ARNO3, A HUMAN Sec7-DOMAIN GUANINE NUCLEOTIDE EXCHANGE FACTOR SPECIFIC FOR ARF1, CONTROLS MEMBRANE STRUCTURE OF THE GOLGI COMPLEX

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Budding of transport vesicles in the Golgi apparatus requires the recruitment of coat proteins onto Golgi membranes. This process is regulated by the ADP ribosylation factor 1 (ARF1) and requires guanine exchange factors (GEFs) which promote the transition to the active GTP-bound form of ARF1. We have recently identified a human protein, ARNO (ARF Nucleotide Opener) which shares a conserved domain with the yeast Sec7 protein, as a GEF acting on ARF1. Yet, the function of ARNO-like GEFs in the regulation of membrane dynamic in the secretory pathway had not been analyzed. We describe a novel human Sec7 domain-containing GEF referred to as ARNO3. ARNO and ARNO3, as well as a third GEF called cytohesin-1, form a family of highly related proteins with identical structural organization that consists of a central Sec7 domain and a carboxy terminal pleckstrin homology domain. We show that all three proteins act as GEF specific for ARF1 *in vitro*, while they are ineffective onto ARF6, an ARF protein implicated in the early endocytic pathway.

To investigate its role *in vivo*, we have expressed ARNO3 in baby hamster kidney cells. Overexpression of ARNO3 results in the fragmentation of the Golgi apparatus and causes a redistribution of Golgi resident proteins (p23, giantin, mannosidase II) as well as of the coat component β-COP, while the localization of the plasma membrane AP-2 adaptor complexes is not affected. This study provides the first indication that Sec7 domain containing GEFs control intracellular membrane compartment structure through the regulation of specific ARF proteins in mammalian cells.

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL SEC7 DOMAIN CONTAINING EXCHANGE FACTOR SPECIFIC FOR ARF6

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ADP-ribosylation factors (ARFs) are ubiquitous, small GTP-binding proteins that play a regulatory role in secretory and endocytic pathways of eukaryotic cells. ARF1 triggers the recruitment of cytoplasmic coat proteins that are instrumental for vesicle budding at the Golgi membrane. Activation of ARF1 requires its conversion from an inactive GDP to an active GTP-bound conformation, a step promoted by a guanine nucleotide-exchange factor (GEF). Recently, a genetic approach in *S. cerevisiae* has led to the identification of two related GEFs for yeast ARF1 which contain a 200 aa long domain showing sequence similarity with a region of the yeast Sec7 protein (Peyroche *et al.* (1996), *Nature*, **384**, 479-481). In mammals, three closely related proteins called ARNO, Cytohesin-1 and ARNO3 contain also a Sec7 domain and catalyze GDP/GTP exchange specifically on ARF1 (Chardin *et al.* (1996), *Nature*, **384**, 481-484; Franco *et al*, submitted).

We are interested in characterizing the function of another member of the ARF subgroup, ARF6, in the early endocytic pathway. The identification of an ARF6-specific GEF would definitively be an essential step toward this aim. By screening for new Sec7-domain containing proteins, we have identified and cloned a cDNA encoding a new ARF-GEF, EFA6. EFA6 expression is restricted to the brain. Like ARNO-like GEFs, EFA6 contains a Sec7 domain and a pleckstrin homology (PH) domain which might target EFA6 to the membrane. But in contrast to ARNO related proteins, EFA6 promotes GDP/GTP exchange on ARF6 while it is inactive on ARF1 or ARL3, a more distantly related ARF-like protein.

Overexpression studies in BHK-21 and HeLa cells show that EFA6 is associated with the cytoplasmic face of the plasma membrane. Overexpressed EFA6 induces a dramatic reorganization of cortical actin cytoskeleton and we are currently analyzing the involvement of the different domains of EFA6 on cytoskeletal rearrangements. In addition, we are investigating whether EFA6 plays a role in regulating ARF6 function in endocytosis.

