Int. J. Dev. Biol. 43: 425-433 (1999)

Vesicular transport and kidney development

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ABSTRACT Vesicular transport processes play crucial roles in the biogenesis of cellular membranes and in the polarized transport functions of epithelial cells. During the 1990's we have witnessed major progress in elucidation of the machineries responsible for the intracellular membrane trafficking. The components of these machineries are abundant in tissues with a high content of epithelial cells, such as the kidney. However, the developmental role of the membrane trafficking apparatus in higher eukaryotes has been addressed hardly at all. We summarize here data on the presence and the functional role of vesicle transport proteins in the kidney, and describe work addressing the developmentally regulated expression and localization of three molecules suggested to be involved in polarized trafficking in kidney epithelia, Rab17, syntaxin 3, and Munc-18-2. The results show that specialized transport machinery is induced during differentiation of renal epithelia. However, the expression levels of the components under study are highest in the mature structures, indicating that the proteins are predominantly required for the function of mature epithelia and possibly for the maintenance of the polarized phenotype of specific epithelial cells. The proteins are, however, detected at low levels already in earlier, differentiating structures, and could thus also be involved in the differentiation of kidney epithelia.

KEY WORDS: *collecting duct, proximal tubule, Rab proteins, Sec1-related proteins, SNARE proteins*

Development of the metanephric kidney

Development of the mouse metanephric kidney starts on embryonic day 11 when an epithelial structure, the ureter bud, bulges from the Wolffian duct, invades the metanephric mesenchyme and induces the mesenchyme cells to condensate. The mesenchymal condensates then differentiate into polarized epithelial cells and eventually form the nephrons, the secretory units of the kidney. The tissue interactions are bidirectional: the mesenchyme, in turn, induces the ureter bud to undergo branching morphogenesis and finally form the collecting duct system of the mature kidney. Differentiation of the nephrons proceeds through several intermediate stages (Fig. 1). First the non-polarized mesenchyme cells form an aggregate consisting of cells with an irregular shape and a decreased intercellular space. At the vesicle stage the cells proliferate actively, elongate, and start to polarize. A slit then appears at one end of the vesicle, resulting in the formation of the so called comma-shaped body. Soon another slit emerges at the opposite pole and the structure transforms into an S-shaped body. The proximal part of the structure, where the first slit appeared, develops into a glomerulus, the middle segment into a proximal tubule, and the most distal portion forms the distal tubule. Endothelial cells forming the vascular tuft of the glomerulus start migrating to the glomerular space at the S-shaped stage. The distal tubule is then eventually connected to the collecting duct system (Saxén, 1987).

The morphogenesis of nephrons from uninduced mesenchyme cells to fully polarized epithelial structures can also be followed at the molecular level by staining for marker molecules specific for mesenchymal or epithelial tissue types (Fig. 1). The uninduced mesenchyme cells express uniformly, e.g. collagen type I, collagen type III, and fibronectin (interstitial proteins). After induction, as the mesenchyme cells start to aggregate, the expression of these molecules ceases (Ekblom *et al.*, 1981a) and markers characteristic for epithelial cells become prominent. For example, laminin, the major component of the basement membrane, is present at a low level in uninduced mesenchyme but its localization is diffuse. When the cells aggregate, laminin becomes more prominent in these aggregates and later concentrates to the basement membrane (Ekblom *et al.*, 1980; Sorokin *et al.*, 1997). Other proteins suggested to affect cell adhesion

Abbreviations used in this paper: ARF, ADP-ribosylation factor; MT, microtubule; NSF, N-ethylmaleimide sensitive fusion protein; SNAP, soluble NSF attachment protein; SNAP-25, synaptosomal-associated protein of 25 kDa; SNAP-23, ubiquitous homolog of SNAP-25; SNARE, SNAP receptor; t-SNARE, target SNARE; VAMP, vesicle-associated membrane protein; v-SNARE, vesicle SNARE.

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0214-6282/99/$15.00

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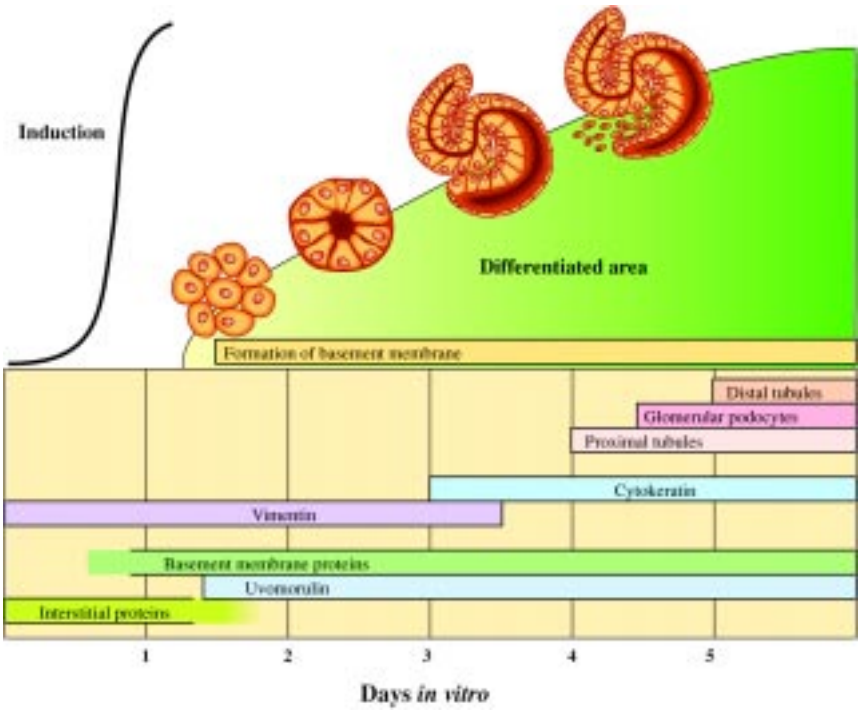

