Arf6 and Arf1 are membrane-bound as well as cytosolic proteins. Fig. 3*D* shows a two-dimensional Western blot analysis of cytoplasmic proteins in the 150,000 × *g* cytosolic fraction of purified proximal tubules. The polyclonal anti-Arf1 antibody recognized a single protein (estimated molecular mass ≈ 20 kDa and pI ≈ 6.0) in kidney proximal tubule cytoplasm. The monoclonal anti-Arf6 antibody also recognized a single protein (estimated molecular mass ≈ 20 kDa and pI ≈ 8.6) in the cytoplasm. There was no cross-reactivity of these antibodies with other Arf isoforms in kidney proximal tubules.

V-ATPase-dependent Recruitment of ARNO from Cytosol to Endosomal Membranes—Because ARNO was present and co-localized with V-ATPase in endosomal vesicles *in situ* (Fig. 1*A*), we next determined whether the acidification status of these vesicles was involved in recruiting ARNO to endosomal membranes *in vitro*. Purified endosomes were capable of significant ATP-dependent acidification, reaching a maximum and steady-state in 10 min (Fig. 4*A*). This acidification was dissipated by the V-ATPase inhibitor folimycin, and/or by the uncoupler, FCCP. Moreover, endosomal acidification was abolished by 0.1 μM folimycin and significantly diminished by 100 μM DCCD (Fig. 4*A*). The amount of ARNO associated with purified, non-acidic endosomes was low and varied among different endosomal preparations (compare Figs. 4, *B*, control, and *C*, control). However, translocation assays revealed that under conditions of maximal endosomal acidification, a significant amount of ARNO was recruited from the cytosol to endosomal membranes (Fig. 4*B*, *+ATP*). ATP-dependent ARNO translocation was completely abolished by folimycin (Fig. 4B, *+ ATP + folimycin*) and by DCCD (Fig. 4*B*, *+ ATP + DCCD*). The E subunit of the V-ATPase was used as an internal standard for endosomal protein loading. The amount of V-ATPase associated with endosomes did not change during ATP-dependent ARNO recruitment (Fig. 4*B*). To demonstrate the specificity of ATP action on ARNO recruitment, we also examined the effect of a non-hydrolyzable analog of GTP in the ARNO translocation assay. Incubation of endosomes in the presence of GTPγS and/or GDPβS did not lead to recruitment of ARNO from the cytosol to endosomal membranes (Fig. 4*C*).

Both Arf6 and Arf1 Are Targeted to Endosomal Membranes during the GTP/GDP Cycle—It is generally accepted that Arf1 is recruited from the cytosol and targeted to Golgi membranes during the GTP/GDP cycle (2–5). However, the cytosol to membrane shuttling of Arf6 is controversial (9, 33) and target membranes for Arf6 recruitment have not yet been identified (45). Fig. 5*A* shows that incubation of early endosomes with 200 μM

