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DISCOVERIES 2014, Jul-Sep, 2(3): e24
DOI: 10.15190/d.2014.16

*REVIEW Article*

# Porosome in Cystic Fibrosis

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*Submitted:* June 09, 2014; *Revised:* Sept. 04, 2014; *Accepted:* Sept. 05, 2014; *Published:* Sept. 28, 2014;
*Citation*: Jena B. Porosome in Cystic Fibrosis. Discoveries 2014, Jul-Sep; 2(3): e24. DOI: 10.15190/d.2014.16

## ABSTRACT

Macromolecular structures embedded in the cell plasma membrane called 'porosomes', are involved in the regulated fractional release of intravesicular contents from cells during secretion. Porosomes range in size from 15 nm in neurons and astrocytes to 100-180 nm in the exocrine pancreas and neuroendocrine cells. Porosomes have been isolated from a number of cells, and their morphology, composition, and functional reconstitution well documented. The 3D contour map of the assembly of proteins within the porosome complex, and its native X-ray solution structure at sub-nm resolution has also advanced. This understanding now provides a platform to address diseases that may result from secretory defects. Water and ion binding to mucin impart hydration, critical for regulating viscosity of the mucus in the airways epithelia. Appropriate viscosity is required for the movement of mucus by the underlying cilia. Hence secretion of more viscous mucus prevents its proper transport, resulting in chronic and fatal airways disease such as cystic fibrosis (CF). CF is caused by the malfunction of CF transmembrane conductance regulator (CFTR), a chloride channel transporter, resulting in viscous mucus in the airways. Studies in mice lacking functional CFTR secrete highly viscous mucous that adhered to the epithelium. Since CFTR is known to interact with the t-SNARE protein syntaxin-1A, and with the chloride channel CLC-3, which are also components of the porosome complex, the interactions between CFTR and the porosome complex in the mucin-secreting human airway epithelial cell line Calu-3 was hypothesized and tested. Results from the study demonstrate the presence of approximately 100 nm in size porosome complex composed of 34 proteins at the cell plasma membrane in Calu-3 cells, and the association of CFTR with the complex. In comparison, the nuclear pore complex measures 120 nm and is comprised of over 500 protein molecules. The involvement of CFTR in porosome-mediated mucin secretion is hypothesized, and is currently being tested.

**Keywords**: Porosome Complex, CFTR, mucin secretion

## SUMMARY



## 1. Introduction

It is well established that cup-shaped macromolecular lipoprotein structures called *porosomes* are secretory portals embedded in the cell plasma membrane in cells, where membrane-bound secretory vesicles transiently dock and fuse to expel intravesicular contents during secretion[1-10]. Porosomes have been isolated from a number of cells, including the exocrine pancreas[5,6] (Figure 1), neurons[3] (Figure 2), and in the mucin-secreting human airway epithelial cell line Calu-3 (Figure