Fig. 1. A schematic presentation of nephron differentiation. *The time schedule represents an in vitro differentiation process induced by using the transfilter technique (Grobstein, 1956). Day 0 in this in vitro time schedule corresponds to the time of setting up the transfilter cultures using tissue pieces isolated from 11-day embryos. The sequence of events corresponds closely to that observed in vivo. The top part illustrates the early morphogenesis of the nephrons and the bottom part the time of expression of some structural proteins and specific markers of the different segments of the nephron. (After Lehtonen and Saxén, 1986; Saxén, 1987).*

during this process include N-CAM (Thiery *et al.*, 1982, 1984) and uvomorulin or E-cadherin (Vestweber *et al.*, 1985), both of which are upregulated at an early stage of cell aggregation. Also, expression of cytoskeletal proteins changes during epithelialization: Vimentin, characteristic of mesenchymal cells, is replaced by epithelial-specific cytokeratins (Lehtonen *et al.*, 1985). Later, the different parts of the nephron can be distinguished by staining for marker molecules specific for each segment (Ekblom *et al.*, 1981b; Lehtonen *et al.*, 1983).

At what stage can a differentiating epithelial cell be considered as functionally polarized? The basement membrane already starts to form around the early mesenchymal aggregates and is completed when the structure reaches a vesicle stage. However, the basement membrane as such is apparently not a polarizing factor. The cells in the mesenchymal aggregate establish stable cell-cell contacts and assemble intercellular junctional complexes, followed by basal accumulation of basement membrane proteins and apical formation of lumen. Later, the role of the basement membrane could be to maintain the polarized state of the cells by providing the necessary adhesive support (Saxén, 1987). It is evident that the cells reach a morphologically polarized state much before they become functionally polarized.

Function of the kidney

The function of the kidney (see the corresponding chapters in Brenner, 1996) is to maintain the salt, water and acid-base balance of the body and to secrete waste products in the form of urine. The functional unit of the kidney, the nephron, consists of the renal corpuscle, the proximal tubule, the thin limbs of Henle's loop, the distal tubule, and the connecting segment, which links the nephron to the collecting duct system (Fig. 2).

All these structures are composed of highly differentiated, well-polarized epithelial cells carrying out specialized functions in absorption and secretion. The function of the renal corpuscle is to produce a large quantity of ultrafiltrate from the plasma, which is then modified as it passages through the other segments of the nephron. More than half of the ultrafiltrate volume produced by the glomerulus is reabsorbed already in the proximal convoluted tubule; also the descending thin limb of Henle's loop is highly permeable to water. Fluid reabsorption through the cell membrane is a passive process connected to ion reabsorption and the osmolarity of the interstitium surrounding the tubules. The final concentration and composition of urine is modified in the most distal portion of the nephron and in the collecting duct under strict hormonal control.

The glomerular ultrafiltrate resembles plasma but has a lower protein content. Albumin and other remaining plasma proteins in the ultrafiltrate are reabsorbed mainly by receptor-mediated endocytosis in the proximal convoluted tubule, the epithelial cells of which contain a large number of lysosomes involved in degradation of the endocytosed proteins. Also, some secretory activity is detected in this part of the proximal tubule: the lysosomes containing indigestible residual material empty their contents into the tubular lumen. The tubules also secrete certain organic ions into the urine.

To create and maintain their polarized phenotype, the highly absorptive kidney tubule epithelial cells need a complex membrane transport machinery (see following section: intracellular membrane trafficking). This is required, in addition to endocytosis of proteins from the lumenal space, for transcytosis and recycling of the endocytosed membrane components (e.g. receptor, channel, cotransporter and ion pump proteins) back to the correct plasma membrane domains as well as for exocytic delivery of newly synthesized membrane proteins to their appropriate destinations.

Intracellular membrane trafficking

Compartmentalization of eukaryotic cells into membrane-bounded organelles necessitates tightly controlled transport of biomolecules from their sites of synthesis to their destinations, and mechanisms preventing deleterious mislocalization of organelle constituents. One of the major processes responsible for the localization of molecules within the cell is called membrane trafficking or vesicular transport. Here, membranous carrier structures bud off a donor membrane compartment and fuse with a recipient one, thus delivering their membrane-associated and soluble lumenal constituents to the target. The major vesicular trafficking routes in cells are the biosynthetic or secretory pathway and the endocytic

pathway. In addition, transcytosis of material from one surface of polarized cells to the other is an important transport function of epithelia.

The molecular machinery of membrane trafficking

Sorting of cargo to membranous carrier vesicles or tubules is the first step of a transport process. Increasing evidence shows that this is an active process that leads to concentration of specific cargo molecules and the necessary targeting machinery in the transport intermediates. Cytosolic coat protein complexes, which assemble on specific membrane sites, form a major machinery responsible for both cargo sorting and formation of the membrane curvature required for transport vesicle pinch-off (Robinson, 1997; Nickel *et al.*, 1998). Membrane association of cytosolic coat complexes is regulated by small Ras-related GTPases belonging to the ADP-ribosylation factor, ARF, or Sar1 groups. Further, the lipid composition of membranes plays an important role in coat recruitment and transport vesicle formation (Roth, 1999).