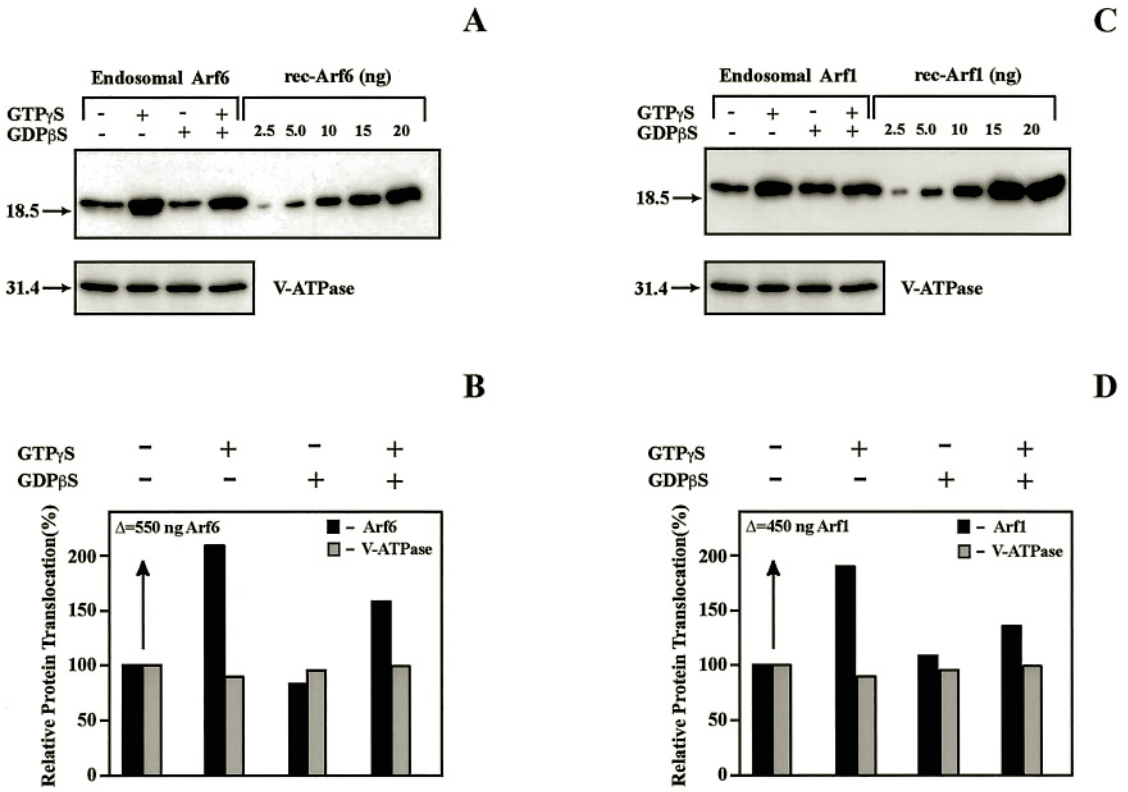

FIG. 5. **GTP/GDP cycle dependent recruitment of both Arf6 and Arf1 from cytosol to endosomal membranes *in vitro*.** Cytosolic Arf6 is targeted and recruited to kidney proximal tubule endosomes during the GTP/GDP cycle (*A*). Quantification of GTPγS-dependent Arf6 translocation using recombinant Arf6 (rec-Arf6) as a standard (*B*). Cytosolic Arf1 is also targeted and recruited to kidney proximal tubule endosomes during the GTP/GDP cycle (*C*). Quantification of GTPγS-dependent Arf1 translocation using recombinant Arf1 (rec-Arf1) as a standard (*D*). The amount of subunit E of V-ATPase on endosomal membranes does not change during the experiment and serves as a protein loading control. Molecular sized (kDa) of protein markers are indicated. Both cytosol and endosomes were prepared from purified kidney proximal tubules. Each *in vitro* translocation experiment was performed at least three times.

GTPγS but not 200 μM GDPβS led to recruitment of Arf6 from the cytosol to endosomal membranes. GTPγS-mediated Arf6 recruitment to endosomes was reduced by GDPβS (*lane 4*). However, the recruitment of Arf6 was not completely abolished by GDPβS, probably because the ratio of GTPγS:GDPβS in the incubation medium was 1:1. A similar pattern of recruitment during the GTP/GDP cycle was also seen with Arf1 (Fig. 5*C*). In these experiments, the E subunit of V-ATPase was also used as an internal standard for protein loading and the amount associated with endosomes did not change during GTP-dependent Arf6 and Arf1 recruitment (Fig. 5, *A, B* and *C, D*). Thus, both Arf6 and Arf1 are targeted to endosomal membranes during the GTP/GDP cycle *in vitro*. These results are consistent with the presence of both Arf6 and Arf1 in the proximal tubule RME pathway *in situ*.

V-ATPase-dependent, Selective and Predominant Recruitment of Arf6 from Cytosol to Endosomal Membranes—Acidification-dependent recruitment of Arf proteins to microsomal membranes has been previously demonstrated (28, 29), but the exact membrane target as well as the precise Arf isoform(s) involved were not identified. We, therefore, examined the role of ATP-dependent acidification on the recruitment of Arf1 and Arf6 to endosomes. A significant amount of Arf6 was recruited from the cytosol to endosomal membranes under conditions of maximal endosomal acidification (Fig. 6*A, +ATP*). A significant parallel disappearance of Arf6 from the cytosol was also observed (not shown). ATP-dependent Arf6 translocation was completely abolished by folimycin (Fig. 6*A, + ATP + folimycin*) and was significantly diminished by DCCD (Fig. 6*A*, + *ATP* + *DCCD*). As described above, the amount of V-ATPase associated with endosomes did not change during acidification (Figs. 4*B* and 6*B*). Quantification using rec-Arf6 as a standard revealed that up to 1.3 μg (per mg of endosomal protein) of Arf6 was recruited to endosomes under conditions of maximum acidification (Fig. 6*B*). In contrast, the V-ATPase-dependent recruitment of Arf1 from cytosol to endosomes in our experimental conditions was quite modest compared with that of Arf6 (Fig. 6*A*). These data indicate that acidification-dependent recruitment of Arf6 is specific, selective, and correlates with recruitment of the Arf-GEF, ARNO (Figs. 4*B* and 6*B*).

