IDENTIFICATION OF CFTR AND SNARE PROTEINS IN SHARK RECTAL GLAND

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A unique feature of the cystic fibrosis transmembrane conductance regulator (CFTR) is that its heterologous expression can confer cAMP-regulated membrane trafficking events (exocytosis and endocytosis) in cells where cAMP-dependent trafficking processes did not previously exist. Effects on CFTR activity of co-expressing individual components of the SNARE membrane fusion machinery indicate that SNARE proteins are involved in CFTR-dependent membrane trafficking (Takahashi, A., S. C. Watkins, M. Howard, R. A. Frizzell (1996) Am. J. Physiol. 271: C1887; Peters, K. W., J.-J. Qi, S. C. Watkins, and R. A. Frizzell (1999) Am. J. Physiol. 277: C174). Recent results suggest hat physical interactions between CFTR and components of the SNARE machinery may provide a mechanism by which phosphorylation of CFTR's R domain initiates insertion of membrane vesicles containing CFTR with the plasma membrane (Peters, K. W., J. Qi, S. C. Watkins, and R. A. Frizzell (2000) Med. Clinics of N. A. 84: 633). The CFTR N-terminus has been found to interact with the target membrane SNAREs, syntaxin 1A and SNAP-23/25, and the CFTR R domain with the vesicle associated SNARE, VAMP-2 (Naren, A. P., Quick, J. F. Collawn, D. J. Nelson, K. L. Kirk (1998) Proc Natl. Acad. Science USA. 95: 10972; Peters, K. W., J. Qi. R. J. Dudley, R. S. Edinger, J. P. Johnson, and R. A. Frizzell (2000) Pediatric Pulmonology #2).

Mammalian epithelia in which CFTR is normally expressed are complex tissues comprised of multiple cell types. For example, the mammalian proximal airway is composed of at least 11 distinct cell types (Ayers, M. M. and P. K. Jeffery (1988) Eur. Respir. J. 1: 58) and significant levels of CFTR expression are observed in only two of these, surface columnar epithelial cells and submucosal gland serous cells. These properties render mammalian tissues insufficient for biochemical studies designed to identify subcellular CFTR containing compartments. Yet, characterization of the protein complement of the regulated compartment containing CFTR in the basal secretory state is an important goal of our efforts to determine the mechanism of regulated CFTR trafficking. For this purpose, the shark (*Squalus acanthias*) rectal gland (SRG) presents potential advantages. It is well known, for example, that the epithelium of secretory tubules of the gland is comprised of a single cell type that functions in the secretion of approximately half molar NaCl. Accordingly, there are not cells with absorptive properties that modify the secretory product as is the rule in exocrine glands, nor are there cells secreting macromolecular products (mucins or defense molecules). These additional cell types would dilute efforts to identify this compartment biochemically. The goals of the initial stage of this project were two-fold. First, to determine whether antibodies used previously to identify SNARE components in mammalian cells would be useful for this purpose in the shark. Second, to determine whether the SRG was a candidate for subcellular fractionation that would permit identification of a regulated compartment of the secretory pathway that contains CFTR, as we would like to identify its other constituents.

For the first goal, we performed immunoblot analyses using relatively crude SRG cell homogenates. Glands were opened lengthwise with a blade and tubule clumps were scraped from the capsule. After homogenization, nuclei and large membrane fragments were pelleted at ~700g and the supernatant obtained by spinning at ~160,000g was concentrated for further studies. This membrane fraction was separated by SDS-PAGE and the proteins transferred to nitrocellulose for immunoblotting using commercially available antibodies that successfully detected mammalian syntaxin 1A, SNAP-23 and VAMP-2 in positive control experiments. We detected VAMP-2 at the predicted molecular weight in SRG membranes, but the detection of syntaxin 1A and SNAP-23 was sporadic. A representative blot for VAMP-2 is illustrated in Figure 1. Presumably, the epitopes recognized by these antibodies are not sufficiently conserved in the shark proteins. In separate studies, we are attempting to clone the core proteins of the SNARE fusion complex in the shark so that shared epitopes can be identified or shark-specific antibodies generated.