After a transport intermediate has detached from a donor membrane, it has to be moved to the vicinity of the appropriate target membrane. In mammalian cells, microtubules (MT) form a major apparatus responsible for the location and movement of transport vesicles and membrane organelles (Lippincott-Schwartz, 1998). The polarized nature of microtubules, and the MT-based motor proteins belonging to kinesins and cytoplasmic dyneins, play important roles in determining the directionality of membrane trafficking events. The distinct organization of MT in epithelial cells is one of the factors controlling the vectorial trafficking events characteristic of these cells (Mays *et al.*, 1994; Lafont and Simons, 1996). In addition to the microtubules, cytoskeletal elements consisting of actin, spectrin and ankyrin are involved in membrane trafficking: actin and spectrin apparently participate in structures that connect membrane organelles and transport vesicles with microtubules. Furthermore, actin microfilaments are suggested to be involved in transport vesicle budding, and to form tracks for short-distance vesicle movement. Reorganization of the cortical actin cytoskeleton plays an important role in both exo- and endocytic processes in mammalian cells. Unconventional myosins serve as the motors driving organelle movement along actin microfilaments (Mermall *et al.*, 1998).

The protein machinery responsible for the contact between a transport vesicle and its target membrane (initial tethering and more intimate docking) and the following fusion of bilayers has been subject to intense research efforts in the 1990s. Identification of the so-called SNARE (soluble NSF attachment protein, SNAP, receptor) machinery in 1993 was a major breakthrough in the field (Söllner *et al.*, 1993; Rothman, 1994). These membrane-anchored proteins were discovered as receptors for α-SNAP, a factor mediating the binding of N-ethylmaleimide sensitive fusion protein (NSF) onto membranes. NSF is an ATPase required for most of the membrane trafficking events assayed. The “prototype” SNAREs first identified were the synaptic vesicle synaptobrevin/VAMP proteins as well as syntaxin 1 and SNAP-25 (synaptosomal-associated protein of 25 kDa), predominantly localizing at the presynaptic plasma membrane in neurons. SNAREs present on transport vesicles were denoted as vesicle SNAREs, v-SNAREs (related to VAMP), and those present on the acceptor membranes as target SNAREs, t-SNAREs (related to syntaxins or SNAP-25). The finding that SNAREs are the targets of the proteolytic action of

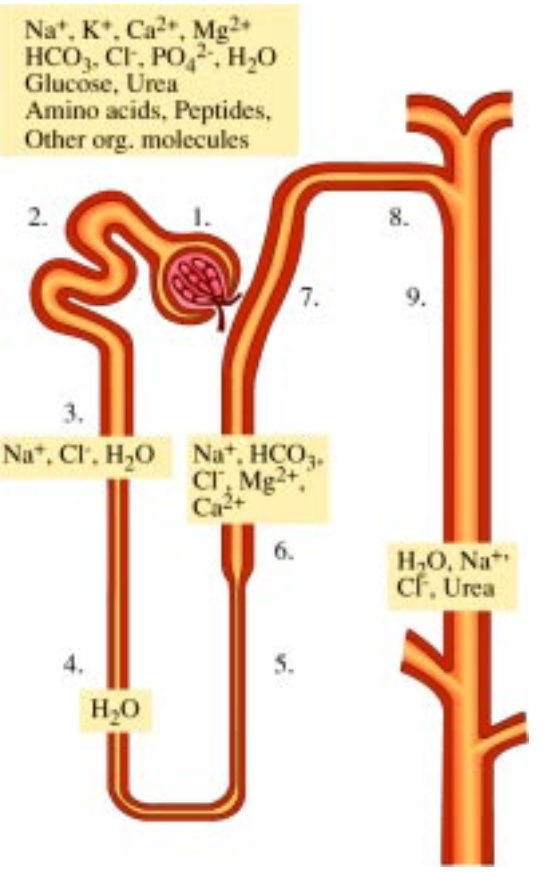

Fig. 2. Sites of water and solute trafficking in different parts of the nephron and the collecting duct. *1, glomerulus; 2, proximal convoluted tubule; 3, proximal straight tubule; 4, descending thin limb of Henle's loop; 5, ascending thin limb of Henle's loop; 6, ascending thick limb of Henle's loop; 7, distal tubule; 8, connecting segment; 9, collecting duct.*

clostridial neurotoxins illustrates well their central role in synaptic vesicle fusion and other intracellular transport events (Montecucco, 1998). SNARE proteins have since then been identified in all eukaryotic organisms and all mammalian cell types studied, thus constituting a universal protein apparatus responsible for membrane docking/fusion. The SNAREs carry sequences with a high propensity to form coiled-coil complexes, and the energetically favorable assembly of four-stranded coiled-coils consisting of parallel helices from proteins on the transport vesicle and, on the other hand, on the target membrane, is suggested to be a process actually driving the bilayer fusion event (Fig. 3; Poirier *et al.*, 1998; Sutton *et al.*, 1998; Weber *et al.*, 1998). The ATPase NSF is most probably required to resolve such "used" SNARE complexes, thus allowing recycling and reuse of their components, and priming the syntaxin-related SNAREs for a new round of vesicle docking/fusion (Hay and Scheller, 1997).