Translocation of ARNO and Arf6 Correlate with the Level of Intra-endosomal Acidification—To further demonstrate the role of endosomal acidification in ARNO and Arf6 recruitment, experiments with different uncouplers of endosomal acidification were performed. Endosomal acidification was completely abolished by 1 μM FCCP or 1 μM nigericin and was greatly diminished by 10 mM NH_4Cl (Fig. 7*A*). ATP-dependent Arf6 and ARNO recruitment was prevented or significantly reduced by all uncouplers tested (Fig. 7*B*). Both intra-endosomal acidification and ARNO, Arf6 translocation were reduced in parallel by different structurally unrelated uncouplers, and the efficiency of these uncouplers to dissipate the endosomal proton gradient was correlated with their capacity to prevent translocation of both ARNO and Arf6 (Fig. 8, *A* and *B*). As shown above, the amount of E subunit of the V-ATPase associated with endosomal membranes was not affected under these experimental conditions.

DISCUSSION

Proximal tubule epithelial cells have an extensive apical endocytotic apparatus that is critical for the reabsorption and degradation of proteins that traverse the glomerular filtration

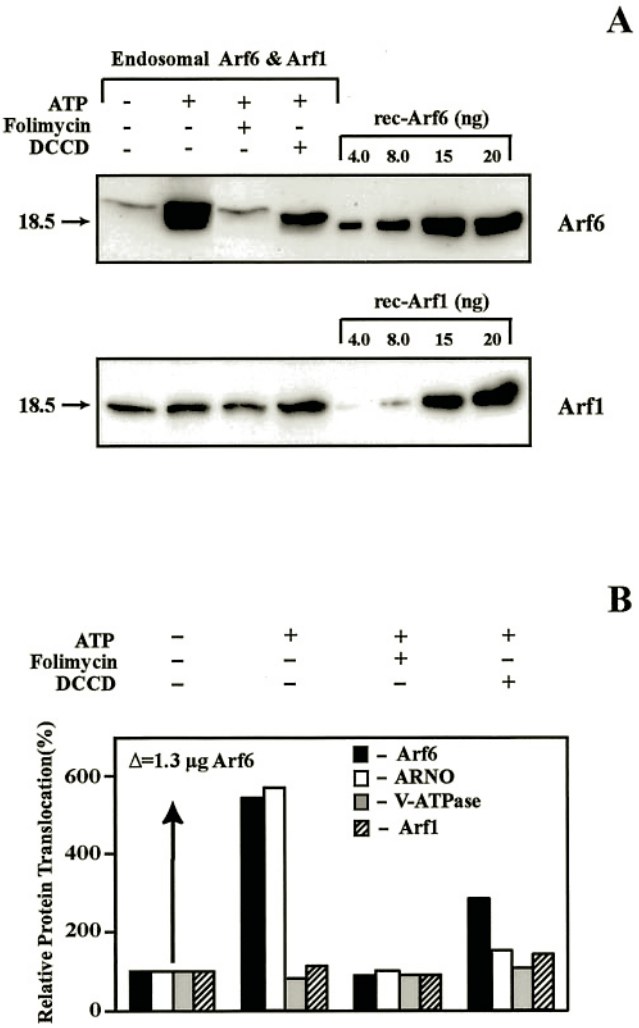

FIG. 6. **Selective V-ATPase-dependent translocation of Arf6 from cytosol to endosomal membranes correlates with ARNO recruitment.** The translocation of Arf6 from cytosol to endosomes is induced in the presence of ATP and this translocation is abolished by inhibitors of the V-ATPase folimycin and DCCD (*A*). Quantification of selective V-ATPase-dependent Arf6 translocation and its correlation with ARNO recruitment (*B*). Quantification was performed using recombinant Arf6 (*rec-Arf6*) and Arf1 (*rec-Arf1*) as standards. Molecular sizes (kDa) of protein markers are indicated. Each *in vitro* translocation experiment was performed at least three times.

