FRACTIONATION OF SHARK RECTAL GLAND CELLS FOR IDENTIFICATION OF CFTR TRAFFICKING COMPARTMENTS

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The shark (*Squalus acanthias*) rectal gland (SRG) is composed of secretory tubules in which the cystic fibrosis transmembrane conductance regulator (CFTR) is highly expressed. Several studies suggest this model is useful for studying translocation of CFTR from its site of synthesis to its functional location in the plasma membrane. A membrane vesicle fraction, distinct from the basolateral membrane, was isolated using a sucrose gradient and differential centrifugation (Dubinsky and Monti, Am J. Physiol. 251: C721-C729, 1986). Antibody labeling studies have demonstrated that CFTR translocates and concentrates at the apical membrane in response to cAMP stimulation of chloride secretion (Lehrich *et al.*, J. Clin. Invest. 101: 737-745, 1998). We have observed SNARE proteins, e.g. vamp, snap, and syntaxin 1A in SRG (Peters *et al.*, MDIBL Bull. 40: 55-57, 2001) thus components of the membrane fusion machinery are present. Accordingly, SRG should allow us to evaluate our hypothesis that CFTR and VAMP-2 are present in cytoplasmic vesicles under basal conditions and that upon stimulation, CFTR is depleted from this compartment.

Rectal glands were removed and perfused under basal or stimulated (10 μM forskolin) conditions (Lehrich *et al.*, 1998). Tissues were homogenized and subjected to a series of differential centrifugations (Dubinsky and Monti, 1986). The 33,000g pellet was suspended in iodixanol (OptiPrep™) to a 15% final concentration, then centrifuged in a vertical rotor (Vti65.2) for three hours at 50,000 rpms. Under these conditions a self forming gradient is generated which should allow separation of organelles and cytoplasmic vesicles. This method was chosen because the hyperosmotic concentration of sucrose is believed to coincide with the density of some intracellular mem-

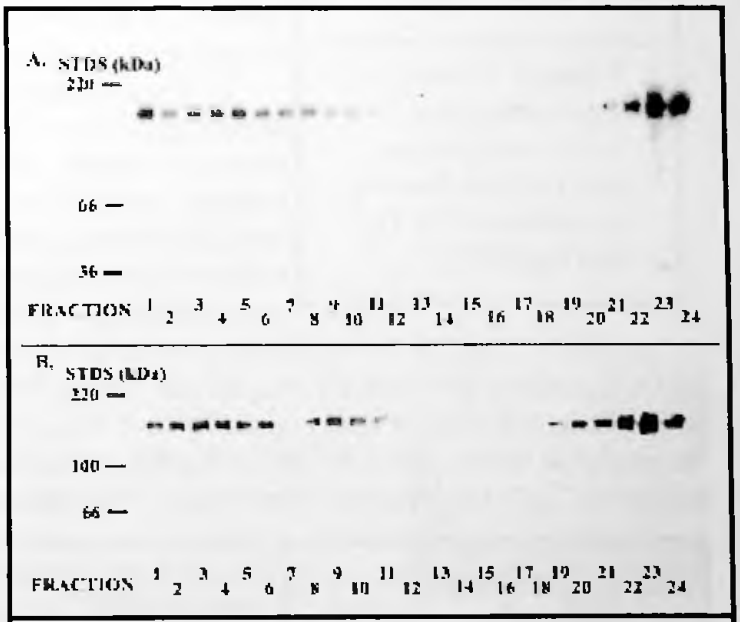

Figure 1. Shark rectal glands were perfused under basal (A) or stimulated (B) conditions and subjected to a series of differential centrifugations. The 33,000g pellet was suspended in a final concentration of 15% iodixanol and centrifuged in a vertical rotor to form self-generated gradients. After electrophoresis and transfer, proteins were subjected to chemiluminescence in which R-3195 anti-CFTR polyclonal antibodies were utilized.