Recent data suggest that the SNAREs form a minimal apparatus for the actual bilayer fusion (Weber *et al.*, 1998), and it is evident that upstream of the SNAREs there has to be additional machinery responsible for the initial vesicle-target interaction (tethering the vesicles) and for regulation of SNARE function. The best characterized protein families implicated as upstream regulators of SNARE

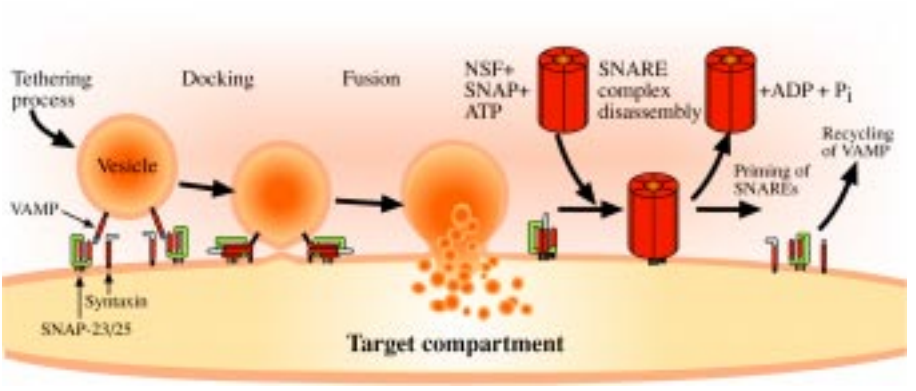

Fig. 3. A model for the function of SNARE proteins in transport vesicle docking/fusion. *After initial contact of the transport vesicle with its target membrane, denoted as tethering, vesicle SNAREs (v-SNAREs) homologous to VAMP/synaptobrevin interact with target SNAREs (t-SNAREs) homologous to syntaxin and SNAP-23/SNAP-25. The coiled-coil forming segments of the different SNAREs wind together in an energetically favorable process, which facilitates fusion of the membrane bilayers. The SNARE complexes are thereafter untangled by the action of NSF, an ATPase which functions in conjunction with soluble NSF attachment proteins, SNAPs (not related to SNAP-23 and SNAP-25). The action of NSF also serves to conformationally prime SNARE proteins for another round of vesicle transport, which also requires recycling of v-SNAREs to the donor compartment.*

function are the Sec1-related proteins and the Rab GTPases belonging to the superfamily of Ras-related proteins. The Sec1 proteins (Halachmi and Lev, 1996) are cytosolic proteins capable of binding with high affinity to syntaxin-related t-SNAREs. This interaction regulates the ability of syntaxins to participate in SNARE complex formation. Sec1-related proteins have been suggested to play an inhibitory role in vesicle docking/fusion, but a wealth of evidence shows that the proteins have also a positive role in vesicle transport, perhaps through stabilization of a conformation of syntaxin that is competent to interact in a productive fashion with the other SNAREs or shielding syntaxin from promiscuous, non-productive interactions.

The Rab proteins (Novick and Zerial, 1997; Olkkonen and Stenmark, 1997) were already in the 1980's found to play a crucial role in intracellular membrane trafficking. These GTPases localize to distinct membrane compartments, and numerous studies using protein variants with the GTP binding-hydrolysis cycle disturbed by mutations have confirmed that each protein controls quite specific vesicular transport steps. The GTP binding-hydrolysis cycle of Rab proteins as well as their membrane association are modulated by a multitude of accessory protein factors. Although the mechanism of action of Rab GTPases is not yet understood in detail, it is well established that Rabs function as regulatory devices, "molecular switches", that oscillate between GTP- and GDP-bound states displaying distinct conformational differences. The GTP-bound forms of Rabs recruit specific effector proteins on the membrane (Fig. 4). The Rab effectors identified so far represent diverse types of proteins: they have in some cases been implied as factors involved in the vesicle tethering process (Christoforidis *et al.*, 1999; Guo *et al.*, 1999), while others are thought to connect membrane vesicles to cytoskeletal elements (Echard *et al.*, 1998) or to regulate the tethering/docking process by other mechanisms.

The protein apparatus responsible for vesicular transport in the kidney

The machineries of membrane trafficking characteristically constitute families of related proteins, where some family members are expressed ubiquitously while others display cell type-specific expression patterns. In the same cell, different family members are involved in distinct intracellular trafficking steps, only very few components being common for all trafficking events. The kidney has a high content of epithelial cells, which are specialized in carrying out vectorial transport of ions and other solutes. Accordingly, a markedly high number of the known vesicle transport proteins are relatively abundant in the kidney as judged from Northern analysis. In addition, there are mRNAs which are expressed in distinct subsets of tissues including the kidney, in many cases reflecting predominant expression in specific types of polarized epithelial cells. Among the central components of vesicle transport machineries, these molecules include the t-SNARE syntaxin 3 (Bennett *et al.*, 1993), the v-SNARE VAMP8 (Advani *et al.*, 1998), the Sec1 homolog Munc-18-2 (Hata and Südhof, 1995; Katagiri *et al.*, 1995; Tellam *et al.*, 1995; Riento *et al.*, 1996), and a number of Rab GTPases: Rab 17 (Lütcke *et al.*, 1993), 19 (Lütcke *et al.*, 1995), 25 (Goldenring *et al.*, 1993), 26 (Wagner *et al.*, 1995), and 29 (Massmann *et al.*, 1997). Without detailed analysis of the cell-type specific expression pattern and subcellular localization of the proteins in the kidney by *in situ* hybridization and immunofluorescence/immunoelectron microscopy, however, not much can be concluded from this data. The analysis of the expression, localization, and function of these proteins surmised to be highly important for kidney transport processes has been extended to a higher level of resolution only in very few cases. Furthermore, there are no published reports on the use of transgenic or knock-out animal models to elucidate the role of the vesicle transport machinery in kidney function or development.

