IDENTIFICATION OF SNARE PROTEINS IN THE RECTAL GLAND OF *Squalus acanthias*

Kathryn W. Peters[1], Simon C. Watkins[1], John N. Forrest, Jr[2]. Christopher R. Marino[3], and Raymond A. Frizzell[1]

[1]Department of Cell Biology and Physiology,
University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261

[2]Department of Medicine,
Yale University, School of Medicine, New Haven, Connecticut 06510

[3]Department of Medicine,
University of Tennessee, Memphis, Tennessee 38163

The intracellular localization of CFTR under basal conditions and its regulated movement into the apical plasma membrane upon stimulation have been the subject of numerous investigations in a variety of cell types and tissues. Some systems may yield opposing conclusions because they lack appropriate traffic regulatory proteins; alternately, CFTR over-expression in heterologous systems can lead to saturation of trafficking pathways and deposition of CFTR in the plasma membrane (Howard, M., *et al.*, *Am. J. Physiol.* **279**: C375, 2000). The shark rectal gland (SRG) is a good system for trafficking studies because copious amounts of endogenous CFTR are expressed in a single cell type. Concentration of CFTR at the apical pole with stimulation has been observed in perfused SRG (Lehrich, R. W., *et al.*, *J. Clin. Invest.* **101**: 737, 1998). Therefore, we asked whether this trafficking process could be mediated by interactions among the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) family of proteins. These proteins function in intracellular membrane fusion events (Rothman, J.E. *Nature* **372**: 58, 1994) and aid in the establishment of cell polarity through differential their expression or localization (Li, X., *et al.*, *Am. J. Physiol.*, **283**: F1111, 2002).

Rectal glands were removed, perfused under basal or stimulated (1 μM forskolin) conditions, homogenized and centrifuged as described (Dubinsky, W.P. and L.B. Monti. *Am. J. Physiol.*, 251: C721, 1996). We utilized the 33,000 x g pellet to obtain a plasma membrane vesicle fraction, then performed immunoblots (IBs) or immunoprecipitations (IPs), to monitor the presence of proteins known to be involved in trafficking in other epithelial systems. To observe expression of proteins *in situ* by immunofluorescence (IF), glands were perfusion-fixed in 300 mM Na HEPES, 3 % sucrose, and 4 % paraformaldehyde, flash frozen in liquid nitrogen cooled isopentane, then sectioned (5 μm), and incubated with specific antibodies followed by fluorescein-conjugated secondary antibodies.

We observed CFTR using polyclonal antibodies raised against amino acids 1464-1476 of the rat C-terminal sequence (French, P. J. et al., *J. Clin. Invest.* **98**: 1304, 1996). The rat sequence, is considerably homologous to that of the shark, and the

rat	K E E T E E E V Q E T R L
shark	Q E E A E E D L Q E T R L

antibodies proved to be useful tools in recognition of CFTR by both IB and IP in SRG (Figure 1).

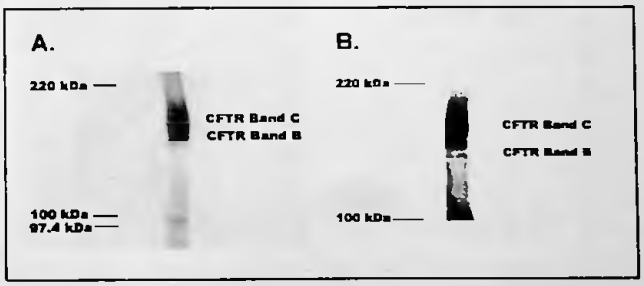

Figure 1. ***Observation of CFTR in SRG.***

The 33.000 x g SRG pellet was utilized for (A) immunoblot or (B) immunoprecipitation. After electrophoresis and transfer, proteins were subjected to chemiluminescent labeling with R3195 anti-CFTR polyclonal antibodies.

Numerous isoforms of the t-SNARE, syntaxin, have been identified and are unique to specific cellular compartments. Those syntaxins, 1 through 4, residing in the plasma membrane, appear to participate in epithelial secretion and the maintenance of epithelial polarity. We observed syntaxins 1 and 3 by IB, IP, and IF (Figure 2) routinely. Syntaxins 1 and 3 are apically polarized. Expression of syntaxin 2 was weak and not routinely observed; syntaxin 4 was not detected.

We observed a protein of approximately 17 kDa using a commercially available IgM antibody (Babco) known to react with recombinant VAMP-1 or VAMP-2. As assessed by IB and IF, this protein is expressed abundantly in SRG (Figure 3); it is most likely VAMP-2, but this result requires confirmation.

Studies in our laboratory have examined cysteine string protein (Csp), a membrane associated protein that we have shown interacts with CFTR and

