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Expression and distribution of adenosine diphosphate-ribosylation factors in the rat kidney[1]

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Background. Adenosine diphosphate (ADP)-ribosylation factors (ARFs) are small guanosine triphosphatases involved in membrane traffic regulation. Aiming to explore the possible involvement of ARF1 and ARF6 in the reabsorptive properties of the nephron, we evaluated their distribution along the different renal epithelial segments.

Methods. ARFs were detected by immunofluorescence and immunogold cytochemistry on renal sections, using specific anti-ARF antibodies.

Results. ARF1 was detected in proximal and distal tubules, thick ascending limbs of Henle's loops, and cortical and medullary collecting ducts. By immunofluorescence, labeling was mostly localized to the cell cytoplasm, particularly in Golgi areas. By electron microscopy, the Golgi apparatus and the endosomal compartment of proximal and distal tubular cells were labeled. ARF6 immunofluorescence was observed in brush border membranes and the cytoplasm of proximal convoluted tubular cells, whereas it was restricted to the apical border of proximal straight tubules. ARF6 immunogold labeling was detected over microvilli and endocytic compartments of proximal tubular cells.

Conclusions. This study demonstrates the following: (*a*) the heterogeneous distributions of ARF1 and ARF6 along the nephron, (*b*) the existence of cytosolic and membrane-bound forms for both ARFs, and (*c*) their association with microvilli and endocytic compartments, suggesting an active participation in renal reabsorption.

Adenosine diphosphate (ADP)-ribosylation factors (ARFs) are a family of small guanosine triphosphatases (GTPases), first described as protein cofactors in the ADP-ribosylation of the $G\alpha_s$-subunit of trimeric GTP-binding proteins by cholera toxin [1]. ARFs have been shown to play important roles in vesicular membrane traffic and organelle assembly [2–5]. ARF proteins comprise a family of 21 kDa proteins with highly conserved amino acid sequences from yeast to man [6, 7]. Six mammalian ARF isoforms, which are all expressed in rats [8] and mice [9], have been described. They are divided into three classes, based on their size and amino acid sequence homology. Class I includes ARF1, ARF2, and ARF3. Class II includes ARF4 and ARF5, and class III includes only ARF6 [10]. Like other GTPases, ARFs exist in two states, depending on the guanine nucleotide bound, the inactive guanosine diphosphate (GDP) form, and the active GTP one [3, 4, 7]. ARFs are activated by nucleotide exchange and are inactivated by GTP hydrolysis catalyzed by specific nucleotide exchangers and ARF GTPase-activating proteins, respectively [11–13].

ARF1, the most abundant ARF isoform, has been shown to be involved in the transport along the secretory pathway by controlling the assembly of specific cytosolic coat proteins onto Golgi membranes [8–10]. Indeed, ARF1 mediates the recruitment of either coatomer proteins (COPs) to the *cis*-Golgi membrane for the formation of COPI-coated vesicles [5, 14, 15] or adaptins for the formation of clathrin-coated vesicles from the *trans*-Golgi network [16]. ARF1 binding to the Golgi membrane is regulated by the GDP to GTP exchange and is inhibited by the fungal metabolite Brefeldin A [3, 11, 12]. The membrane-bound ARF1 triggers the binding of coat proteins to Golgi membranes [2, 5] and probably induces local lipid changes that initiate vesicle budding [4, 17, 18]. Interestingly, ARFs are potent activators of membrane-bound phospholipase D [19, 20], which catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. Phosphatidic acid has been proposed to

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have a role on vesicular transport acting as a fusogenic lipid [4], and it also stimulates the ARF GTPase-activating protein [21]. Although ARF1 cycles between the cytosol (inactive form) and the Golgi membranes (active form) according to its nucleotide bound, ARF6 had been suggested to cycle between the plasma membrane and the endosomal compartment [22–25]. ARF6 has been proposed to act as a regulator of membrane traffic between the plasma membrane and the endosomes [22–25] and as modulator of cytoskeletal reorganization [24, 26, 27]. However, ARF6 has been recently found in the cytosol of different mammalian cells [28–30].

Renal epithelial cells have been extensively used as a model for the study of protein trafficking and endocytosis [31–33]. The highly specialized epithelia lining the nephron is involved in the maintenance of body fluid and acid-base homeostasis by processes including protein sorting and membrane recycling [34]. Although ARF proteins have been largely studied to determine their specific role in membrane traffic, their precise localization and *in situ* distribution in epithelial cells along the nephron and particularly in the proximal tubule are largely unknown. Previous studies from this laboratory using Western blot analysis on subcellular fractions of human, dog, and rat isolated renal proximal tubules have revealed the expression of ARF1 and ARF6 in plasma membrane and early endosomal compartments [28, 29]. In this study, the *in situ* localization of ARF proteins was performed on rat renal tissue under normal physiological conditions and on isolated proximal tubules. The immunofluorescent detection of ARF1 and ARF6 on renal sections allowed us to study their distribution along the different nephron segments, whereas the immunogold technique on ultrathin sections of renal cortex and isolated tubules revealed the ARF proteins in precise cellular and subcellular compartments. This morphological study demonstrates the following: (*a*) the heterogeneous distributions of ARF1 and ARF6 along the nephron; (*b*) the existence of cytoplasmic and membrane-bound forms for both ARF1 and ARF6; and (*c*) the association with microvilli and endocytic compartments, suggesting their participation in renal reabsorption.