Membrane recycling through endocytosis/exocytosis processes is an important means of regulating the function of the distinct channel and ion pump proteins in the collecting duct epithelium (see Brown and Sabolic, 1993; Knepper and Inoue, 1997). Of these vesicular transport events, the vasopressin-regulated trafficking of water (aquaporin) channels has received most attention in terms of the protein machinery involved. The t-SNARE syntaxin 4 has been localized to the apical plasma membrane of renal collecting duct principal cells, where it is suggested to regulate the fusion of aquaporin-2 containing vesicles (Mandon *et al.*, 1996). SNAP-23, a homolog of the neuronal t-SNARE SNAP-25, is also found at the apex of the same cells, where it could function as a partner of syntaxin 4. SNAP-23 is also present in proximal tubule epithelial cells and in thick ascending limb of Henle's loop (Inoue *et al.*, 1998). The cognate v-SNARE partner of syntaxin 4 and SNAP-23 in the principal cells is obviously a VAMP2-like protein present on the water channel-containing vesicles (Jo *et al.*, 1995; Nielsen *et al.*, 1995). Furthermore, the small GTPase Rab3 is enriched in these vesicles (Liebenhoff and Rosenthal, 1995). A functional role of the SNAREs in the fusion of aquaporin-containing vesicles, as well as in the trafficking of proton pumps responsible for H^+ secretion by inner medullary collecting duct (intercalated type A) cells, is suggested by the sensitivity of the processes

to clostridial toxins (Jo *et al.*, 1995; Alexander *et al.*, 1997). Also the general components of the intracellular membrane trafficking machinery, NSF and α-SNAP, as well as the ubiquitous v-SNARE cellubrevin, are detected in the inner medullary collecting duct epithelial cells (Franki *et al.*, 1995).

Rab 17 (Lütcke *et al.*, 1993) as well as the ubiquitously expressed GTPases Rab 18 and 20 (Lütcke *et al.*, 1994) have been localized to apical dense tubules of proximal tubule epithelial cells. In addition, Rab17 was detected on the basolateral plasma membrane. The multitude of Rab GTPases present on the apical dense tubule compartments indicates a central role of these membrane structures as organizing centers for membrane trafficking in the kidney proximal tubule epithelium. Receptor-mediated endocytosis of albumin and other proteins from the glomerular ultrafiltrate is a central transport process in proximal tubules (Marshansky *et al.*, 1997a). This occurs mainly through clathrin-coated pits which are highly abundant in proximal tubule cells (Rodman *et al.*, 1984). ARF1 and ARF6 GTPases were found to be predominantly expressed in these tubules. ARF1 has a well established function in Golgi transport, whereas ARF6, which was localized to brush border membranes and early endosomes, may play an important role in the regulation of protein reabsorption by proximal tubules (D'Souza-Schorey *et al.*, 1995; Marshansky *et al.*, 1997b). Data on the expression and localization of syntaxin 3, Munc-18-2, and Rab17 during development of mouse kidney is discussed below.

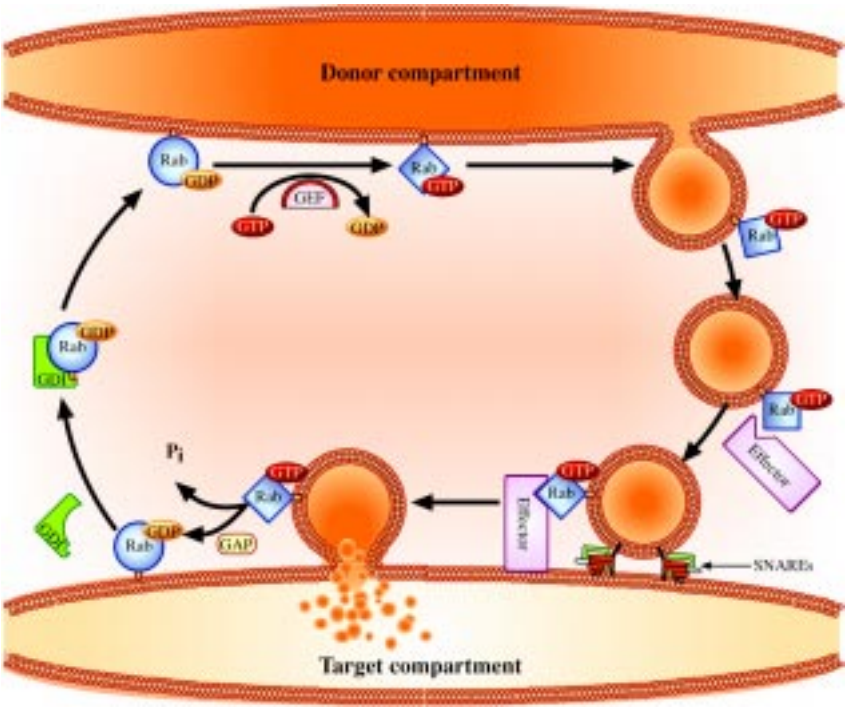

Fig. 4. A model for the function of Rab GTPases in vesicular transport. *The Rab protein in its active, GTP-bound form is included in a transport vesicle, and binds effector molecules involved in tethering of the vesicle at a specific target membrane. The tethering is followed by more stable docking and finally by fusion, which is suggested to be mediated by the SNARE machinery. A GTPase activating protein (GAP) facilitates hydrolysis of the bound GTP by the Rab protein, and the resulting GDP-bound Rab is detached from the target membrane by GDP dissociation inhibitor (GDI) protein. GDI mediates recycling of the Rab back to the donor compartment, where it is reactivated through action of a guanine nucleotide exchange factor (GEF) and can enter another cycle of vesicular transport.*

The developmental significance of membrane trafficking processes

Surprisingly little is known of the role of membrane trafficking functions in mammalian development. However, the central developmental role of the vesicle transport machineries becomes evident when studying the data from simpler eukaryotic organisms such as the budding yeast *Saccharomyces cerevisiae* and the fruitfly *Drosophila melanogaster*, in which the easily applicable genetic methodology has allowed detailed analysis of the topic. In the context of development, generation of cell polarity (see Eaton and Simons, 1995; Drubin and Nelson, 1996) and biogenesis of membranes are central processes involving membrane trafficking. In yeast, there is convincing evidence for an important function of the transport machinery components in establishing cell polarity, as manifested by selection of the bud site, and thereafter in septum formation and cytokinesis. An important factor connecting cell polarity and vesicular transport here is the Sec3 protein, a component of the so-called exocyst complex (Finger and Novick, 1998; Finger *et al.*, 1998). In the context of kidney development and epithelial differentiation, it will be highly important to assess the role of the mammalian counterparts of these yeast proteins.

