



Pierre J. COURTOY, Member
Christophe PIERREUX, Associate Member

Patrick VAN DER SMISSEN, Assistant Member

Donatienne TYTECA, Assistant Member

Sarah CARPENTIER, Graduate Student

Ludovic D'AURIA, Graduate Student

Anne-Christine HICK, Graduate Student

Wânia Rezende LIMA, Graduate Student

Thierry MEDTS, Graduate Student

Thanh LAC, Technician (half-time)

Michèle LERUTH, Technician (half-time)

Benoît MARIEN, Technician

Francisca N'KULI, Technician

Yves MARCHAND, Secretary



ENDOCYTOSIS AND EPITHELIAL DIFFERENTIATION

Endocytosis, a central activity of all eukaryotic cells, allows for nutrition, regulates plasma membrane and controls macromolecules transport across epithelial barriers. This research group has made significant contributions in the dynamic structure of endocytosis, delineation of pathways and physiopathology. Our current investigations focus on endocytosis at the apical membrane of polarized kidney proximal tubular cells (PTC). We surmise that this most active apical endocytic surface will allow to identify rate-limiting actors and elucidate diseases when these become defective. These molecular actors should also emerge from the study of genetic or acquired mouse models causing low-molecular weight proteinuria. Recent achievements include: (i) delineation of the signalling cascade whereby the paradigmatic oncogene, v-Src, and the master catalyst, Rab5a, impact on the apical actin cytoskeleton, so as to induce macropinocytosis, like enteropathogens (1, 6); (ii) elucidation of defective apical endocytosis in CIC5-KO mice mimicking Dent's disease, a genetic predisposition to kidney stones (2) and CFTR-KO mice, a model of cystic fibrosis in kidneys (5); (iii) serendipitous discovery that apical endocytosis of ultrafiltrated lysosomal proteases is a central mechanism for PTC lysosomes biogenesis (4); and (iv) identification of the transcription factor, ZONAB, as a key component for the switch between proliferation and apical differentiation in various epithelia (9). In view of the distinct composition of apical plasma membrane lipids, we have also undertaken to address whether their micrometric organization impacts on distinct endocytic rate and fate. We thus provided the first evidence that sphingomyelin forms temperature-dependent micrometric domains in living cells (10). Biogenesis of epithelia is the second line of investigations. We focus on epithelial tubulogenesis and differentiation, using developing pancreas and salivary glands in vivo and explants, as complementary models of controlled interconversion between multilayered cell masses and polarized monolayers, with emphasis on paracrine and transcriptional control (8). Our group offers expertise in structural biology and cellular and tissular imaging with a versatile platform, from confocal and multiphoton microscopy (7), to transmission and scanning electron microscopy.

REGULATION OF APICAL ENDOCYTOSIS BY v-SRC IN POLARIZED EPITHELIAL CELLS

M. Mettlen, Ph de Diesbach, T. Medts, S. Carpentier, P. Van Der Smissen, D. Tyteca, P.J. Courtoy

Oncogenic transformation is well known to accelerate the endocytic activity, but the underlying mechanisms remained poorly understood. We originally reported that v-Src causes a profound remodelling of actin cytoskeleton in Rat-1 fibroblasts, resulting in stress fiber disappearance, cortical actin polymerisation, ruffling and macropinocytosis (Amyere et al., Mol Biol Cell 2000;11:3453-67). Since most cancers (i) are of epithelial origin, (ii) and frequently overexpress or overactivate Src; and since (iii) apical endocytosis depends on actin, (iv) which is a major target of Src, we examined whether v-Src would similarly trigger apical endocytosis in MDCK cells. Because stable cell transformation abolishes epithelial polarity due to epithelio-mesenchymatous transition, we resorted to MDCK cells bearing a thermosensitive (ts) v-Src kinase.

When MDCK/tsLA31 cells were plated at high density on a permeable support and cultured at the non-permissive temperature (40°C), a polarized epithelial monolayer could be established, with ~2-fold faster fluid-phase endocytosis at the basolateral than at the apical surface. Shifting to the permissive temperature (34°C) rapidly activated v-Src kinase but preserved a tight monolayer for at least 6 h, allowing to study the effect of Src on polarized endocytosis. During this interval, Src kinase induced apical circular ruffling (Fig. 1) and selectively accelerated apical fluid-phase endocytosis (up to 6-fold). This was accompanied by the induction of macropinosomes, merging into a huge (> 5 µm) apical endocytic vacuole, generated by overflow into the apical recycling compartment (ARE). Preservation of ARE tubulation and of apical polarity indicated that the overall function of this essential compartment was