METHODS

Antibodies

The monoclonal 1D9 antibody was provided by Dr. Richard A. Kahn (Emory University School of Medicine, Atlanta, GA, USA). This antibody recognizes human and rat ARF1, ARF3, ARF5, ARF6, and, to a lesser extent, ARF4 by Western blot analysis [35]. The polyclonal antibody SYL1 was raised in rabbit by using bovine recombinant ARF1 as an immunogen. It cross-reacts with ARF5 but not with ARF6 [20, 28]. Monoclonal antibodies SYL6A, SYL6B, and SYL6C were produced using purified human recombinant ARF6 as immunogen [28]. These antibodies are specific for ARF6 and showed no cross-reactivity with ARF1, ARF3, and ARF5 [28]. The monoclonal antibody against Rab5 was a gift from Dr. Angela Wandinger-Ness. This antibody specifically recognizes the C-terminus of Rab5a and has been already applied on mouse kidney cryosections by confocal immunofluorescence [36].

Immunofluorescence

Normal rat kidney was fixed in Bouin's fixative and embedded in paraffin. Tissue sections were deparaffinized in xylol and hydrated in a series of decreasing concentrations of ethanol. Sections were then washed twice in 10 mmol/liter phosphate-buffered saline (PBS) and incubated with the antibodies as follows: 1D9, 1/20, two hours, room temperature; SYL1, 1/20, two hours, room temperature; SYL6 A, B, and C, pures, 18 hours, room temperature; anti-Rab5, 1/30, 18 hours, room temperature. After washing in PBS, the sections were incubated with the corresponding secondary antibody coupled to fluorescein isothiocyanate for one hour (FITC goat anti-rabbit or FITC goat antimouse IgG; Sigma Chemicals, St. Louis, MO, USA). Before incubation with the SYL6 and anti-Rab5 antibodies, a pretreatment with sodium metaperiodate (saturated solution, 5 min) was performed. Control experiments, omitting the primary antibody, were performed for each labeling protocol. The sections were observed on a Leitz DM RB microscope equipped with a Leitz Vario-Orthomat E camera (Leica Canada, Willowdale, Ontario, Canada).

Immunogold labeling

Tissues. Samples from normal rat renal tissue were fixed by immersion in 1% phosphate-buffered glutaraldehyde for two hours at room temperature. The tissues were dehydrated in graded methanol and were embedded in Lowicryl resin (J.B. EM Services, Pointe-Claire, Quebec, Canada) at −30°C according to previously described procedures [37]. Isolated cortical tubules (80 to 90% proximal) deriving from normal rat kidneys were prepared by collagenase digestion, as previously described [38], fixed in 1% glutaraldehyde, and embedded in Lowicryl resin, as indicated earlier here. Thin tissue sections were cut and mounted on Parlodion- and carbon-coated nickel grids.

Labeling protocol. The tissue sections were successively incubated on (*a*) 150 mmol/liter glycine in PBS for 10 minutes, (*b*) the primary antibody solution (SYL1, 1/200; 1D9 1/200, SYL6 A, B, C, pures) for three hours at room temperature or overnight at 4°C, and (*c*) protein A-gold (10 nm, OD_{525} = 0.5) for 30 minutes. Control experiments were performed to assess the specificity of the different labelings. After uranyl acetate staining, sections were observed in a Philips 410 electron microscope.

RESULTS

Immunofluorescence

Similar labeling patterns were observed with the SYL1 polyclonal antibody (Fig. 1) and the 1D9 monoclonal antibody (not shown) on renal tissue sections. This was expected because of the specificity of the antibodies and the high abundance of ARF1 among the ARF isoforms (90% of total ARF proteins) [21, 35]. Immunofluorescence was observed over proximal and distal tubules, as well as cortical collecting ducts (Fig. 1 A, B). Distal tubules and collecting ducts were more intensely labeled than the proximal ones. The signal was mainly cytoplasmic, with higher intensities around the nucleus (Fig. 1C), suggesting a Golgi association. On the other hand, glomeruli showed a weak immunolabeling (Fig. 1 A, B). In the outer medulla (Fig. 1 D, E), the thick ascending limbs of Henle's loops were labeled. In the inner medulla and the papilla (Fig. 1E), collecting ducts were labeled but not the thin (ascending or descending) limbs of Henle's loops. The labeling was cytoplasmic with occasional higher intensities around the nucleus (Fig. 1 F, G).

Renal tissues were labeled with SYL6A, which specifically recognizes ARF6. Fluorescence was distributed over proximal convoluted tubules in the cortex and over proximal straight tubules in the outer medulla. In the cortex (Fig. 2 A, B), the labeling was intense over the brush border membranes and moderate over the cytoplasm, thus revealing cytosolic and membranous forms for ARF6. In the pars recta (Fig. 2 C, D), a strong labeling was found associated with the proximal straight tubule brush border. Collecting ducts appeared negative. In the inner medulla, toward the papilla (Fig. 2 E, F), numerous single labeled cells, either epithelial or interstitial, presented an intense cytoplasmic labeling. Identification of these cells was not achieved. They could constitute particular cells of the descending thin limbs of Henle's loops because a sparse labeling was also observed in the outer medulla, among unlabeled thick ascending limbs of Henle's loops and collecting ducts. Conversely, they might represent medullary interstitial cells known to be sparse in the outer medulla but abundant in the inner medulla [39].