The *Drosophila* t-SNARE syntaxin 1 plays a vital role throughout the development: initially, it is necessary for cellularization of the syncytial blastoderm, a specialized form of cell division in which the 6000 nuclei are enclosed into separate cells, a process requiring extensive membrane biogenesis (Burgess *et al.*, 1997). At later stages of *Drosophila* development, syntaxin 1 has an important role in the larval imaginal discs, affecting the development of the eye and the wing, as well as in cuticle secretion and neurotransmission (Schulze *et al.*, 1995; Schulze and Bellen, 1996). Similarly, the *Drosophila* Sec1 protein Rop has, in addition to a well established function in neurotransmission, an important role in general secretion processes necessary for normal embryonic development: the *Rop* null mutant animals die at a late embryonic stage (Harrison *et al.*, 1994). One can envision that in analogy, membrane biogenesis, secretion, endocytosis, and the molecular machineries involved most certainly play vital roles also in mammalian development.

Epithelial-enriched proteins involved in polarized vesicular transport in the kidney

Expression of Rab17 in developing mouse kidney

A partial *Rab17* cDNA was cloned from mouse kidney by using a PCR approach (Chavrier *et al.*, 1992). Further analysis showed that the *Rab17* mRNA is present only in the kidney, liver, and intestine, indicating a predominant function in polarized epithelial cells (Lütcke *et al.*, 1993). This notion is supported by *in situ* hybridization data revealing that the mRNA is restricted to epithelial cells in the developing kidney. Here, the mRNA is detectable from day-14 on (Fig. 5A-D). Interestingly, *Rab17* first appears in the central branches of the ureter tree in the medulla, whereas the

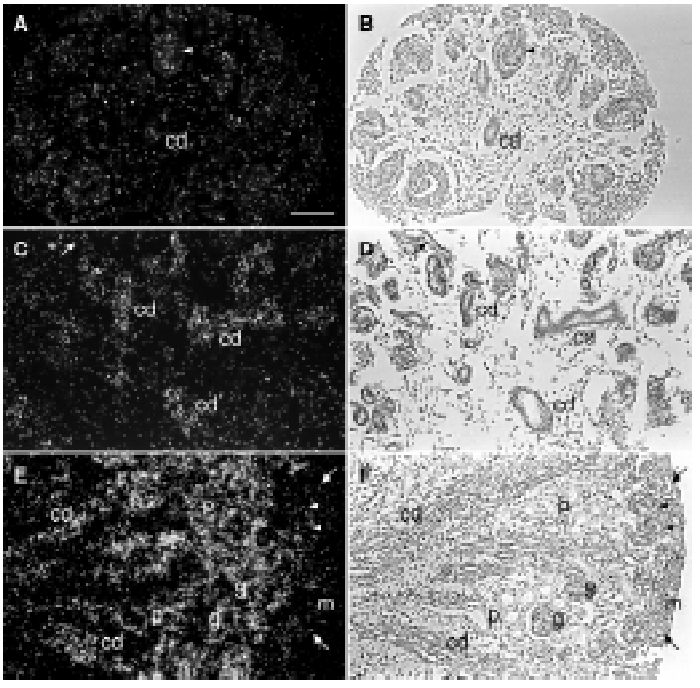

Fig. 5. *In situ* hybridization of embryonic mouse kidneys with *Rab17* probe. (A,C *and* **E)** *Darkfield images*; **(B,D** and **F)** *corresponding brightfield images.* **(A-B)** *Embryonic day-13 kidney shows hardly any signal. cd, collecting duct; arrowhead, early differentiating epithelial structure.* **(C-D)** *On day-14, Rab17 is upregulated in the central branches of the collecting duct (cd), whereas the tips (arrow) remain negative.* **(E-F)** *In day-17 kidney Rab17 is prominent in the proximal tubules (p) and central branches of the collecting duct (cd). Tips of the collecting duct (arrow), early differentiating epithelial structures (arrowhead), and undifferentiated mesenchyme cells (m) in the cortical region of the kidney remain negative. Glomeruli (g), similarly, show no signal. Bar, 100 μm.*

branching tips are negative (Fig. 5C-D). At the day-14 stage of development, the epithelial ureter bud has already branched several times and formed the ureter tree. As kidney development proceeds, the more mature structures locate in the medullary region of the kidney, while proliferation, nephrogenesis and branching morphogenesis of the ureter tree still continue in the cortical region. Apparently, although the cells of the ureter bud bulging into the metanephric mesenchyme on embryonic day 11 appear morphologically well differentiated and polarized, they gain or create their functional polarization at a later stage. It thus seems that Rab17 does not appear until the cells reach a certain developmental stage, or alternatively, Rab17 is involved in creating this state of polarization of the collecting duct cells.

During nephronogenesis Rab17 also appears at a fairly late stage. *In situ* hybridization reveals no signal in the early stages of differentiating epithelia, such as comma- or early S-shaped bodies, which are present from day-13 on (Fig. 5A-B). Similarly, on day-18, the outermost region of the kidney cortex, containing undifferentiated mesenchyme cells and differentiating early epithelial structures, appears negative. A faint signal can be detected in late S-shaped bodies, and later upon nephron differentiation *Rab17* becomes prominent in proximal tubules (Fig. 5E-F).