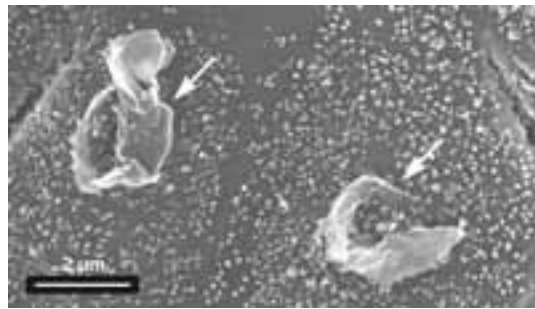


Figure 1. Src causes circular apical ruffling (arrows; scanning electron microscopy, from Ref 3).

however not affected. Macropinosomes and the ARE were labelled for v-Src, Rab11, and rabankyrin-5, but not for early endosome antigen-1, thus distinguishing two separate Rab5-dependent apical endocytic pathways. Ruffling and macropinocytosis depended on an amplification cascade involving PI3K, PLC and PLD, as shown by inhibition by wortmannin, NCDC and 1-butanol, respectively.

These data demonstrate that v-Src selectively affects the dynamics of the apical plasma membrane, where microdomains known as “lipid rafts” are abundant. The mechanisms of Src-induced apical ruffling and macropinocytosis may provide important insights for the apical entry into enterocytes triggered by enteroinvasive pathogens and on the apical differentiation of osteoclasts (3).

DIFFERENTIAL MEMBRANE RECRUITMENT OF SRC MAY SPECIFY ITS DOWNSTREAM SIGNALLING TO MAPK AND PI3K PATHWAYS

Ph. de Diesbach, T. Medts, S. Carpentier, L. D'Auria, P. Van Der Smissen, D. Tyteca, P.J. Courtoy

Most Src family members are diacylated and constitutively associate with membrane “lipid rafts” that coordinate signalling. Whether the

monoacylated Src also localizes at “rafts” was controversial. Using polarized MDCK cells expressing the thermosensitive v-Src/tsLA31 variant, we addressed how Src tyrosine-kinase activation may impact on its (i) membrane recruitment, in particular to “lipid rafts”; (ii) subcellular localization; and (iii) signalling. The kinetics of Src-kinase thermoactivation correlated with its recruitment from the cytosol to sedimentable membranes where Src largely resisted solubilisation by non-ionic detergents at 4°C, and floated into sucrose density gradients like “lipid rafts”. By immunofluorescence, activated Src showed a dual localization, at the apical plasma membrane and adjacent endocytic vesicles. The plasma membrane Src pool did not colocalize with caveolin-1 and flotillin-2, but extensively overlapped with GM1 labelling by cholera toxin. Severe (~70%) cholesterol extraction with methyl- β -cyclodextrin (M β CD) did not abolish “rafts” floatation, but strongly decreased Src association with floating “rafts” and abolished its localization at the apical plasma membrane.

Src activation independently activated first the MAP-kinase-ERK1/2 pathway, then the PI3-kinase-Akt pathway. ERK1/2 activation was insensitive to M β CD, which suppressed Akt phosphorylation and apical endocytosis induced by Src, both depending on the PI3K/Akt pathway. We therefore suggest that activated Src is recruited at two distinct membrane compartments allowing differential topological signalling, first via ERK1/2 at “non-raft” domains, then via PI3-kinase-Akt on a distinct set of apical plasma membrane “rafts”. Whether this model is applicable to c-Src remains to be examined (6).

CONTRIBUTION OF APICAL ENDOCYTOSIS TO THE BIOGENESIS OF LYSOSOMES IN KIDNEY PROXIMAL TUBULAR CELLS

P.J. Courtoy, M. Leruth, F. N’Kuli and W.R. Lima, in collaboration with E.I. Christensen and his colleagues (Aarhus, DK), M. Jadot (FUNDP, BE) and O. Devuyst (NEFR, UCL)

Recruitment of acid hydrolases to lysosomes generally occurs by intracellular sorting based on recognition of a common mannose 6-phosphate signal in the trans-Golgi network and selective transport to late endosomes/lysosomes. We have obtained direct evidence for an alternative, efficient secretion-recapture pathway mediated by megalin, exemplified by cathepsin B in kidney proximal tubular cells (PTC). We found that in mouse kidneys with defective megalin expression [megalin knockout (KO)] or apical PTC trafficking (CIC-5 KO, see below), the (pro)cathepsin B mRNA level was essentially preserved, but the protein content was greatly decreased and the enzyme was excreted in the urine as mannose 6-phosphate-devoid species.