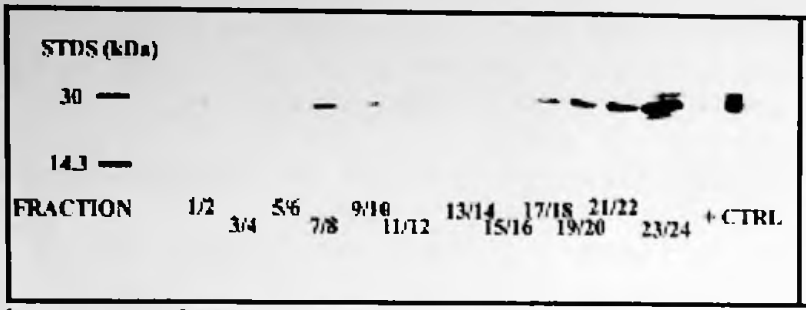

Figure 2. Samples were prepared as described for Figure 1B. Chemiluminescence was conducted using VAMP-2 monoclonal antibodies (Synaptic Systems).

branes, e.g. GLUT4-containing vesicles, making it more difficult to resolve distinct fractions (Hashiramoto and James, Mol. Cell. Biol. 20: 416-427, 2000). Twenty-four 200 µl fractions were collected from the top of the tube, proteins were resolved by SDS-PAGE, then immunoblotted using R-3195 antibodies generated against the C terminus of CFTR (French *et al.*, J. Clin. Invest. 98: 1304-1312, 1996). Immunoblot of gradient fractions from a basal gland (Figure 1A) reveals that the majority of CFTR is in the lighter fractions (1 and 3-5) and in the heaviest fractions (22-24). In contrast, in the stimulated gland, CFTR is observed in fractions of a medium density (8-11). Confirmation of cellular compartments associated with these fractions awaits immunoblots with organelle specific markers. Interestingly, VAMP-2, a vesicle associated protein, that we have shown previously to co-immunoprecipitate with CFTR as well as cause a potentiation of Cl^- currents when co-expressed with CFTR in oocytes (Peters *et al.*, Amer. J. Physiol. 277: C174-C180, 1999), is present in these fractions (Figure 2).

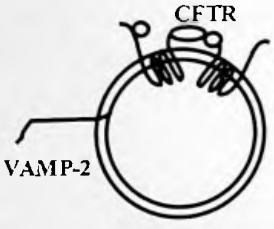

Figure 3. Schematic representation of inside out cytoplasmic vesicles showing orientation of CFTR and VAMP-2.

The co-sedimentation of CFTR and VAMP-2 suggests that it may represent a vesicle population of CFTR en route to the plasma membrane. If this hypothesis is correct, CFTR would be oriented (Figure 3) such that cytoplasmic sites would be accessible to antibodies for immunoprecipitation of these vesicles in buffers without detergents. We began to answer this question using immunoisolations. The starting material for our initial studies was the 33,000g pellet which was suspended in either HES (20mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4; Hashiramoto and James, 2000) or HE buffer (100 mM mannitol, 5 mM K^+-HEPES, pH 7.6; Dubinsky and Monti, 1986). Approximately 300 µg of the sample was suspended in buffer, commercially available monoclonal antibodies generated against the C terminus of CFTR (Chemicon), were added, and samples were rotated for several hours at 4 °C.

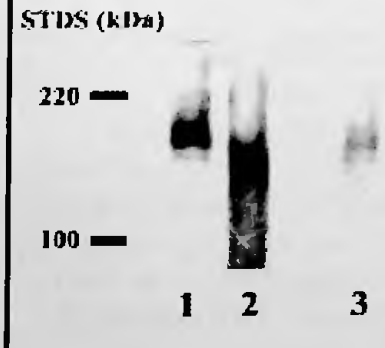

Figure 4. The 33,000g pellet from a non-perfused gland was utilized in immunoisolations. Monoclonal antibodies raised against the C terminus of CFTR (Chemicon) were used in conjunction wtih Protein A to precipitate the immune complex. Proteins were observed through chemiluminescence using 3195 antibodies for the immunoblot. Lane 1--positive control; Lane 2--immunoisolation using HEPES/mannitol buffer; Lane 3--immunoisolation in HEPES/EDTA/sucrose buffer.

These IgG_2 antibodies have a high affinity for Protein A; therefore, 50 µl Dynabeads® Protein A was added so that immune complexes could be precipitated. After several washes, proteins were resolved by SDS-PAGE and immunoblotted with R-3195 polyclonal antibodies. As seen in Figure 4, either band B (lane 2) or band C (lane 3) appears to be resolved with HM or HES buffer, respectively. Similar results were obtained when R-3195 polyclonal antibodies were used for both the immunoprecipitation and immunoblot (data not shown). The yield in this procedure is noticeably low and future experiments will include optimization of sample and antibodies.

These results represent the beginning of a project aimed at identification of proteins in CFTR trafficking compartments. Future studies will include marker analyses of the 24 fractions to identify which organellar or subcellular fractions are represented. The integrity of vesicles will be confirmed through electron microscopy. Immunoisolations will be performed on fractions containing CFTR and VAMP-2. We will attempt to identify other proteins in these vesicles through silver staining and protein sequencing.

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