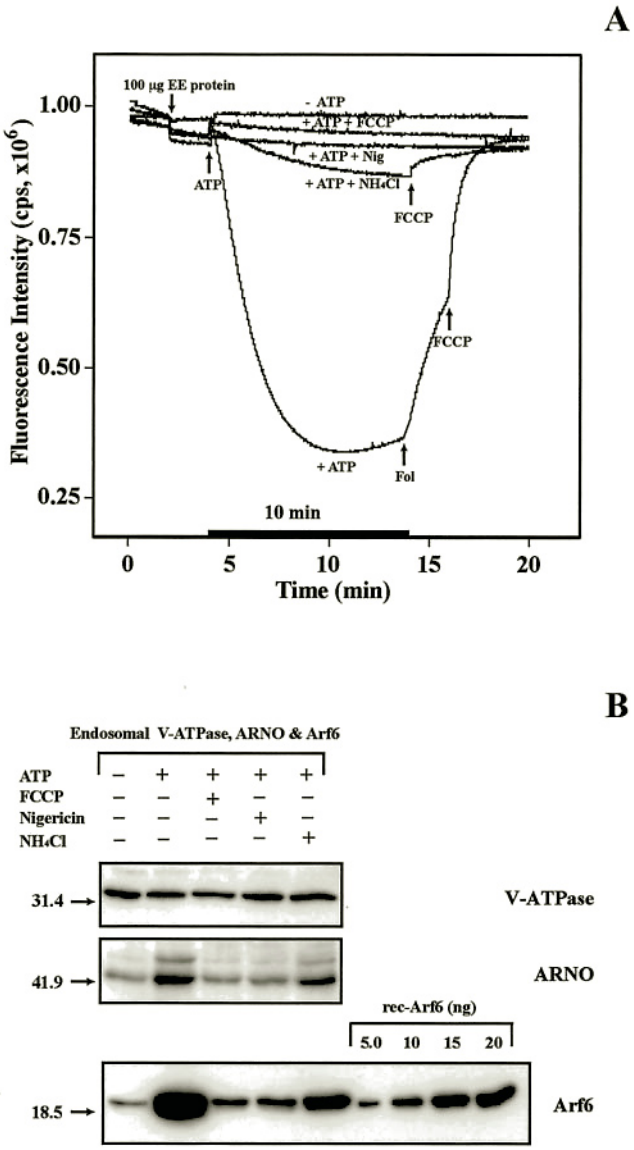

FIG. 7. **Translocation of ARNO and Arf6 from cytosol to endosomal membranes is related to the degree of intra-endosomal acidification.** Acidification of endosomes in the presence of ATP is completely abolished by FCCP and nigericin and is greatly diminished by NH_4Cl (*A*). Acridine orange fluorescence quenching was used to measure the acidification capacity of freshly prepared endosomes *in vitro*. In the presense of ATP both ARNO and Arf6 are translocated from cytosol to endosomes and this recruitment is reduced by various structurally unrelated uncouplers of intra-endosomal acidification such as FCCP, nigericin, and NH_4Cl (*B*). The amount of subunit E of V-ATPase on endosomal membranes does not change during the experiment and serves as a protein loading control. Molecular sizes (kDa) of protein markers are indicated. Both cytosol and endosomes were prepared from purified kidney proximal tubules. Each *in vitro* translocation experiment was performed at least three times.

barrier, as well as for the extensive recycling of functionally important apical plasma membrane proteins (1). The physiological importance of acidification processes in proximal tubule function is underlined by our finding that V-ATPase inhibition in cadmium nephrotoxicity leads to a Fanconi-like syndrome (46) and by our observation that the V-ATPase inhibitor folimycin completely abolishes albumin uptake by proximal tubule cells.[2] Moreover, a mutation in the ClC-5 chloride channel, which together with V-ATPase is believed to be responsible for efficient endosomal acidification in kidney proximal tubules, results in Dent's disease, whose manifestations include a partial Fanconi-like phenotype in humans (47, 48) as well as in clcn5$^-$ knockout mice (49). However, the link between endosomal acidification and regulation of the endocytotic pathway in proximal tubules is poorly understood. Our present data provide new insight into this process by showing a direct correlation between V-ATPase-dependent endosomal acidification and recruitment of the GTP/GDP exchange factor ARNO, and its cognate small GTPase Arf6 to endosomal membranes.