In polarized PTC monolayers, purified cathepsin B was avidly and selectively taken up at the apical membrane; uptake was abolished by the “receptor-associated protein”, a megalin competitor. Direct interaction of cathepsin B with megalin was demonstrated by surface plasmon resonance. Circulating pro-cathepsin B was detected in normal mouse serum. Purified cathepsin B injected into mice was preferentially taken up by kidneys and targeted to lysosomes where it remained active, as shown by autoradiography and subcellular fractionation. A single cathepsin B injection into cathepsin B KO mice could reconstitute full lysosomal enzyme activity in the kidneys. These findings demonstrate a pathway whereby circulating lysosomal enzymes are continuously filtered in glomeruli, reabsorbed by megalin-mediated endocytosis, and transferred into lysosomes to

exert their function, providing a major source of enzymes to PTC. These results also extend the significance of megalin in PTC and have several physiopathological and clinical implications (4).

MOLECULAR MEDICINE: THE CAUSE OF ENDOCYTIC DEFECTS IN KIDNEY FANCONI SYNDROMES

S. Carpentier, W.R. Lima, P. Van Der Smissen, B. Marien, P.J. Courtoy, in collaboration with O. Devuyst (NEFR) and E.I. Christensen (Aarhus, DK)

The highest apical endocytic activity in the body by kidney proximal tubular cells (PTC) allows to recapture all ultrafiltrated low-molecular weight (LMW) proteins. Conversely, defective PTC function in inherited or acquired diseases, collectively referred to as Fanconi syndromes, leads to LMW proteinuria. Dent's disease, also known as X-linked nephrolithiasis, is a paradigm of Fanconi syndromes, due to inactivating mutations of CIC-5. CIC-5 KO mice, which faithfully mimick Dent's disease, showed a severe deficit in PTC endocytosis of a variety of ligands of megalin and cubilin, acting as high-capacity tandem receptors at the apical membrane (brush border) (Fig. 2). Surprisingly, the total megalin pool was not appreciably affected. However, the combination of analytical subcellular fractionation and quantitative ultrastructural immunogold labelling revealed instead that the endocytic receptors were sequestered in apical endosomes and failed to reach the apical membrane, likely as part of a general membrane trafficking defect (2).

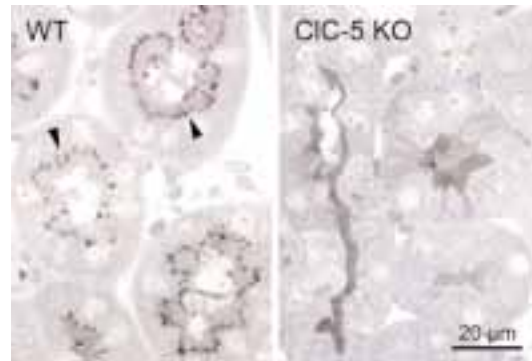


Figure 2. Evidence for a deficit of apical endocytosis of low-molecular weight proteins in kidney proximal tubular cells of CIC-5 KO mice. Arrows indicate cytochemical detection of injected, ultrafiltrated peroxidase. Note the strong labelling by peroxidase of multiple apical PTC endosomes in WT mice, contrasting with poor apical uptake and luminal retention in CIC-5 KO mice.

THE TRANSCRIPTION FACTOR, ZONAB, IS CONTROLLED DURING EPITHELIAL POLARIZATION AND IS A KEY REGULATOR IN THE PROLIFERATION/ DIFFERENTIATION SWITCH

W.R. Lima, C.E. Pierreux and P.J. Courtoy in collaboration with K. Parreira and O. Devuyst (NEFR)

Apical endocytosis is a hallmark of epithelial differentiation. Epithelial polarization depends, and impacts on, gene expression. The transcription factor, ZONAB, can shuttle between tight junctions and the nucleus to promote expression of cyclin D, and thus participate in the control of proliferation. We have examined whether ZONAB simultaneously represses differentiation, using renal proximal tubular cells (PTC) as a model. During both mouse kidney ontogeny and polarization of PTC monolayers in vitro, decreasing ZONAB level inversely correlated with differentiation of the apical endocytic receptors, megalin/cubilin, brush border and primary cilium mar-

kers. Conversely, ZONAB was reexpressed in dedifferentiated renal carcinomas.