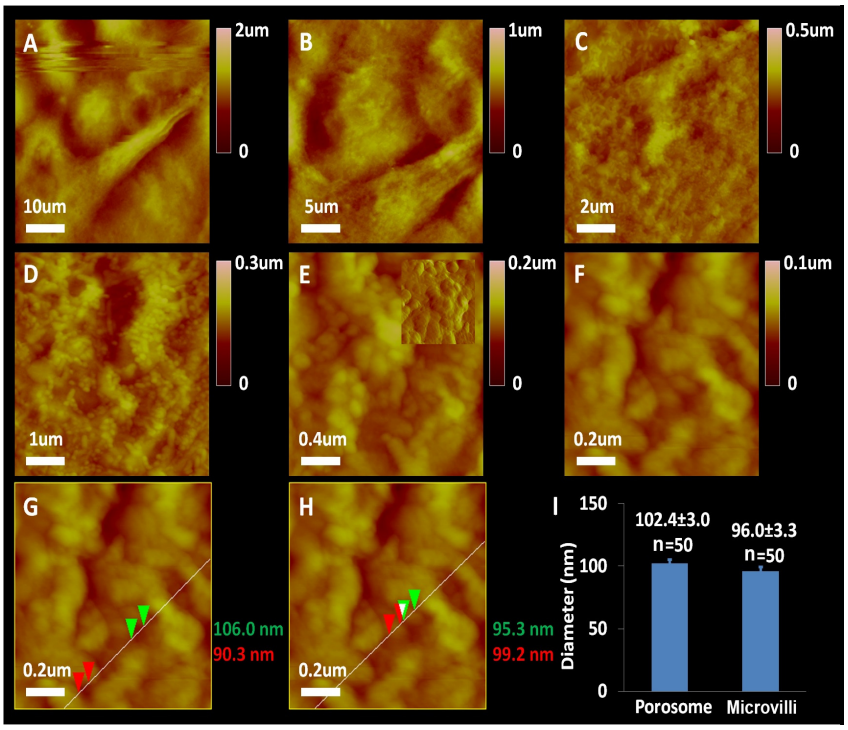


**Figure 1.** Atomic force microscopy (AFM) micrographs demonstrate the presence of microvilli and interspersed mucin-secreting porosomes at plasma membrane in Calu-3 cells[11]. Microvilli measuring on average 96 nm in thickness (mean ± SEM; 96 ± 3.3, n=50) are densely packed at the cell plasma membrane exposed to the medium, and is demonstrated both in low (A-C) and high (D-H) resolution AFM images. Interspersed among the microvilli are the 102 nm in diameter porosome openings (mean ± SEM; 102 ± 3, n=50) shown in figure G (red and green arrowheads). Similarly, the microvilli shown in figure H (red and green arrowheads) demonstrate some that appear coiled around each other, possibly as a consequence of secreted mucus[11]. ©Bhanu Jena.

3,4)[11]. The morphology, composition, and reconstitution of porosomes in the exocrine pancreas (Figure 5-7) and in neurons are well documented[2-12], and the 3D contour map of the assembly of proteins within the structure has also been determined in great detail[10]. This new understanding of the secretory machinery in cells now provides a platform to address diseases resulting from secretory defects. The structure, and composition of the porosome complex in Calu-3 cells expressing cystic fibrosis (CF) transmembrane conductance regulator (CFTR) has been determined for the first time[11], with promise to help better understand cystic fibrosis. CFTR is a plasma membrane chloride selective cyclic AMP-activated ion channel, localized at the apical membrane of secretory epithelial cells, including the conducting airways[13]. Besides mediating the secretion of $Cl^-$, CFTR also regulates several other transport proteins, including $K^+$ channels, aquaporin water channels, anion exchangers, the membrane fusion protein syntaxin-1A, and sodium bicarbonate transporters[14-26]. Accordingly, studies show that CFTR and its associated proteins are present in large macromolecular signaling complexes via scaffolding proteins containing PDZ domains[13,25,27]. The C-terminus of CFTR in humans contains the sequence Asp-Thr-Arg-Leu, that mediate binding to several PDZ domain proteins[13]. For example, ezrin and moesin present in the Calu-3 porosome complex[11] are also known CFTR-PDZ binding protein[13]. In addition, CFTR has several other regions that mediate protein-protein interactions, such as a domain at its N-terminus that binds to syntaxin-1A and SNAP-23[15,23]. CFTR also contains a protein phosphatase-2A (PP2A)-binding, and an AMP kinase (AMPK)-binding domain[12]. Similarly, CFTR has a regulatory domain that is a substrate to both protein kinase A (PKA) and C (PKC)[28]. These interactions facilitate CFTR to form large CFTR-associated macromolecular signaling complexes at the plasma membrane. CF as a disease was first identified as cysts observed in the pancreas and the highly viscous mucus found in the lung of patients. However, since discovery that these observed defects are a result of a dysfunction of the CFTR chloride channel[29.30], there has been little progress in our understanding of the link between CFTR dysfunction and the secretion of such highly viscous mucin in the lung of CF patients[31]. The surface of the airways is coated with a thin film of mucous composed of essentially mucin, salt, proteases, antioxidants, and antibodies[31,32]. Mucin lubricates, trap foreign particles and pathogens, and assists in the clearance of foreign particles from the airways via ciliary transport[31,32]. A key property of mucus is its appropriate viscosity that enables its movement by the underlying cilia. Secretion of