In order to compare the cellular distribution of ARFs in the tubular epithelia with that of an early endosomal marker, Rab5, the immunofluorescence labeling protocol was also applied for this antigen, using a specific anti-Rab5 monoclonal antibody. Labeling was present in all cellular types along the nephron, although with different intensities. Distal tubules and collecting ducts were intensely labeled in the cytosol (not shown) with a pattern similar to that reported by Bucci et al on mouse kidney cryosections [36]. This pattern coincides with that obtained for Syl1 and 1D9 (Fig. 1). In proximal convoluted tubules, on the other hand, labeling was concentrated in the apical side of the cell, particularly at the base of the microvilli (Fig. 3), identifying the early endosomal compartment. Rab5 labeling partially overlapped with the intense apical labeling observed in proximal tubules for ARF6 (Fig. 2 A, B).

No fluorescent signal was detected under control conditions when the tissue sections were incubated with the secondary fluorescent antibody, omitting the primary antibody step (results not illustrated), thus assessing the specificity of the results.

Immunogold labeling

At the electron microscope level, SYL1 and 1D9 antibodies yielded similar immunogold labeling distributions on cellular compartments (Figs. 4 and 5). Both antibodies labeled the Golgi apparatus in every distinct epithelial, mesenchymal, and endothelial cell evaluated in the rat renal cortex and outer medulla, confirming the presence of ARF1 and other Golgi-associated ARFs in renal cells. Significant labelings were detected on the Golgi apparatus of proximal (Figs. 4A and 5A) and distal tubular epithelial cells (Fig. 4C), both *in situ* (Fig. 5A) and in isolated tubules (Fig. 4 A, C). In addition, a moderate labeling was observed on endocytic compartments in the apical region (Figs. 4 A, B, and 5B). The microvilli were also labeled, but to a lesser extent (Figs. 4 A, B, and 5B). Only very few gold particles were detected over other organelles such as nuclei and mitochondria.

The three monoclonal antibodies used SYL6A, B, and C, which specifically recognize ARF6, generated similar immunogold labelings. The results with SYL6A are illustrated in Figure 6. Labeling was restricted to the microvilli and the apical endocytic vesicles as well as possible deep invaginations of the apical plasma membrane of cortical tubules either isolated (Fig. 6A) or *in situ* (Fig. 6B). The Golgi apparatus, as well as other intracellular compartments, including nuclei and mitochondria, was negative. Control experiments, which omitted the primary antibody step of the experimental procedure, resulted in an absence of labeling (not shown).

DISCUSSION

The different regions of the nephron carry out highly specialized functions for the maintenance of body fluid and acid-base homeostasis. The epithelia lining each tubule segment exhibit special cellular architectures and particular compositions of plasma and intracellular membranes, which are consistent with their specific roles in the exchange of fluids, electrolytes, and larger molecules. Protein sorting and membrane traffic events constitute important mechanisms for achieving specific functional properties, as well as for the maintenance of cell polarity [33, 34]. Although small molecules are transported across the plasma membrane into the cell in a controlled man-