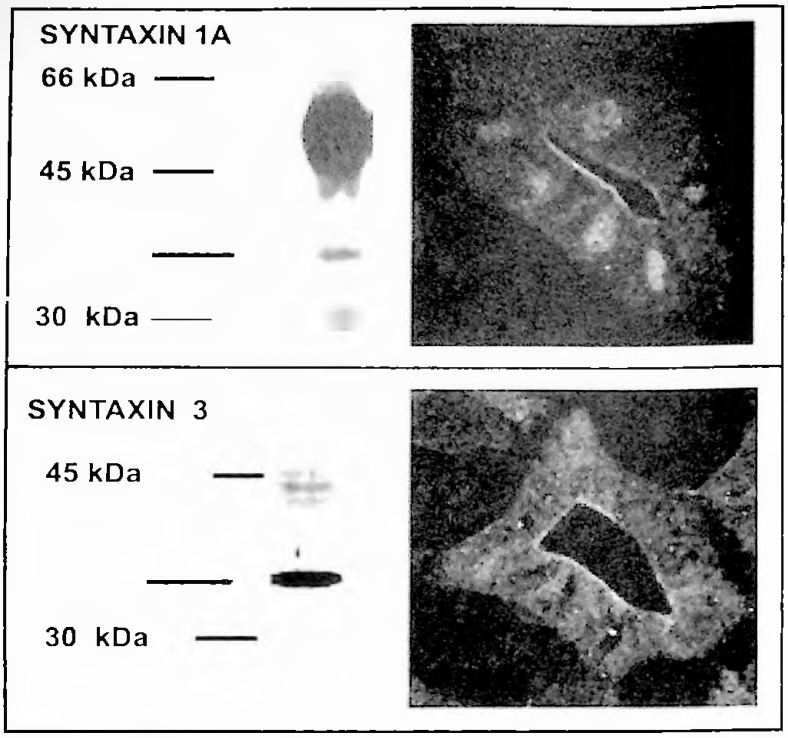

Figure 2. *Syntaxins 1A and 3 are expressed in SRG.*

Syntaxin 1A: Top Left: A 35 kDa band corresponding to S1A was immunoprecipitated by polyclonal antibodies. Top Right: The SRG was stimulated with forskolin, fixed with paraformaldehyde, flash frozen, and sectioned. Apical staining is evident.
Syntaxin 3: Bottom Left: IB of Syntaxin 3. Bottom Right: IF

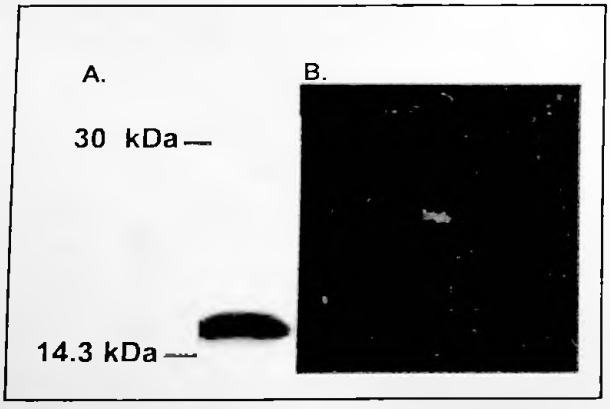

Figure 3. *Vamp 1/2.*

Fifty ug of the 33,000 x g SRG pellet was subjected to IB with an antibody against VAMP-1/2. A prominent 17 kDa band is observed. Immunofluorescent staining is punctate and located apically and basally.

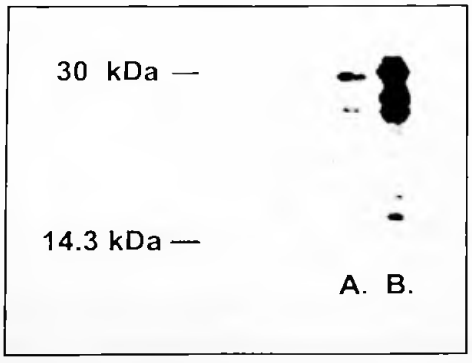

Figure 4. *Csp in SRG.*

After IP, the immune complex was resolved by SDS-PAGE and visualized by chemiluminescence.
A. SRG
B. Transfected HEK cells

results in decreased levels of mature (band C) CFTR when it is overexpressed (Zhang, H. *J. Biol. Chem.*, **277**: 28048, 2002). We detected Csp by immunoblot in SRG only after its immunoprecipitation (Figure 4). This result is probably due to low levels of endogenous Csp expression as the antibody recognizes amphibian Csp also.

To identify physical interactions between proteins, we performed IPs with one antibody and immunoblotted with a second antibody to the protein in question. These data indicate that Csp interacts with CFTR and VAMP in the SRG (Figure 5). Additional experiments will focus on binding partners of syntaxins 1 and 3.

The abundant expression of specific SNARE proteins in the SRG suggests that they may play a role in regulated CFTR trafficking at the SRG apical membrane. Future studies will focus on whether such interactions are sequential and SNARE protein-specific.

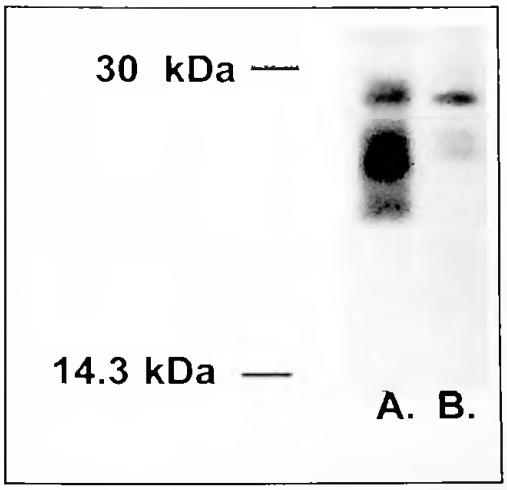

Figure 5. ***Interactions between Csp and CFTR or VAMP.***

One hundred ug of protein was utilized in IP with either CFTR antibodies (A) or VAMP antibodies (B). After proteins were resolved by SDS-PAGE, they were subjected to chemiluminescence using Csp antibodies.