Sparsely plated PTC formed small islands: peripheral cells, necessarily lacking external tight junctions, strongly expressed nuclear ZONAB, proliferated and failed to differentiate; central cells, able to form continuous junctional belts, lost nuclear ZONAB, stopped proliferating and engaged in apical differentiation (see Fig. 3). In confluent PTC monolayers, stable ZONAB overexpression inhibited expression and function of the endocytic receptors and impaired brush border and primary cilium maturation. Reporter and chromatin immunoprecipitation assays demonstrated that megalin and cubilin were ZONAB target genes. ZONAB expression was regulated by polarity at pre- and posttranslational levels (mRNA expression or stability, proteasomal degradation). In PTC islands, proteasome inhibition extended nuclear

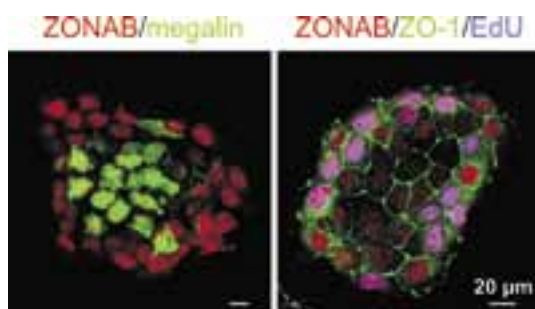


Figure 3. ZONAB is a key regulator in the switch between epithelial proliferation and differentiation. In colonies of kidney proximal tubular cells, ZONAB (immunolabelled in red) is absent in central differentiated cells (expressing megalin, in green, left) but is selectively detected in nuclei of peripheral proliferating cells (DNA synthesis is shown in blue at right).

ZONAB to central cells, which reversed their choice from differentiation to proliferation.

Thus, ZONAB is down-regulated by epithelial polarity at both mRNA and protein levels and acts simultaneously to promote proliferation and repress differentiation. In vitro and in vivo data suggest that ZONAB is a

sensor of epithelial density, involved in their switch from proliferation to differentiation (9).

PLASMA MEMBRANE LIPID FLUORESCENT ANALOGS FORM STRUCTURALLY AND KINETICALLY DISTINCT MICROMETRIC DOMAINS

L. D'Auria, P. Van Der Smissen, P.J. Courtoy, D. Tyteca

Lipids are the most abundant biological membranes constituents. For several decades, plasma membrane lipids were considered to form a homogenous two-dimensional barrier, acting as solvent for membrane proteins. However, it is now well-accepted that some membrane lipids spontaneously cluster into discrete nanometric “lipid rafts” (liquid-ordered domains), thereby creating small-scale lateral asymmetry. In addition, micrometric domains have been reported using fluorescent lipids incorporated into artificial liposomes, erythrocyte ghosts, and in living cells for non-natural lipids, but the natural occurrence of micrometric lipid domains in the plasma membrane of living cells is questioned. We found that insertion of BODIPY-SM into the outer membrane leaflet of living, featureless erythrocytes labelled (sub)micrometric fluorescent plasma membrane

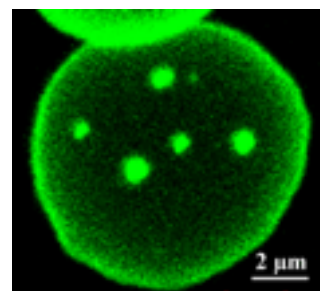


Figure 4. Fluorescent sphingomyelin analogs reveal (sub)micrometric domains on erythrocytes (see Ref 10).

domains (Fig 4). BODIPY-SM domains were randomly distributed in control erythrocytes at

37°C and coalesced upon hypotonic shock, excluding structural surface features and endocytosis and indicating control by surface tension. In CHO cells, either direct plasma membrane insertion of BODIPY-SM or intracellular enzymatic conversion of BODIPY-ceramide into BODIPY-SM produced similar surface BODIPY-SM domains. Domains were extracted by surface back-exchange, resisted endocytosis block (K⁺-depletion) and actin depolymerization (latrunculin B), and clustered upon cholesterol deprivation. BODIPY-SM excimers demonstrated clustering in ordered domains. Depletion of endogenous SM by sphingomyelinase cleavage or biosynthetic inhibition impaired (sub)micrometric domains, strongly suggesting that fluorescent domains of exogenous BODIPY-SM reflected endogenous SM compartmentation. Consistent with domain boundaries, fluorescence recovery after photobleaching revealed restriction of BODIPY-SM lateral diffusion over long-range, but not short-range, and various perturbations affected its mobile fraction as predicted from effects on fluorescent domains viewed by confocal microscopy. Taken together, these data reveal that BODIPY-SM spontaneously clusters at the outer leaflet of the plasmalemma of living cells into ordered micrometric domains, defined in size by membrane tension and cholesterol, which may reveal similar domains of endogenous SM.