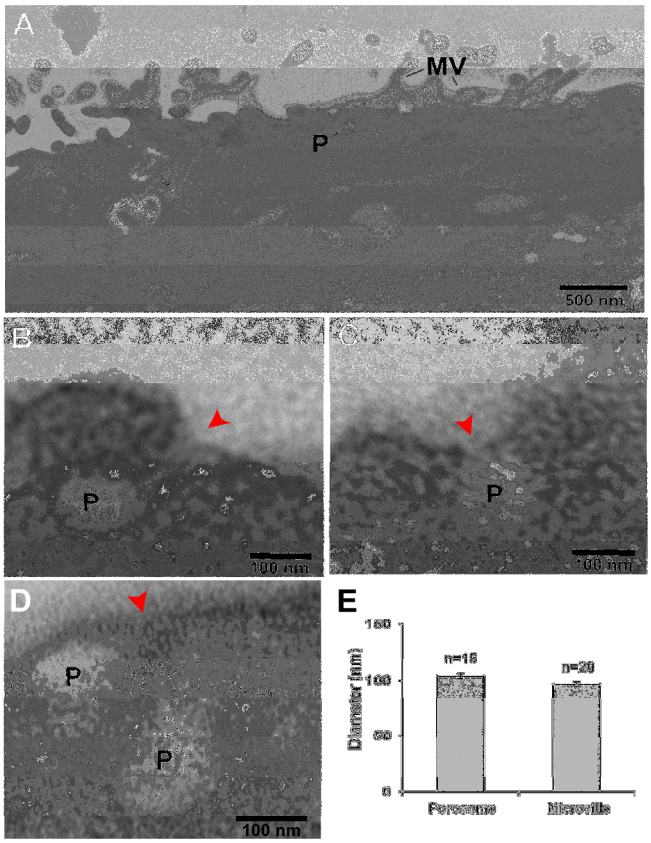


**Figure 2.** Representative electron micrographs of Calu-3 cells in culture demonstrating the presence of microvilli (MV) and porosomes (P) at the cell plasma membrane [11]. (A) Calu-3 cells demonstrate the presence of dense microvilli and scattered porosomes at the cell plasma membrane. (B-D) Note the flask-shaped porosomes measuring nearly 100 nm in diameter (E) and from 200-300 nm in depth, with openings to the cell surface (red arrowhead). Mucus (C), is found at the opening of the porosome to the cell exterior. Of the two porosomes shown in (D), the one to the center appears to be sectioned right through the center of the organelle, where as the porosome to the left, has been sectioned at its base. (E) Similar to the AFM images in Figure 1, the microvilli measure on average 92 nm in diameter [11]. ©Bhanu Jena.

more viscous mucus disallows its proper transport, resulting in chronic and fatal airways disease such as CF[32]. Similar to other secretory cells that undergo secretory vesicle volume increase during secretion[35-45], goblet cells of the airways epithelia that store mucin in a dehydrated state within membrane-bound secretory granules are no exception. Since vesicle swelling is a requirement for cell secretion[41], and both ion channels and water channels or aquaporins regulate this process[42,44], altered chloride transport would impair secretory vesicle hydration and optimal release. Furthermore, recent studies in mice lacking functional CFTR[31] showed that these animals secrete highly viscous mucous that adhered to the epithelium. Since CFTR is known to interact with syntaxin-1A, chloride channel CLC-3, and aquaporins[14-26], which are components of the porosome complex[1,2,5-7,46], the possible interactions between CFTR and the porosome in goblet cells was hypothesized and tested in a recent study[11]. Results from the study demonstrate the presence of approximately 100 nm in size porosomes and microvilli at the surface of the plasma membrane in Calu-3 cells (Figure 1,2)[11].

The t-SNARE SNAP-25 specific antibody conjugated to protein A-sepharose® has been utilized to isolate the porosome complex from Calu-3 cells[11]. For each immunoisolation, 1 mg of Triton-Lubrol-solubilized Calu-3 cells was used. The Triton/Lubrol solubilization buffer contained 0.5% Lubrol, 0.5% Triton X-100, 1 mM benzamidine, 5 mM Mg-ATP, and 5 mM EDTA in PBS at pH 7.5, supplemented with protease inhibitor mix (Sigma, St. Louis, MO). Ten micrograms of SNAP-25 antibody conjugated to the protein A-sepharose® were incubated with the 1 mg of the solubilized cells for 1 h at room temperature followed by three washes of 10 volumes of wash buffer (500 mM NaCl, 10 mM Tris, 2 mM EDTA, pH 7.5). The immunoprecipitated porosome attached to the immunosepharose beads was eluted using low pH buffer (pH 3.5) to dissociate the complex from the antibody bound to the bead, and the eluted sample immediately returned to neutral pH and stored at -80 degrees[11]. A combination of proteomics, Western blot analysis, and immunocytochemistry, were all used to determine the composition and distribution of the porosome complex in Calu-3