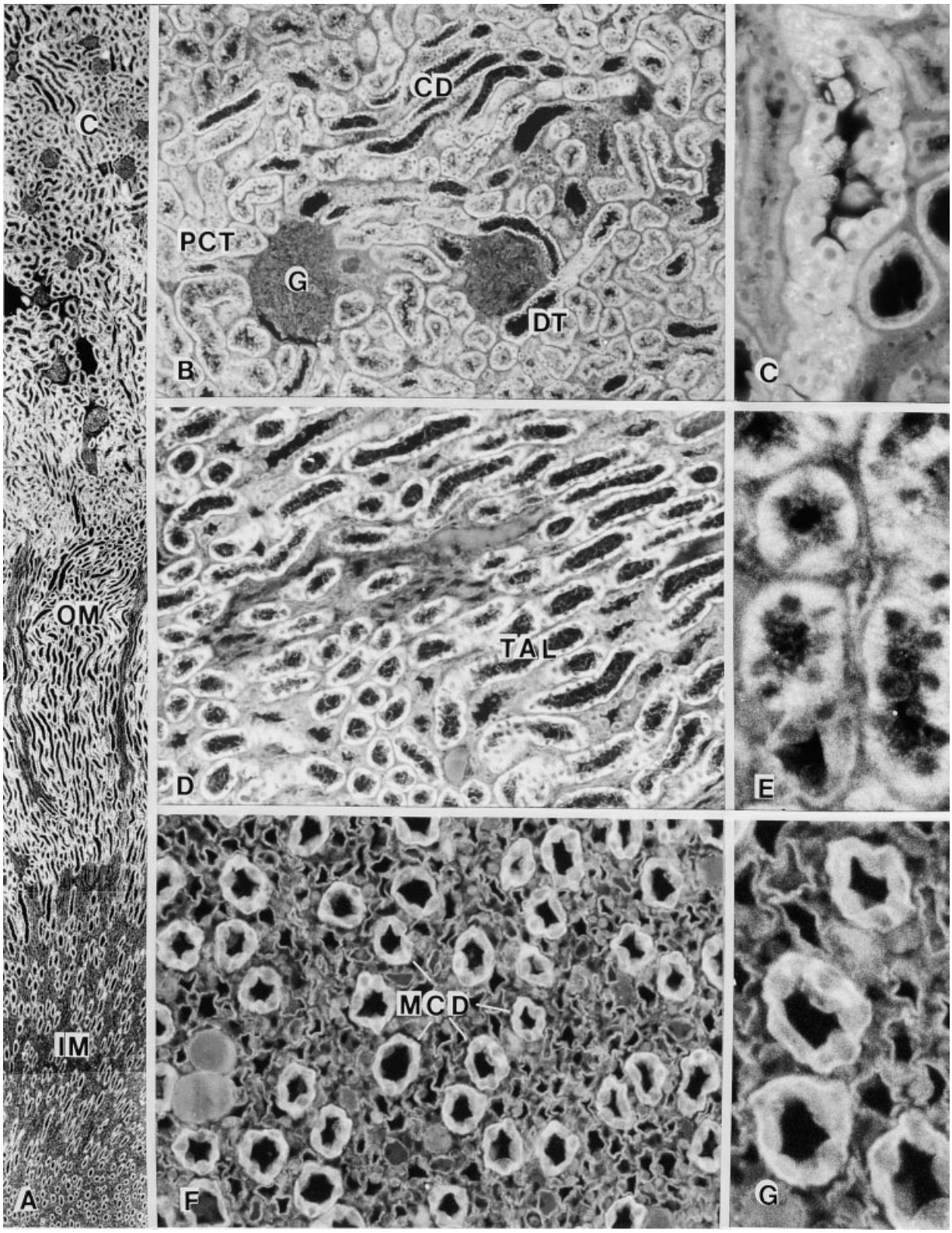

Fig. 1. Light microscopy immunolabeling along the rat nephron using the SYL1 and the 1D9 antibodies. (*A*) Low magnification. C, cortex; OM, outer medulla; IM, inner medulla. (*B*, *C*) In the cortex, proximal convoluted (PCT) and distal tubules (DT), as well as collecting ducts (CD), appear positive, whereas glomeruli (G) show a weak signal. Labeling is mainly cytoplasmic but more intense around the nucleus, suggesting a preferential association with the Golgi area. Thick ascending limbs (TAL) in the outer medulla (*D*, *E*) and medullary collecting ducts (MCD) in the inner medulla (*F*, *G*) are intensely labeled. Labeling is predominantly cytoplasmic (magnifications are: A ×70, B ×300, C ×1000, D ×500, E ×1500, F ×800, G ×2000).