The subcellular localization of a Rab protein usually gives a first hint about its function in the membrane transport machinery. Immunohistochemistry and immunoelectron microscopy of adult mouse kidney revealed tubular structures with a weak signal, apparently distal tubules, and ones showing prominent *Rab17* expression, identified as proximal tubules (Lütcke *et al.*, 1993). The signal in the proximal tubules concentrated to the basolateral plasma membrane, but also staining of tubular structures below the apical brush border was detected. This staining decorated the apical dense tubules, which function in recycling of endocytosed apical plasma membrane components and transcytosis (Christensen, 1982; Nielsen *et al.*, 1985). This is well in accordance with functional studies on *Rab17* performed in cultured epithelial cells, suggesting a role as a regulator of apical recycling and transcytosis (Hunziker and Peters, 1998; Zacchi *et al.*, 1998; Hansen *et al.*, 1999).

Munc-18-2 and syntaxin 3 in developing kidney

The Sec1 protein Munc-18-2 and its interaction partner syntaxin 3 are expressed in distinct subsets of mammalian tissues including the kidney (Bennett *et al.*, 1993; Riento *et al.*, 1996). Syntaxin 3 is a t-SNARE protein whose function is apparently controlled by interaction with Munc-18-2 (Riento *et al.*, 1998). In contrast to *Rab17*, both *syntaxin 3* and *Munc-18-2* mRNAs are present already in the embryonic day-11 kidney, but they are strongly upregulated as the development proceeds, the expression levels being highest in the adult organ (Fig. 6). *In situ* hybridization analysis revealed that their mRNAs concentrate to the same structures in the kidney, the proximal tubules and the collecting ducts, whereas the other syntaxins studied were widely expressed by both epithelial and mesenchymal cells. Furthermore, coimmunoprecipitation from kidney lysates showed that the syntaxin 3 and Munc-18-2 proteins form a physical complex in the kidney (Lehtonen *et al.*, 1999). These results imply that syntaxin

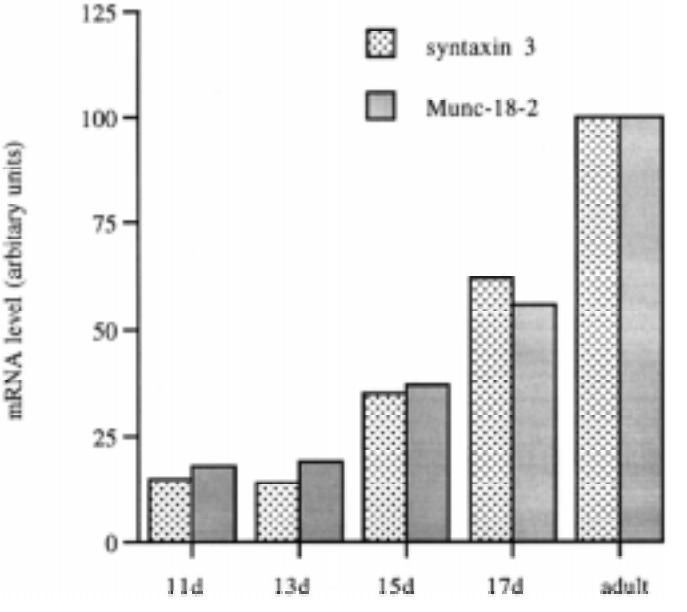

Fig. 6. Quantitation of *syntaxin 3* and *Munc-18-2* mRNAs in the developing kidney. *The syntaxin 3 and Munc-18-2 mRNAs are expressed already on embryonic day-11. The amount of both mRNAs increases upon development and is highest in the adult organ.*

3 and Munc-18-2 are involved in the same membrane transport process in the developing kidney.

Immunohistochemistry revealed that the syntaxin 3 and Munc-18-2 proteins, like their mRNAs, concentrate in epithelial structures in the kidney. Both of them are abundant in the ureter bud of day-11 kidney (Figs. 7A and 8A), and noticeably, the signal shows strong apical concentration in these cells. Apical expression in the collecting ducts persists during embryonic development (Figs. 7B and 8B), but in the case of Munc-18-2, some cytoplasmic staining is also detected (Fig. 8B). Mandon *et al.* (1997) reported that syntaxin 3 is expressed in the adult rat collecting duct type A intercalated cells. However, in contrast to our findings in developing mouse kidney, they detected in these cells a basolateral localization of syntaxin 3. This discrepancy may be due to differences in the cell type- and developmental stage-specific localization of the protein.

In the developing nephrons, syntaxin 3 and Munc-18-2 first appear in well-formed S-shaped bodies. Upon maturation of the nephron, the signal becomes restricted to the proximal tubules (Figs. 7B-D and 8B-D), while the distal tubules and glomeruli remain negative. As in the collecting ducts, the signal is apical. In the mature kidney, most of the proteins and peptides present in the glomerular ultrafiltrate become reabsorbed in the proximal convoluted tubule, and the reabsorption rate is already significantly lower in the following proximal straight tubule. Noticeably, syntaxin 3 is expressed only in the convoluted part of the proximal tubule (Fig. 7C-D). Instead, Munc-18-2 is present also in the straight portion but its localization there is dramatically changed: instead of concentrating apically the protein appears as cytoplasmic vesicular structures mainly in the basal part of the cells (Fig. 8C-D). The changed localization of Munc-18-2 in the straight proximal tubule could be due to absence of syntaxin 3, and suggests that in this part of the tubule Munc-18-2 has interaction partners other than syntaxin 3 and apparently also functions independent of syntaxin 3.