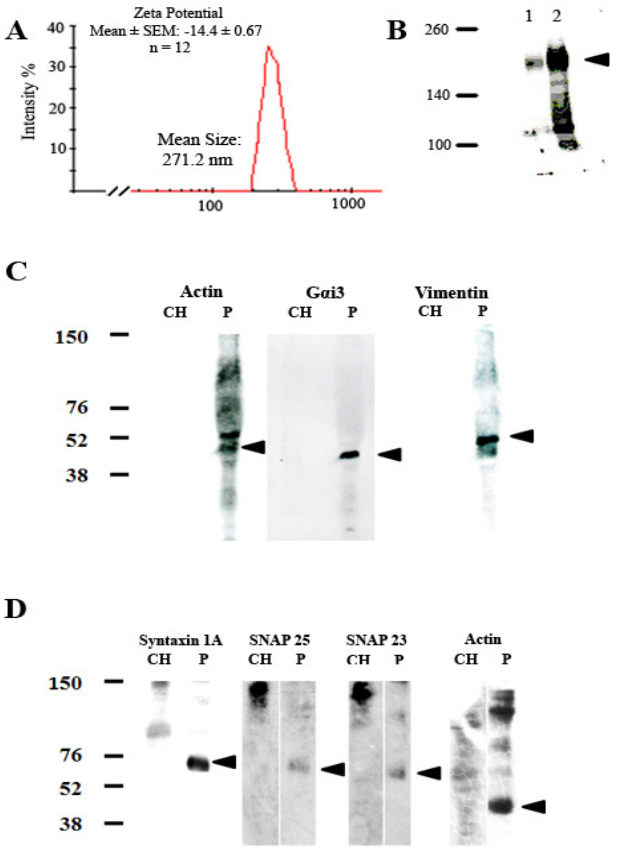


**Figure 3.** Photon correlation spectroscopy (PCS) demonstrate the immunoisolated porosome complex from Calu-3 cells to measure on average 271 nm (trimmers), and both immuno-precipitation and immunoblot analysis demonstrate the interaction of CFTR with the porosome complex in the cell[11]. (A) PCS on isolated porosomes from Calu-3 cells demonstrate an average size of 271.2 nm. (B) Immunoblot analysis using CFTR-specific antibody of CFTR-expressing HEK cell proteins resolved using SDS-PAGE, followed by electrotransfer to nitrocellulose membrane, demonstrates the presence of a 180 kD band representing CFTR (lane 1, positive control). SDS-PAGE resolved immunoisolated porosome complexes also demonstrate immunopositive for CFTR (lane 2). (C) Immunoblot analysis of the total Calu-3 cell homogenate (CH) and isolated porosome complex (P), demonstrate the presence of porosome proteins actin, Gαi3, and vimentin. Note the enriched presence of the proteins in the porosome complex. (D) Similarly, immunoisolated CFTR complex using the CFTR-specific antibody, results in the pull-down of porosome associated proteins such as Syntaxin-1A (present as 70 kDa t-/v-SNARE complex), SNAP-25 (present as 70 kDa t-/v-SNARE complex), SNAP-23, present as 68 kDa t-/v-SNARE complex, and actin[11]. ©Bhanu Jena.

cells[11]. Proteomic analysis of isolated Calu-3 porosomes using mass spectrometry demonstrate the presence of CFTR as well as several proteins found in the neuronal porosome complex, including Syntaxin-1A, actin, rabs, heterotrimeric G-protein, and the GTPase activating protein GAP (Table I)[11]. Immunoblot analysis (Figure 3) of the isolated Calu-3 porosome complex, and immunocytochemistry (Figure 4) further confirms CFTR association with the porosome complex, reflecting important implication of CFTR in both normal mucus secretion in the airway epithelium in health, and in the impaired state in CF disease. In the past two decades, employing a combination of approaches including AFM, biochemistry, molecular biology, electrophysiology, EM, mass spectrometry, SAXS analysis, and database searches such as STRING 9.1 of known functional and predicted protein-protein interactions, further structural details of the porosome complex have been determined[1-12]. Although great progress have been made in our understanding of the porosome, of $Ca^{+2}$ and SNARE-mediated membrane fusion[47-76], and on secretory vesicle volume regulation required for regulated fractional release of intravesicular contents[35-45] during cell secretion, a molecular level understanding of porosome-mediated secretion in mucin-secreting cells remains to be determined. Therefore, a clear understanding of the porosome in mucin secreting in Calu-3 cells, and the role of CFTR in porosome-mediated mucin secretion is critical in revealing how mucin secretion is precisely regulated.