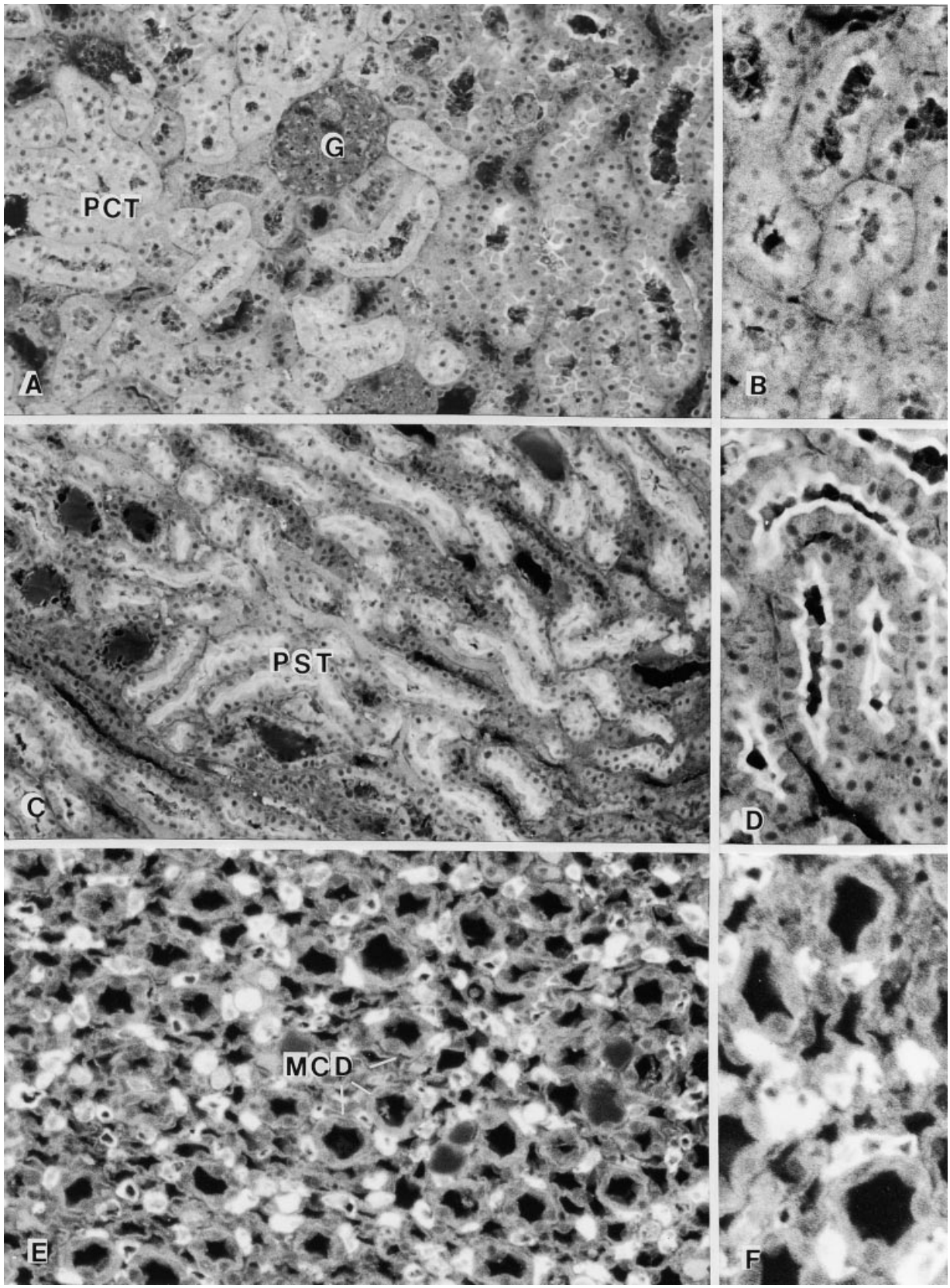

Fig. 2. Light microscopy immunolabeling along the rat nephron using the SYL6 antibody. (*A*, *B*) Labeling is observed over the cytoplasm and the brush border of PCT epithelial cells. (*C*, *D*) In the outer medulla, the brush border membrane of proximal straight tubules (PST) is intensely labeled; collecting ducts and thick ascending limbs (TALs) are devoid of labeling. (*E*, *F*) In the papilla, an intense cytoplasmic labeling is observed in isolated cells, likely interstitial cells laying between collecting ducts (MCD) or cells from thin limbs of Henle's loop. Medullary collecting ducts are negative (magnifications are: A ×600, B ×1000, C ×550, D and E ×1000, F ×1800).

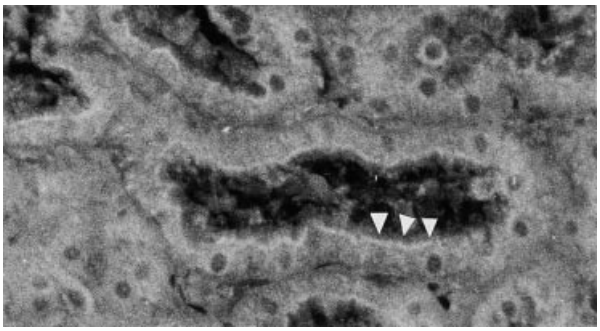

Fig. 3. Light microscopy immunolabeling with anti-Rab5 antibody in proximal tubular epithelial cells. Labeling is very intense in the apical side of the tubular cells, at the base of microvilli (arrowheads). Magnification is ×1300.

ner, macromolecules are selectively incorporated by endocytosis. Endocytosis has also a fundamental role in keeping size and properties of cell compartments by membrane traffic. Of special interest are the proximal segments of the nephron, which perform important endocytotic activities, being responsible for the reabsorption of glomerular-derived ultrafiltrate elements.

ARF proteins [30, 35] and their mRNA transcripts [7–9] have been detected in a large number of tissues and cell lines. Their expression is ubiquitous but varies among tissues, the kidney being one of those with high expression levels [7–9, 40]. Using specific antibodies for ARFs, we have detected their presence in renal epithelial cells. Syl1 and 1D9 immunofluorescent labelings were widely distributed along the nephron, with a particularly high expression over distal tubules and collecting ducts; glomeruli and thin limbs of Henle's loop showed only little labeling. The weak immunofluorescence found for ARFs in glomeruli agrees with the faint signal obtained by Western blot analysis on isolated glomeruli when compared with isolated proximal tubule extracts [28]. Nevertheless, and consistent with the ubiquitous expression of ARFs, Syl1 and 1D9 antibodies demonstrated a labeling by immunoelectron microscopy in all glomerular cell types.

The axial heterogeneity of the ARF distribution along the nephron suggests their participation in particular cellular activities. The nonoverlapping, differential distribution of ARF6 compared with that of ARF1 (and possibly ARF5) stresses the distinct characteristics of the former isoform between the ARF family members [23, 35] and indicates that it has some particular functional properties. Axial heterogeneities in the proximal tubule have also been demonstrated for different apical membrane receptors such as GP330, ion exchangers and pumps, and water channels [34]. A differential segmental distribution has also been reported for certain lysosomal hydrolases, which coincides with distinct degradative capabilities of proximal tubule segments [32]. SYL1 immunofluorescent labeling revealed ARF1 (and probably ARF5) in the cytoplasm and the Golgi apparatus of different epithelial cells in the nephron. Using electron microscopy, Syl1 and 1D9 immunolabelings were detected associated to the Golgi apparatus of different cell types and to the endosomal compartment of proximal and distal tubular cells. This labeling pattern is consistent with previous reports demonstrating both cytosolic and membrane-bound forms for ARF1 in kidney [28, 29, 41]. Indeed, ARFs were originally found to be abundant in the Golgi apparatus of various mammalian cell lines and pancreatic acinar tissue [7, 40, 42] by using the 1D9 antibody. Overexpressed wild-type and GTP-hydrolysis–defective mutant ARF1 was also assigned to a perinuclear Golgi location in transfected cells [9, 22, 23, 43]. The GTP-binding–defective mutant, on the contrary, showed a diffuse distribution throughout the cell [23] similar to that induced by Brefeldin A treatment [23, 41]. In these studies, overexpression of ARF mutants induced drastic cellular changes, thereby suggesting the involvement of these small GTPases in membrane traffic. However, concern was raised about the profound membrane effects on the physiological behavior of ARF-overexpressing cells. The advantage of this study, as compared with those using transfected cells, lies on the detection of endogenous proteins in normal tissue cells under physiological conditions.