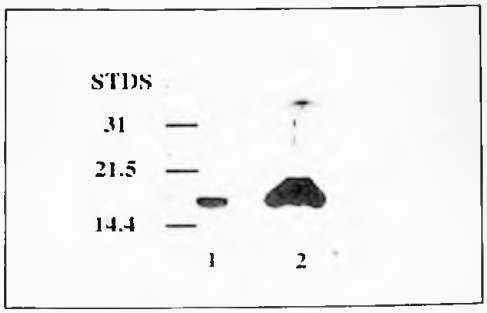

Figure 1. Immunoblot of VAMP-2
A membrane fraction (Lane 1) of the shark rectal gland was resolved by SDS-PAGE and transferred to PVDF membranes. Lane 2 is a positive control.

To identify a compartment containing significant levels of CFTR under basal conditions, differential centrifugation of SRG membranes was attempted (Dubinsky, W. P. and L. B. Monti (1986) Am. J. Physiol. 251: C721). The 33,000g pellet from the 13,000g supernatant was employed for separation utilizing 15% OptiPrep™. A self forming gradient was generated using a VTi65.2 vertical rotor for three hours and 22 fractions were collected. Protein in these fractions was separated by SDS-PAGE and blotted for CFTR using a polyclonal C-terminus CFTR antibody. It is of interest that the CFTR containing fractions were grouped into two regions, and these differed with respect to their content of processed CFTRs (Figure 2).

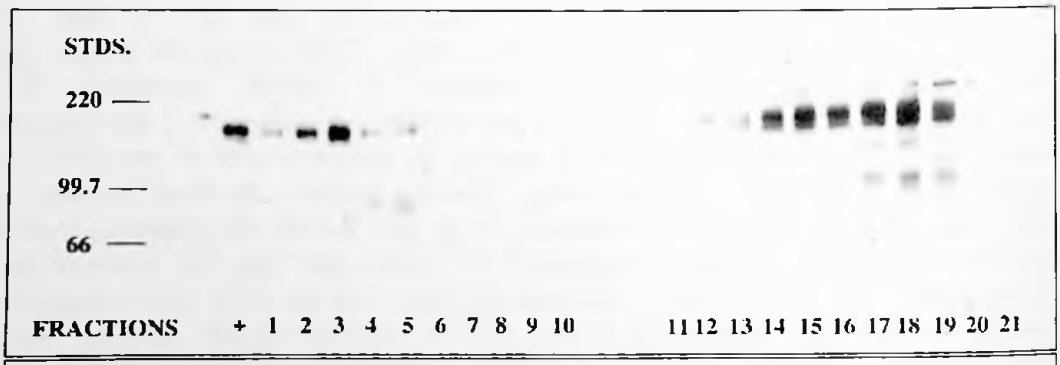

Figure 2. Gradient Fractions of CFTR from shark rectal gland
Glands were homogenized and subjected to differential centrifugation. The final pellet was mixed in 15% OptiPrep™ and fractionated in a vertical rotor. Two hundred ul fractions were collected from the top of the tube, resolved by SDS-PAGE and immunoblotted.

The heavier CFTR containing fractions exhibited the presence of the core glycosylated B band and the mature, fully glycosylated C band. The lighter fractions containing CFTR exhibited the presence of a single CFTR protein band, possibly the mature C band of CFTR. If so, these fractions would consist of post-Golgi membrane compartments that contain only mature CFTR protein. We anticipate that it may be possible to observe a shift in the density of these fractions when they are isolated from cAMP-stimulated rectal glands, which would suggest that at least a component of this CFTR is in a regulated secretory compartment. Similar results have been obtained for insulin stimulated GLUT-4 compartments in adipocytes (Hashiramoto, M. and D. E. James (2000) Mol. Cell. Biol., 20: 416), and this possibility will be an object of future studies.

The present results indicate that we have reasonable reagents for detecting shark VAMP-2 and perhaps other SNARE components in SRG membrane fractions. In future studies, we will determine whether we can detect the subcellular localization of VAMP-2, SNAP-23, and syntaxin 1A by immunofluorescence in SRG sections. In addition, we can separate the intracellular membranes of SRG and obtain fractions of CFTR in pre- and post-Golgi compartments. It will be interesting to determine whether VAMP-2 is a component of the light membrane fraction that contains mature CFTR.

These studies were supported by NIH DK56490.