Because recruitment of Arf small GTPases to membranes is an early step in the regulation of both exo- and endocytotic pathways, this process could be a potential site at which vesicle trafficking and protein reabsorption in proximal tubules are modulated by pH gradients. Previous studies have shown that Arf proteins undergo acidification-dependent recruitment to microsomal membranes (28, 29), but the precise Arf family members that were translocated, the origin of the target vesicles/membranes as well as the molecular mechanism(s) of this recruitment remained unknown. Earlier experiments were performed on mixed microsomal vesicles (containing Golgi, TGN,

[2] V. Marshansky, D. A. Ausiello, and D. Brown, unpublished data.

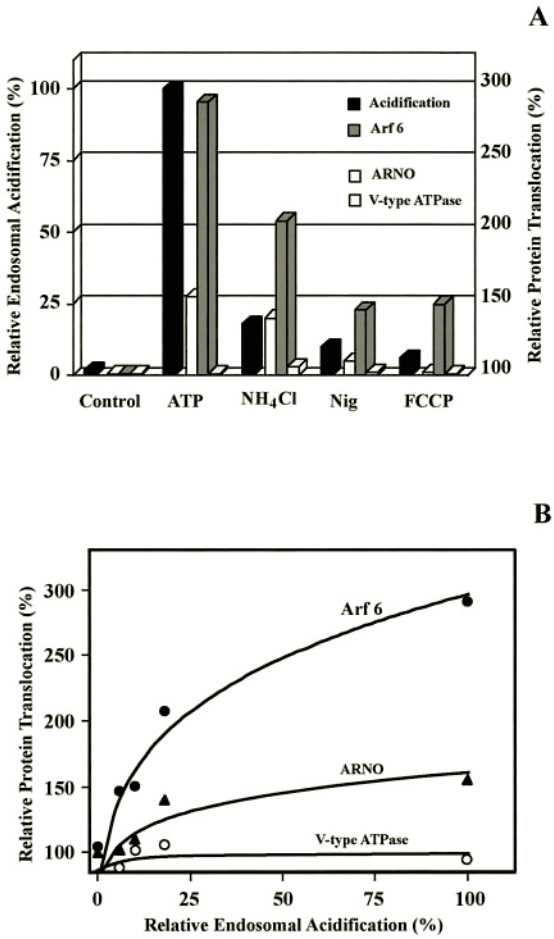

FIG. 8. **The amount of ARNO and Arf6 recruited from cytosol to endosomal membranes correlates with the magnitude of intra-endosomal acidification.** *A,* relative endosomal acidification and relative ARNO, Arf6 translocation in the presence of ATP and various intra-endosomal pH uncouplers. *B,* relative ARNO and Arf6 translocation from cytosol to endosomal membranes as a function of relative intra-endosomal acidification. Experimental conditions were identical to those used in Fig. 7.

endoplasmic reticulum, and endosomal membranes) and Arf proteins were detected using the monoclonal 1D9 antibody which recognizes all members of the Arf family except Arf4 (9). Furthermore, while acidification dependent recruitment of two coat proteins (β-COP, ϵ-COP) to endosomes from BHK-21 cells was also reported (30–32), the parallel translocation of Arf isoforms was not demonstrated in these studies.

Arf-GEF (ARNO) and both Arf6 and Arf1 were detected in the apical RME pathway in kidney proximal tubules *in situ*. This localization pattern partially overlapped with that of megalin, a proximal tubule apical receptor involved in endocytosis of a broad range of luminal ligands (33, 35, 49) as well as with the V-ATPase and an endocytosed marker FITC-dextran. Furthermore, Rab5 a component of early endosomes and Rab11, a marker of recycling endosomes were also concentrated in apically located vesicles in these cells. Arf1 was also found in the perinuclear region (probably the Golgi complex) of proximal tubule cells *in situ*. The presence of Arf1 in the Golgi complex of cultured cells is well documented (2–5, 44). Thus, these components are poised to participate in the proximal tubule endocytotic pathway, and this possibility was explored further using endosomes and cytosol purified from proximal tubules.