ENDOCYTOSIS AND MEMBRANE HOMEOSTASIS; A FLUORESCENCE STUDY ON L929 MOUSE FIBROBLASTS

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This work is based on a quantitative kinetic analysis of membrane endocytosis. Membrane recycling from endocytosis is considered now to mainly arise from early compartments (see for instance Mayor S., Presley J.F., Maxfield F.R. (1993) *J. Cell Biol.*, **121**, 1257-1269). Recycling from late compartments is much less documented. Our previous results (Illinger D., Kuhry J.G. (1994) *J. Cell Biol.*, **125**, 763-794) indicated that, actually, a part of the internalized membranes remained *sequestred* in late compartments. This point has now been confirmed and we showed that the non-recycled membrane fraction was proportional to the endocytosis duration (12-14 % of the plasma membrane area per hour of endocytosis). This effect was found to be devoid of any concomitant change in the cell surface area. There must, nevertheless, be a corresponding default in membrane and this default has to be balanced in some way. We showed that the complementary membrane supply quantitatively came from the secretory pathway. Brefeldin A allowed to modify this equilibrium in almost totally reducing the latter contribution. Still, homeostasis was maintained by a simultaneous decrease in endocytosis and an increase in recycling.

The experiments were performed with the fluorescent membrane probe TMA-DPH (1-(4-trimethlammonium)-6-phenyl-1,3,5-hexatriene). This molecule is characterized by an instantaneous water-membrane partition equilibrium. It is fluorescent only in membranes. In interaction with intact cells, it remains localized in the plasma membrane, being only internalized by endocytosis (review in Illinger D., Duportail G., Mély Y., Poirel N., Gérard D., Kuhry J.G. (1995) *Biochim. Biophys. Acta*, **1239**, 58-66). Thanks to these properties, and according to the conditions of its use, it is possible with TMA-DPH to monitor quantitatively the kinetics of the various aspects of the intracellular membrane traffic, and also the evolution of the cell surface area. The mouse L929 cell line is currently used in the laboratory for its easiness to culture in a reproducible way, and its ability to built homogeneous monolayers, which was useful in this study.

SETTLEMENT OF MEMBRANE POLARITY AND VESICULAR TRANSPORT OF PROLACTIN IN MAMMARY EPITHELIAL CELLS

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Milk prolactin, which regulates differentiation and maturation of neuroendocrine, reproductive and immune systems of infant, is transported from maternal serum to the lumen of mammary acini using vesicular compartments characterized by electron microscopy (Ollivier-Bousquet M. *et al* (1997) J. lipid Res., **38**, 913-925).

With the aim to precise the membrane signals controlling the polarized transport of prolactin in mammary epithelial cells (MEC), we took in hand the characterization of membrane compartments involved in the hormone transport upon the settlement of cell polarization.

We first bring evidence that upon differentiation, MEC gains membrane characteristics of a polarized cell. This stems from results obtained by immunocytochemistry which indicates that in pregnant rabbits, annexin 4 signals the contour of MEC. In lactating rabbits, the fluorescent labelling is restricted to the basolateral domain of MEC plasma membrane.

Then, we show that prolactin transcytosis differs upon polarization of MEC plama membrane. This issues from experiments conducted on mammary explants incubated 30 minutes at 15°C with prolactin tagged with I^{125} or rhodamine and then chased 15 minutes at 37°C. Radioactivity and fluorescence intensity are measured in membrane compartments obtained by sedimentation on sucrose gradients. At 15°C, 50% of internalized prolactin is recovered in low-density compartments of non-polarized MEC. This percentage only slightly changes at 37°C. By contrast, 30% of the hormone is detected at 15°C in low-density compartments of polarized MEC. During the chase, this percentage drops to 20%. At the same time, 60% of the hormone is present in high-density fractions.

The characterization of membrane sorting signals that could be involved in this prolactin trafficking is conducted by analyzing Triton X100-insoluble membrane microdomains prepared from the different fractions of the gradients. Preliminary results indicate that in polarized MEC, annexin 6 (reported as a potent sorting signal of vesicular traffic), is largely recruited into membrane microdomains of high-density compartments. This is not observed in non-polarized MEC.