The ultrastructural localization of ARFs in apical vesicles of proximal and distal tubules coincides with their detection in isolated brush border membrane vesicles and endosomes by Western blot analysis [28, 29] and preembedding immunocytochemistry [29]. It likely accounts for the punctuate immunofluorescence labeling previously found for ARFs [41, 43], in addition to the predominant Golgi staining. The presence of ARFs in endocytic compartments and, to a lesser extent, in microvilli is suggestive of a role in endocytosis. Because ARF1 acts in two different compartments (*cis*-Golgi and *trans*-Golgi network), participating in the recruitment of different cytosolic proteins (COPs and adaptins) for the formation of coated vesicles, its involvement in other membrane traffic events such as recycling from endosomes to plasma membrane and transcytosis from basolateral to apical membranes has also been proposed [2, 5]. Indeed, treatment with Brefeldin A, an ARF1 activation inhibitor, induced dramatic alterations in endosome morphology and function [44, 45]. On the other hand, the overexpression of mutant-defective ARF1 has been shown to inhibit fluid-phase endocytosis, although without particularly affecting endosome morphology [22, 43].

Concerning the distribution of ARF6 in the nephron, the immunofluorescent labeling was almost restricted to the first segments, being localized in the cytoplasm and at the apical plasma membrane of proximal convoluted tubules, as well as at the brush border membrane of proximal straight tubules. The predominant labeling of

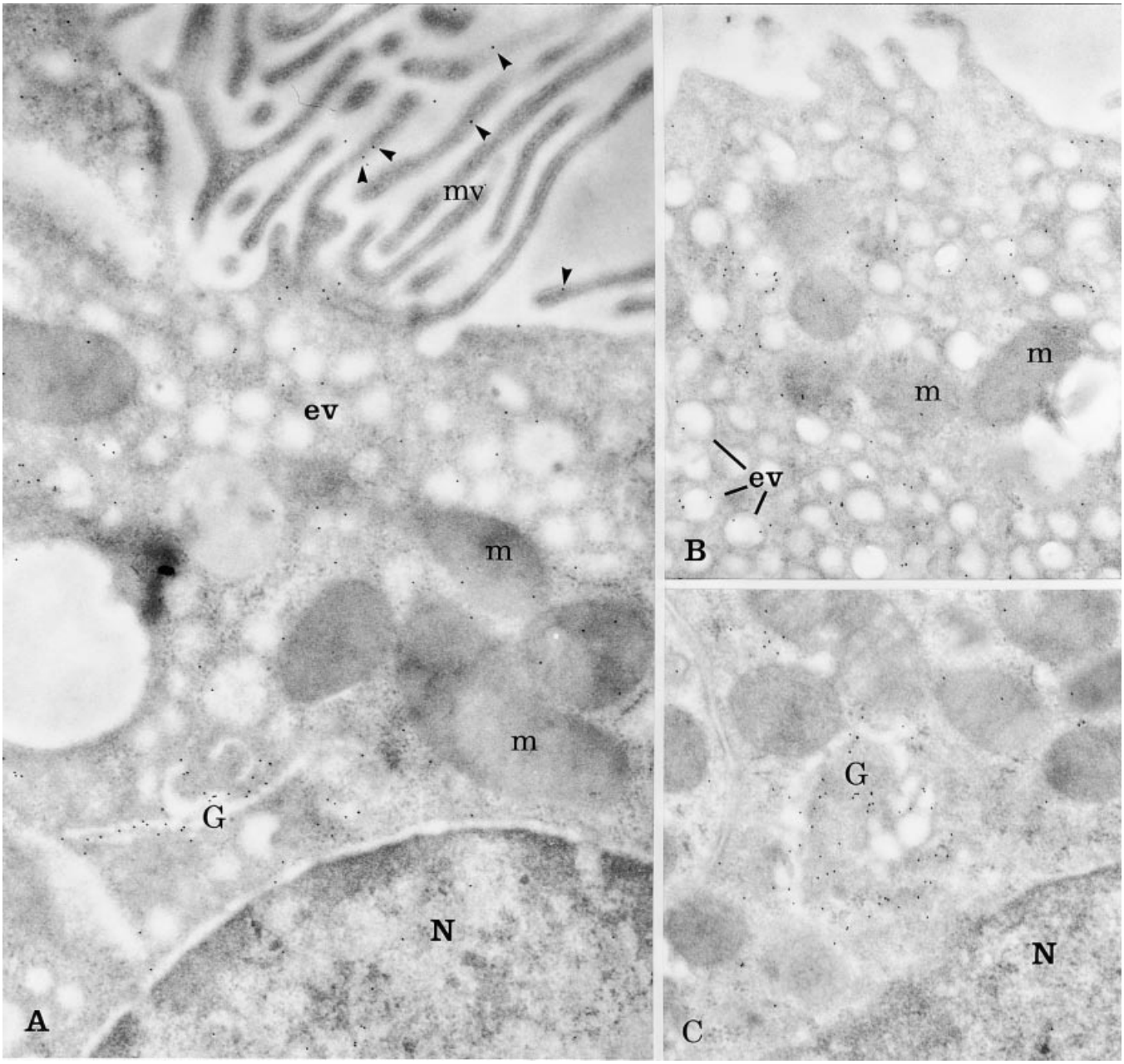

Fig. 4. Electron microscopy immunolabeling with SYL1 antibody in proximal and distal tubular epithelial cells. (*A*) An intense labeling by gold particles is observed over the Golgi apparatus (G), endocytic vesicles (ev), and microvilli (mv, arrowheads) of isolated proximal tubules. (*B*, *C*) In distal tubules, the endocytic vesicles (ev) and the Golgi apparatus (G) are labeled by gold particles. Nuclei (N) and mitochondria (m) are devoid of labeling (magnifications are: A ×32,000 and B ×30,000).