Our data show that ARNO and Arf6 are recruited from cytosol to endosomal membranes *in vitro* in an acidification-dependent manner. Recently, recruitment of ARNO from cytosol to the adipocyte plasma membrane upon activation of the insulin receptor has been reported (21). Insulin receptor-dependent recruitment of ARNO to plasma membranes is dependent on activation of phosphatidylinositol 3-kinase, production of phosphatidylinositol 3,4,5-P_3, and interaction of ARNO with phospholipid through its C-terminal plekstrin homology domain (50). However, using specific V-ATPase inhibitors as well as structurally unrelated uncouplers we found that ATP-dependent ARNO recruitment is intra-endosomal pH-dependent. ARNO recruitment was prevented both by V-ATPase inhibitors (folimycin and DCCD) and endosomal acidification uncouplers (FCCP, nigericin, and NH_4Cl) but not by the phosphatidylinositol 3-kinase inhibitors wortmannin and LY-294002 (data not shown). Also, an effect of endosomal membrane potential on the recruitment of ARNO could be ruled out since the generation of membrane potential was minimal under our experimental conditions. All translocation experiments were performed in the presence of chloride ions, which due to the presence of as yet unidentified chloride channels on these endosomal membranes effectively clamp the generation of membrane potential by the electrogenic V-ATPase, and allows the development of maximal intra-endosomal acidification (41). Thus, the ATP effect on ARNO recruitment is most likely to be due to an acidification-triggered event taking place inside the endosomal lumen and/or the interior leaflet of the endosomal membrane, and not to a membrane potential-dependent lipid rearrangement.

This ATP-dependent recruitment of ARNO was accompanied by the selective, pH-sensitive and predominant recruitment of Arf6 from cytosol to endosomal membranes, while recruitment of Arf1 was very modest under the same experimental conditions. A strong correlation between intra-endosomal lumenal pH and recruitment of both cytosolic ARNO and Arf6 to endosomes was found. The amount of translocated ARNO also correlated well with Arf6 recruitment to endosomal membranes. ARNO is 4 times more effective in activation of Arf6 than Arf1 as a substrate for GDP/GTP nucleotide exchange (23), providing a potential explanation for the specificity of the pH effect on Arf6 recruitment.

In contrast to the pH-specific recruitment of Arf6 to endosomal membranes, both Arf6 and Arf1 were effectively translocated from cytosol to endosomal membranes by GTPγS. While both cytosolic (GDP-bound) and membrane (GTP-bound) Arf1 have been found in a variety of cell types, consistent with its shuttling between the cytosol and Golgi membranes during the GTP/GDP cycle (4–8), Arf6 was thought to be an "unconventional" member of the Arf family that was found exclusively in plasma membranes, but not in endosomes and cytosol, from Chinese hamster ovary cells (9). Our present data, however, establish that Arf6 is both a cytosolic and a membrane-bound protein *in situ*, and that it cycles from cytosol to endosomal membranes during the GTP/GDP cycle in a similar manner to Arf1. This is supported by recent data demonstrating that release of membrane-bound Arf6 into the cytosol depends upon the presence of physiological concentrations of magnesium in incubation buffers and that, under appropriate conditions, Arf6 cytosol-to-membrane shuttling occurs during the GTP/GDP cycle in variety of cells (45). However, the precise target membrane for Arf6 was not previously identified (45) and we now show that early endosomes are a target for both Arf6 and Arf1 recruitment in kidney proximal tubule epithelial cells.

We propose, therefore, that recruitment of ARNO may be an early step in the pH-dependent translocation of Arf6 from cytosol to early endosomal membranes for the following reasons: 1) recruitment of ARNO is dependent only on intra-endosomal acidification but not on the GTP/GDP cycle; 2) intra-