## 2. Porosome in Calu-3 cell

High resolution imaging using atomic force microscopy (AFM) (Figure 1) reveal in great detail the surface topology of Calu-3 cells, demonstrate the presence of approximately 102 nm in diameter porosome openings (mean ± SEM; 102.4 nm ± 3.0 nm), and 96 nm thick (mean ± SEM; 96 nm ± 3.3 nm) microvilli, at the cell plasma membrane. Nearly the entire cell surface is covered with the

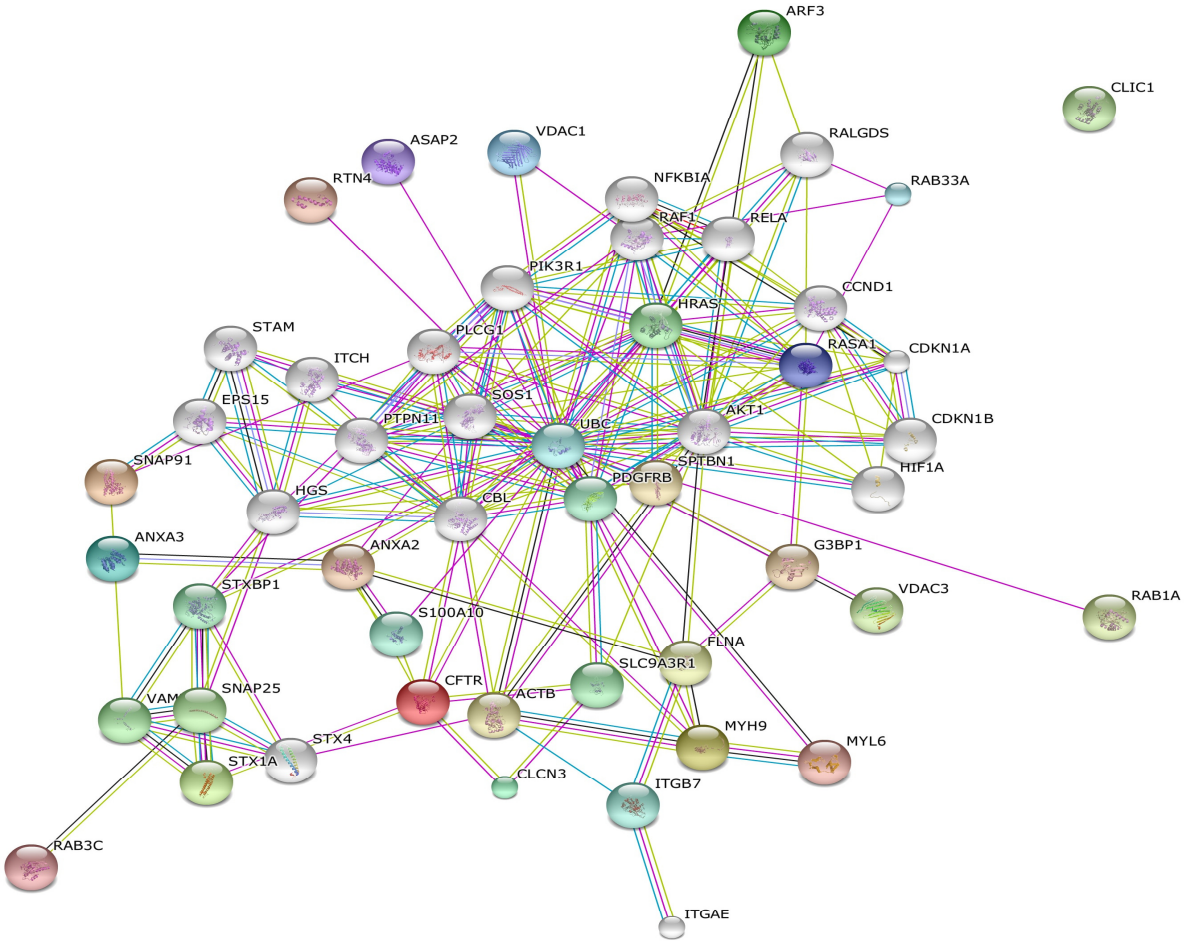


**Figure 4.** Schematic drawing depicting the evidence view of predicted interactions between identified proteins within the mucous-secreting Calu-3 porosome proteome and other regulatory proteins. These interactions are generated from inputs of the identified proteins in the Calu-3 porosome, using STRING 9.1[7,11]. STRING 9.1 is a database of known functional and predicted protein-protein interactions. The interactions include direct (physical) and indirect (functional) associations derived from genomic, high-throughput, conserved co-expression, and earlier knowledge. Note the two clusters of protein-protein interactions identified in the porosome complex. The one cluster to the top, and most likely present at the apical end of the porosome cup are cytoskeletal structure and signalling proteins. The bottom cluster represents proteins that are primarily involved in membrane fusion including SNARE proteins and CFTR, and therefore their location would be at the base of the porosome complex facing the cytosol. The confidence of the predicted functional interactions shown are >99%. ©Bhanu Jena.

microvilli, with interspersed porosome openings. In certain areas of the cell surface devoid of microvilli or porosome openings, cytoskeletal structures underlying the cell plasma membrane are observed (Figure 1B)[11]. Transmission electron microscopy (TEM) performed on Calu-3 cells confirms the AFM results (Figure 2), demonstrating the presence of dense microvilli (Figure 2A), and cup-shaped