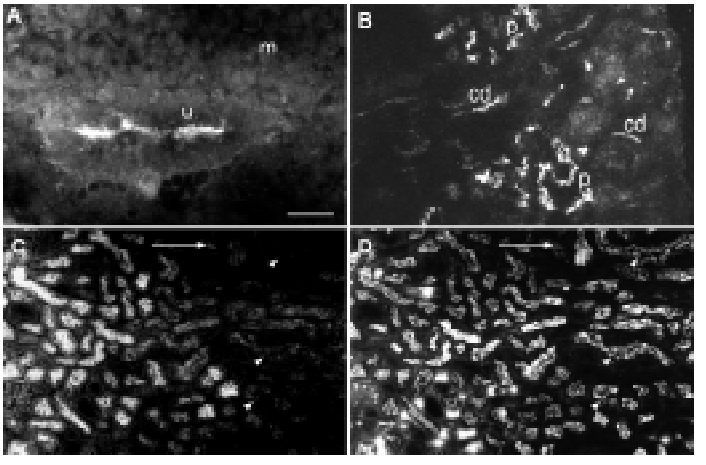

Fig. 7. Immunofluorescence localization of syntaxin 3 in the developing kidney. (A-C) *Staining with syntaxin 3 antibody.* **(D)** *The field in C double labeled with an antibody against megalin decorating proximal tubules.* **(A)** *On embryonic day-11, syntaxin 3 is prominent in the apical domain of the epithelial ureter bud (u); the surrounding mesenchyme cells (m) show no signal.* **(B)** *At day-17, kidney syntaxin 3 is strongly expressed in proximal tubules (p) and collecting ducts (cd).* **(C-D)** *In the adult, syntaxin 3 concentrates in the apical domain of proximal tubules. The expression ceases in the straight portion of the tubule still showing prominent staining for megalin (arrowhead in D). Arrow indicates direction from cortex to medulla. Bar, (A) 30 μm; (B-D) 100 μm.*

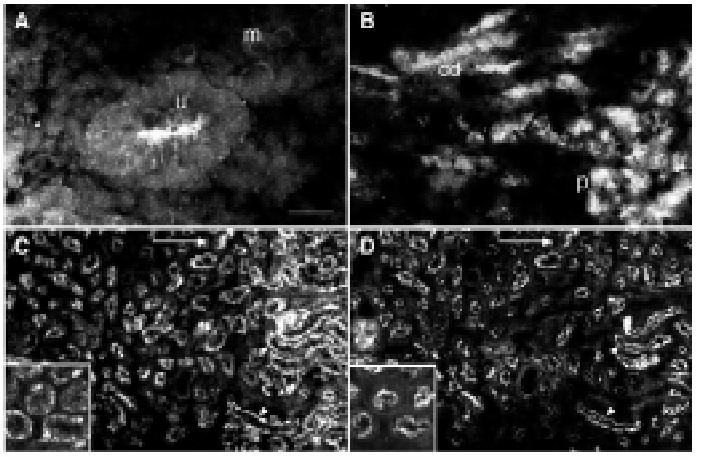

Fig. 8. Immunofluorescence localization of Munc-18-2 in the developing kidney. (A-C) *Staining with Munc-18-2 antiserum.* **(D)** *The field in C double labeled for megalin.* **(A)** *At day-11, kidney Munc-18-2 localizes apically in the ureter bud (u) bulging into the metanephrogenic mesenchyme (m).* **(B)** *On day-17, the protein is abundant in the collecting ducts (cd) and proximal tubules (p).* **(C-D)** *In the adult organ, Munc-18-2 is prominent both in the convoluted and straight portions of the proximal tubule. In the straight portion, however, the localization of the protein changes: Instead of apical distribution, the protein appears in vesicular structures concentrating to the basal domain of the cells (arrowheads). The inserts show a higher magnification of the straight proximal tubule with the vesicular distribution of Munc-18-2. Arrow indicates direction from cortex to medulla. Bar, (A) 30 μm; (B) 75 μm; (C-D) 100 μm.*

What is the function of syntaxin 3 and Munc-18-2 in the developing kidney? Thus far the SNARE machinery involved in endocytosis, the major membrane transport event in proximal convoluted tubules, is poorly known. On the other hand, the closest homologs of these proteins, syntaxin 1A and Munc-18/n-Sec1, respectively, have been shown to function in exocytosis at the synaptic termini (Pevsner, 1996; Robinson and Martin, 1998).

Overexpression of syntaxin 3 in cultured epithelial cells was shown to disturb apical recycling and exocytosis, but not transcytosis (Low *et al.*, 1998). Taken together, these results imply that syntaxin 3 and Munc-18-2 most likely function in apical exocytic events or in recycling of endocytosed apical membrane constituents. The two proteins are present in the ureter bud epithelium already at the 11-day stage. This suggests that these proteins belonging to the SNARE apparatus are required at an earlier stage in the ureter tree development than Rab17. Even though syntaxin 3 and Munc-18-2 are detectable at low levels in early developmental stages of the nephron, their expression is highest in mature proximal tubule epithelia. This indicates that that their primary function is not in the early inductive events leading to epithelial differentiation, but they are rather associated with the maintenance of the polarized transport functions of specific epithelial cell types (see Drubin and Nelson, 1996). Nevertheless, they could also be involved in generation of the differentiated phenotype of the collecting duct and proximal tubule epithelia. The precise developmental role of the proteins under study and their relatives remains to be solved by future work employing gene knock-out animal models.

Acknowledgments

Ms Ulla Kiiski is acknowledged for skilled technical assistance and Mikko Jalanko (Duodecim, Helsinki, Finland) for expert work in making the schematic figures. This work was supported by the Emil Aaltonen Foundation (S.L.), the Finnish Cultural Foundation (S.L.), and the Clinical Research Fund of Helsinki University, Finland (E.L.; grant TYH 9312). V.M.O. is a senior scientist of the Academy of Finland and supported by grants 36282, 42163.

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