ARF6 in the apical brush border membrane of both epithelial cells concurs with the proposition that this protein plays a role in the modulation of plasma membrane organization and cell shape [24, 25]. In recent years, growing pieces of evidence also suggest a role for ARF6 in regulating actin-cytoskeletal organization [24, 26]. Because actin cytoskeleton has been implicated in endocytosis, the participation of ARF6 as cytoskeletal modulator in proximal tubular reabsorption in the nephron could also be proposed. Isolated cells in the medulla and the papilla showed an intense cytoplasmic labeling. The cytoplasmic labeling in these cells and in proximal convoluted epithelial cells had a diffuse appearance, in contrast to the punctuate labeling observed in mammalian cells overexpressing the wild-type [9, 23] or the GTP-binding defective mutant forms of ARF6 [23]. At the electron microscope level, however, ARF6 was only detected when associated with the plasma membrane and

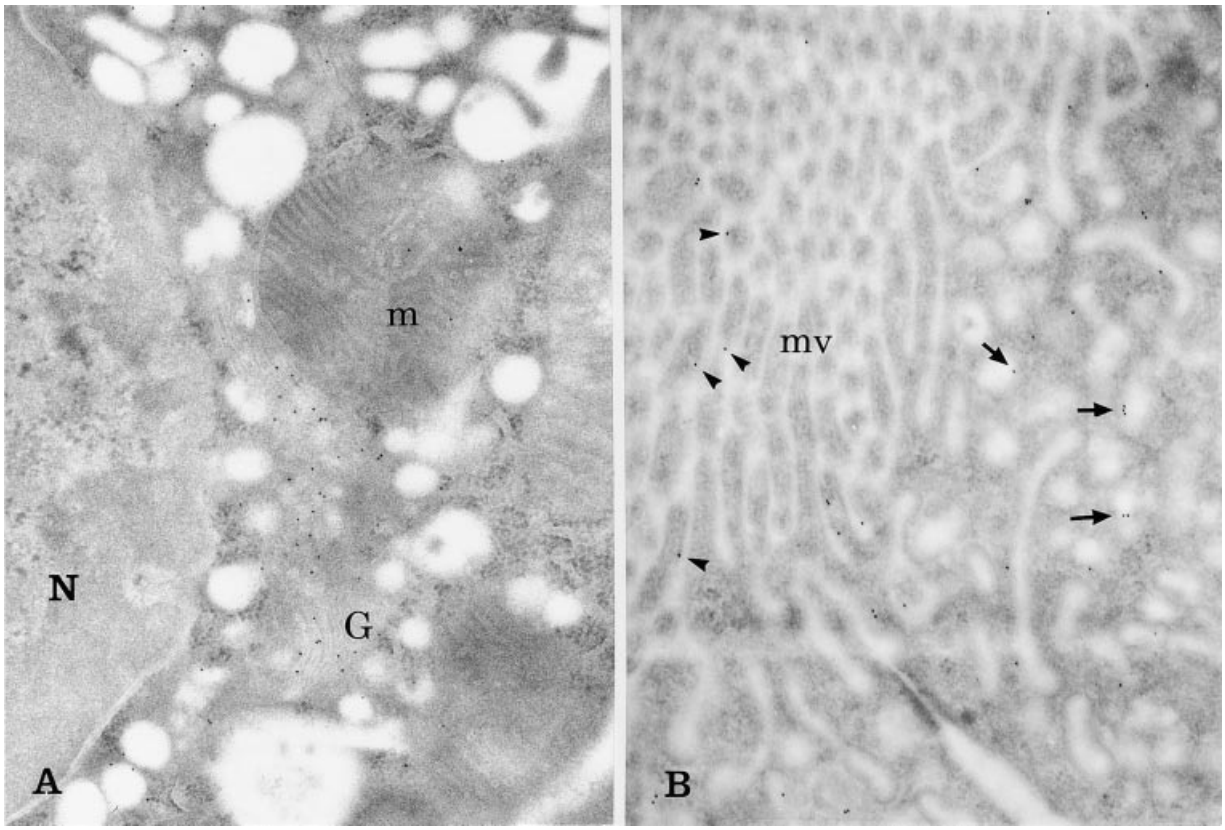

Fig. 5. Electron microscopy immunolabeling with the 1D9 antibody in renal cortical tubules. (*A*, *B*) Gold particles, revealing antigenic sites, are detected in the Golgi apparatus (G) at the surface of microvilli (mv, arrowheads) and on endocytic vesicles (arrows) of proximal tubular epithelial cells. Abbreviations are: N, nucleus; m, mitochondria. Magnifications are A ×22,000, B ×31,000, and C ×30,000.