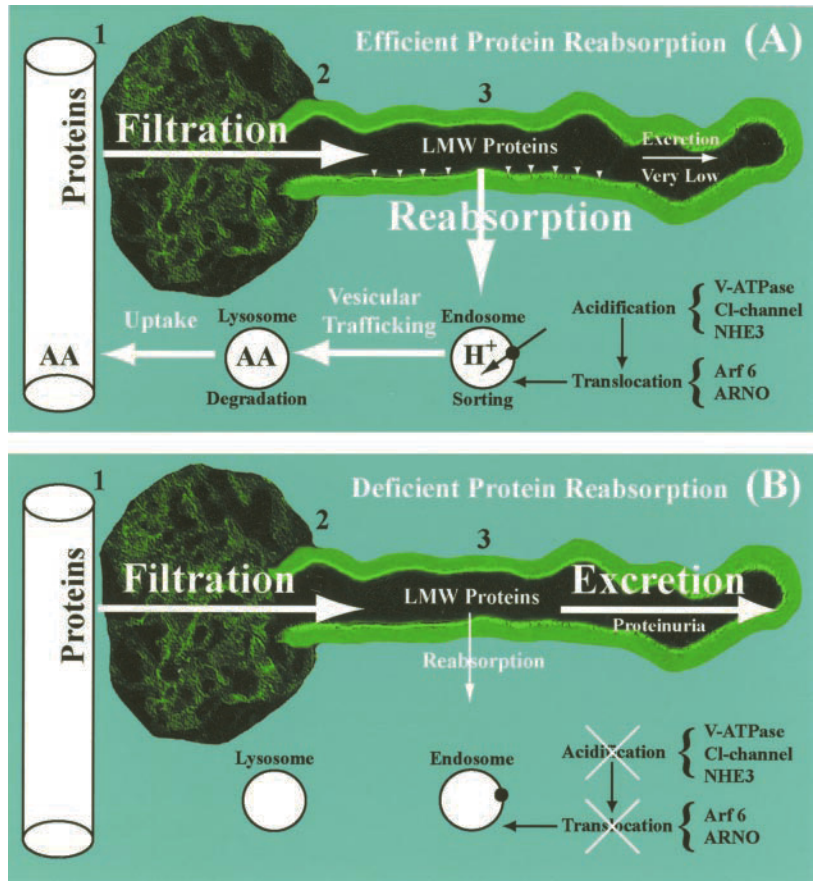

FIG. 9. **Schematic representation of protein reabsorption through the apical endocytotic pathway in kidney proximal tubules and the potential role of endosomal acidification in the physiological regulation of this process by ARNO and Arf6 recruitment.** Intra-endosomal pH-sensitive recruitment of ARNO and Arf6 to kidney proximal tubule endosomes as a possible link between impaired endosomal acidification and protein reabsorption in Dent's disease and Fanconi syndrome. *A,* illustrates efficient protein reabsorption under normal conditions. ARNO and Arf6 recruitment, driven by the acidic endosomal pH signaling, is required for sorting in endosomes, and for more distal processes including trafficking of some internalized proteins to lysosomes for degradation. The glomerulus/proximal tubule shown in *panels A* and *B* is a photomontage taken from tissue immunostained for Arf6 (*arrowheads*). *LMW proteins,* low molecular weight proteins; *AA,* amino acids. *1,* blood vessel; *2,* glomerulus; *3,* S1 segment of proximal tubule). *B,* illustrates defective protein reabsorption resulting from deficient endosomal acidification due to inhibition of the V-ATPase and/or chloride channels. Apical protein reabsorption is blocked due to deficient ARNO and Arf6 recruitment upon diminishing endosomal acidification signaling. Reduced apical protein reabsorption leads to increased protein delivery to more distal segments of the urinary tubule and to proteinuria, which occurs in Dent's disease and Fanconi syndrome.

endosomal pH-dependent Arf recruitment is selective and predominant for Arf6; 3) acidification-dependent translocation of Arf6 is quantitatively greater than GTP/GDP cycle-dependent Arf6 recruitment; 4) recruitment of Arf6 correlates both with intra-endosomal acidification and ARNO translocation.

Previously, based on the acidification-dependent recruitment of two coat proteins (β-COP and ϵ-COP) to endosomes from cultured BHK-21 cells, the presence of a hypothetical pH-sensitive protein (PSP) in endosomal membranes was proposed by Gruenberg and co-workers (30–32). However, neither Arf isoform nor Arf-GEF protein translocation was reported, and a direct interaction of PSP with β-COP was proposed. To explain our present observations, we hypothesize that endosomes from kidney proximal tubules also contain a transmembrane PSP with which ARNO could directly interact upon endosomal acidification. Arf-GEF proteins including ARNO, cytohesin-1, GRP1, and EFA6 share common domains that are involved in protein-protein and protein-lipid interactions as well as GDP/GTP exchange (17–19, 51). V-ATPase-dependent intra-endosomal low pH could trigger a conformational change of PSP on an intra-endosomal site that is transmitted to the cytosolic side of the endosomal membrane, resulting in signaling and interaction with and recruitment of ARNO. The existence of such a hypothetical PSP and its signaling in the endosomal pathway obviously remains to be demonstrated, but various proteins have been reported to undergo conformational transitions due to pH changes (52–55). Furthermore, direct interaction of the transmembrane integrin β_2-receptor with an Arf-GEF family member (cytohesin-1) through its Sec7 domain, leading to the recruitment of cytohesin-1 followed by Arf1 from cytosol to plasma membrane, has also been reported (56). It has also been shown that the association of intra-Golgi ligands with the KDEL receptor promotes interaction of the cytoplasmic tail of this receptor with an Arf1-specific GAP (Arf1-GAP1), providing another example of modulation of the Arf pathway by putative conformational changes in a transmembrane protein (57, 58).