EPITHELIAL DIFFERENTIATION

A.-C. Hick, P.J. Courtoy, C.E. Pierreux, in collaboration with F.P. Lemaigre (HORM)

Several organs, such as the exocrine pancreas and salivary glands, are made of polarized epithelial monolayers with a branched tubular organization. In contrast to the iterative bifid division of the bronchial tree, their tubular organization requires the integration of three developmental processes : (i) formation of blunt apical cavities within a single compact

multicellular mass by epithelial polarization; (ii) confluence of these cavities to create tubules; combined with (iii) branching expansion of tubules and glands (see Fig. 5). Once fully differentiated, epithelial monolayers possess three distinct membrane domains, each able to transmit signals to the nucleus. First, the basal domain ensures adhesion to the extracellular matrix. Next, the lateral domain allows direct interactions with adjacent cells via junctional complexes. Finally, the free apical domain is

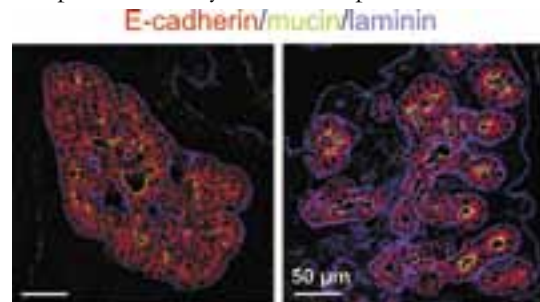


Fig. 5. Branching morphogenesis in exocrine glands. Reorganization in the early pancreatic bud of the multicellular mass of epithelial cells labelled for E-cadherin (left) into polarized monolayers with distinct apical domains (mucin) and their merging to create tubules (right). Laminin delineates basement membranes.

now facing a lumen and bears a mechano-sensory cilium.

Epithelial monolayers formation requires a coordinate and dynamic interaction with their environment, composed of mesenchymal and endothelial cells. This process is gland-autonomous, as it is faithfully reproduced in explant cultures. We have shown that embryonic salivary and pancreatic epithelial cells highly express receptors for SDF-1 (Stromal cell-Derived Factor-1), which was highly expressed by mesenchymal cells immediately adjacent to the epithelial buds. Pharmacological inhibition of SDF-1/receptor interaction in explants of the pancreas or salivary glands fully abrogated the reorganization of the epithelial masses into monolayers. These data demonstrate that paracrine epithelio-mesenchyme interaction is crucial for branched glandular morphogenesis

and bring a proof-of-principle that molecular components of this interaction can be readily dissected in explant cultures (8).

COLLABORATIONS ON MEMBRANE TRAFFICKING AND BRIEF REPORT ON THE CELL AND TISSUE IMAGING PLATFORM

P. Van Der Smissen, D. Tyteca, T. Lac, P.J. Courtoy

We have pursued our long-term commitment to promote collaborations by sharing expertise in endocytosis and cellular imaging. For recent years, we may cite contribution to the study of endocytic trafficking of syndecans and its role in signalling by its heparin-sulfate ligands (Zimmermann et al., *Dev Cell* 2005;9:377-88); of biogenesis of vacuolar H⁺-ATPase and the role of CFTR in kidney (Jouret et al., *J Am Soc Nephrol* 2005;16:3235-46); subcellular trafficking of the thrombopoietin receptor (Royer et al., *J Biol Chem* 2005;280:27251-61; see report by S. Constantinescu) and the amyloid precursor protein, APP (Feyt et al., *J Biol Chem* 2005;280:33220-7); elucidation of the disputed subcellular localization of aspartate N-acetyltransferase (NAT8L) and its congener, NAT8 (Wiame et al., *Biochem J* 2010;425:127-36; Veiga-da-Cunha et al., *J Biol Chem* "in press"; see report by E. Van Schaftingen); first evidence for dispersion of the actin cytoskeleton in epithelial cells by AMP-activated kinase (Horman et al., *Biochem Biophys. Res. Commun* "in press" see report by M. Rider); ultrastructural analysis of differentiating hepatoblasts (Clotman et al., *Genes Dev* 2005;19:1849-54; see report by F. Lemaigre) and the biogenesis of glycosomes in *Trypanosoma brucei* (Galland et al., *Biochim. Biophys Acta Mol Cell Res* 2007;1773:521-35; see report by P. Michels), or the morphological evidence by FRET of the interaction between key players of CTL that is interrupted during their anergy in cancer (Demotte et al., *Immunity* 2008;28:414-24; see report by P. van der Bruggen).

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Pierre Courtoy
 de Duve Institute
 Av. Hippocrate 74-75
 B - 1200 Brussels

[T] +32 02 764 75 69
 [F] +32 02 764 75 43
 [E] pierre.courtoy@uclouvain.be
 [W] http://www.deduveinstitute.be/endocytosis_epithelial.php