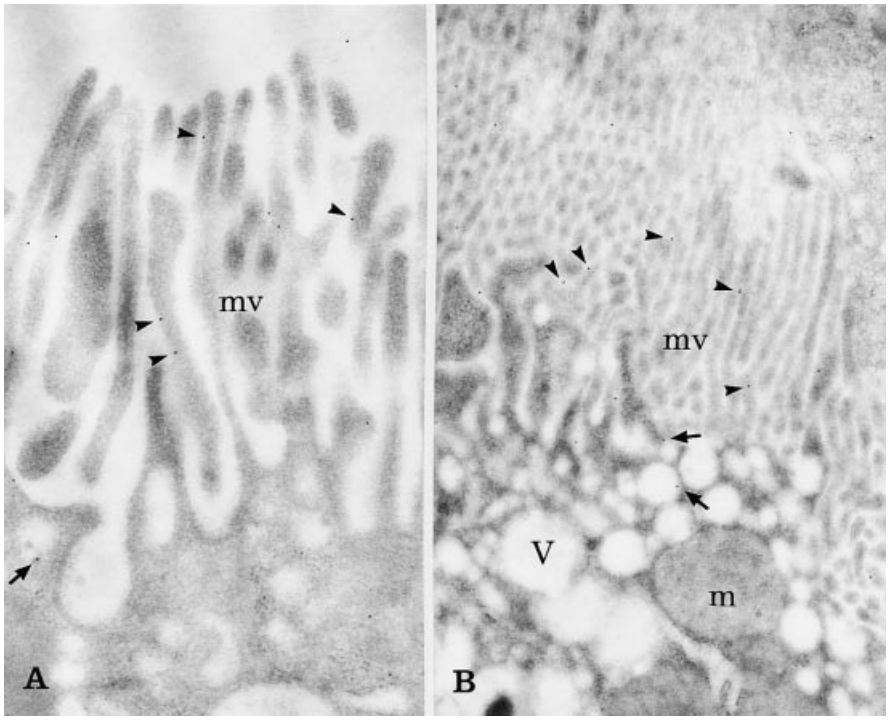

Fig. 6. Electron microscopy immunolabeling with SYL6 antibody in proximal tubular cells. Gold particles are mainly localized over microvilli (mv, arrowheads) and on endocytic vesicles (arrows) of isolated tubules (*A*) and *in situ* (*B*). Abbreviations are: V, endocytic vesicle; m, mitochondria. Magnifications are A ×32,000 and B ×25,000.

endocytic compartments, which contrasts with the cytoplasmic expression demonstrated by light microscopy. The relative scarcity of this ARF isoform [35, 40] and the potential fragility of this antigen to electron microscopy tissue preparation procedures could account for the absence of significant ultrastructural labeling in the cytosol. By Western blot analysis [28, 29] and pre-embedding immunocytochemistry [29], we have previously demonstrated the presence of ARF6 in plasma membrane and endosomal fractions as well as in a cytosolic fraction of isolated proximal tubules. Yang et al have established a cell type-dependent distribution of ARF6 [30]. These authors have demonstrated in different cell types that cytoplasmic, plasma membrane, and postnuclear subcellular fractions vary in their relative ARF6 abundance. Accordingly, in isolated proximal tubules from human kidney, we detected different amounts of ARF6 in brush border vesicular, early endosomal and cytosolic fractions, as estimated by quantitative Western blot analysis [28].

To date, it had been proposed that ARF6 cycles between the endosomal compartment, in a GDP-bound form, and the plasma membrane, in a GTP-bound form [3, 22–24]. This was based on overexpression of GTP-hydrolysis–defective (active form) and GTP-binding–defective (inactive form) ARF6 mutants [22–24]. However, our data demonstrate the presence of cytosolic ARF6 in renal proximal tubule epithelial cells, suggesting that ARF6 can cycle between membrane compartments and the cytosol. Preliminary results of reconstitution studies indicate that ARF6 can be recruited from the cytoplasm to the endosomal fraction of isolated proximal tubules, depending on its GTP/GDP status and could cycle, according to its nucleotide status, between endosomal membranes and the cytosol [29].

Although ARF6 does not colocalize with mannose-6-P receptor (a late endosome marker) or with LAMP-1 and LAMP-3 (lysosome markers), its participation in the vesicular traffic between early and late endosomal compartments has been proposed [46, 47]. The immunofluorescent distribution obtained for another small GTPase, the Rab5, has demonstrated partial overlapping with ARF distributions. Consistent with these results, isolated endosomal and brush border membrane vesicle fractions of proximal tubular cells, which are positive for ARF proteins, also display Rab4 and Rab5 GTPases, which are known markers for the early endosomal compartment [29]. This goes along with the proposition of ARFs as regulators of proximal tubular cell endocytosis.

Recent studies from Galas et al also suggest a role for ARF6 in the exocytotic pathway of endocrine cells [48]. ARF6 was found to be localized to the membrane of chromaffin granules by subfractionation procedures and to be translocated to the plasma membrane after cell stimulation, concomitant with an increase in the phospholipase D (PLD) activity [49]. By analogy with the lipid-modifying activity of ARF1, ARF6 could act by recruiting coat proteins from the cytosol [23, 25] and/or via PLD and its metabolites to induce vesicular formation [4, 23, 25, 50]. In spite of all of these data and the fact that ARF6 (and ARF1) has been proposed to work concertedly with the H^+-pump-ATPase as regulators of membrane traffic pathways in renal tubular endocytosis [24, 25, 29], the precise roles of ARFs either in tubular reabsorption or as catalysts of phospholipid metabolism regulating the endosomal vesicle traffic remain to be elucidated.

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