During preparation of this report, Gu and Gruenberg (59) demonstrated that Arf1 could regulate pH-dependent COP functions in the early endocytic pathway of BHK-21 cells. GTPγS had a very small, if any, effect on translocation of Arf6 to endosomal membranes. Recruitment of Arf1, however, was significantly stimulated during the GTP/GDP cycle and correlated with recruitment of $\beta-$COP but not $\delta-$COP onto endosomal membranes. GTPγS-dependent recruitment of Arf1 was partially (50%) diminished by preincubation of endosomes with a high concentration (50 μM) of nigericin. However, a direct effect of ATP and specific V-ATPase inhibitors (without GTPγS and/or nigericin) on the translocation of Arf isoforms to BHK-21 cell endosomes was not shown (59). Furthermore, a correlation between endosomal acidification and Arf1 translocation was not demonstrated. In contrast, we show that both endogenous Arf1 and Arf6 (but not ARNO) are recruited from cytosol to endosomal membrane during the GTP/GDP cycle. These data suggest that Arf1 could play a GTP/GDP cycle-dependent regulatory role in the apical endocytotic pathway of kidney proximal tubules, perhaps at an early step in endocytotic vesicle budding from the apical membrane, prior to vesicle acidification. In our experiments both endosomes and cytosol were purified from the same source (kidney proximal tubules), they were not concentrated (fractionated) and only endogenous proteins were used. In this way, we show that endogenous ARNO and Arf6 are specifically and selectively recruited from cytosol to purified endosomal membranes in a pH-dependent manner: translocation is promoted by ATP (without addition of GTPγS but in the presence of endogenous GTP/GDP) and is reversed either by specific V-ATPase inhibitors or by various structurally unrelated uncouplers of endosomal acidification.

Moreover, translocation experiments were performed simultaneously and under identical conditions with acidification assays. We hypothesize that the first protein to be translocated in an acidification-dependent (GTP/GDP-independent) manner is ARNO, which triggers the selective GDP/GTP exchange and recruitment of Arf6. Thus our data suggest that in endosomes of proximal tubule epithelial cells, the hypothetical "pH sensitive protein" previously proposed by Gruenberg and co-workers (30–32) initially interacts with ARNO and/or with Arf6.

We did not address downstream events resulting from the acidification-dependent recruitment of ARNO and Arfr6 to endosomal membranes. However, the important role of Arf proteins in the regulation of exocytosis and endocytosis in cultured cells is well documented (2–11). Arf1, the best studied isoform, is involved in the recruitment of coat complexes to different organelles of the exocytotic pathway (60) and to early endosomes (59). Arf1 also regulates binding of spectrin to Golgi membranes (61) and promotes selective recruitment of phosphatidylinositol 4-OH(β) and phosphatidylinositol 5-OH kinases (62). Arf1 directly interacts with $\beta-$COP (63) and regulates the activity of phospholipase D1 (12) and phospholipase D2 (13). Overexpressed Arf6 plays a role in regulating the receptor-mediated endocytotic pathway of cultured Chinese hamster ovary (10, 64) and Madin-Darby canine kidney (65) cells as well as the secretory pathway of bovine adrenal chromaffin cells (66, 67). Arf6 has been also implicated in the regulation of actin cytoskeleton rearrangements (11) as well as in the regulation of phospholipase D activity. Any or all of these activities could ultimately be important in the regulation of the RME pathway by pH-dependent and/or GTP/GDP cycle-dependent Arf recruitment to proximal tubule endosomes. Diminished recruitment of ARNO and Arf6 to endosomes with deficient acidification, for example, during cadmium intoxication (46) and Dent's disease (47–49) might be a crucial molecular event leading to development of Fanconi syndrome as depicted in Fig. 9. Thus we propose that intra-endosomal pH-sensitive recruitment of ARNO and Arf6 to kidney proximal tubule endosomes could be one possible missing link between impaired endosomal acidification and defective protein reabsorption in Dent's disease and Fanconi syndrome. While these and previous data reveal a relationship between transmembrane signal transduction and vesicular trafficking, the precise functional roles of ARNO, Arf6, and Arf1 and their downstream effectors in the RME pathway of kidney proximal tubules *in situ* remain to be determined.

Acknowledgments—We thank Dr. Sylvie Breton for providing chicken polyclonal anti-V-ATPase antibodies used in co-localization experiments. We also thank Mary McKee for technical support with the *in vivo* FITC-dextran uptake experiments.

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