porosomes (Figure 2B-D) at the cell plasma membrane. Immunoisolated Calu-3 porosome complexes demonstrate a particle size of approximately 300 nm (Figure 3A) using photon correlation spectroscopy (PCS), possibly a result of trimerization in their isolated state in suspension[11]. Immunoblot analysis of isolated Calu-3 porosome complex (Figure 3B), demonstrate the co-association of CFTR with the porosome complex that contains among other proteins Gαi3, actin, and vimentin (Figure 3C). Similarly, when CFTR is immunoisolated from solubilized Calu-3 cells, the porosome complex is co-immunoisolated with CFTR, as demonstrated by the presence of

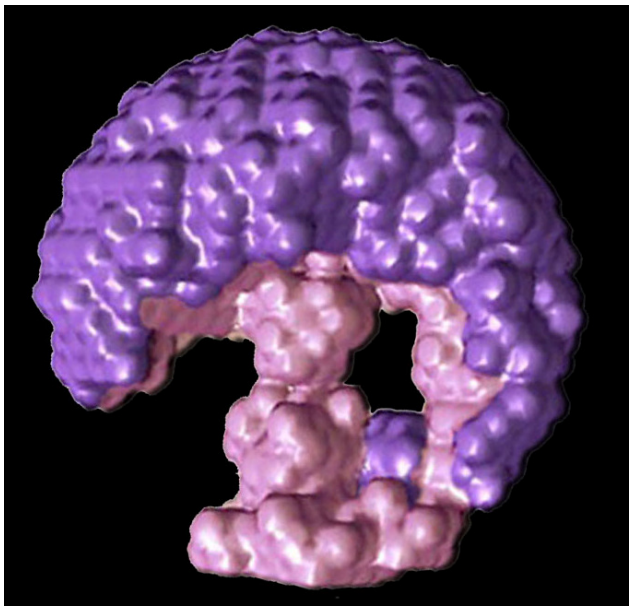

**Figure 5.** Small angle X-ray solution scattering structure of a native 35 nm synaptic vesicle (violet) docked with a 15 nm neuronal porosome complex (pink) at the presynaptic membrane. Note the prominent central plug of the porosome, which has been implicated in the rapid closing and opening of the complex[80]. ©Bhanu Jena.

porosome-associated proteins Syntaxin-1A, SNAP-25, SNAP-23, actin, and vimentin (Figure 3D). Mass spectrometry on the immunoisolated porosome complex from Calu-3 cells, demonstrates the presence of various porosome-associated proteins (found in the neuronal and pancreatic porosome complexes) as well as CFTR[11]. Furthermore, mass spectrometry results confirm the interaction between CFTR and the porosome complex in Calu-3 cells, as determined using imunoprecipitation and immunoblot analysis. Using the STRING 9.1 database search[77] similar to the neuronal porosome complex[12], two clusters of protein-protein interactions within the mucin-secreting Calu-3 porosome have been identified (Figure 4). The cluster to the top-right in figure 4, represent primarily cytoskeletal and signaling proteins, whereas the bottom-left cluster represents proteins that are primarily involved in membrane fusion, including SNAREs, ion channels and CFTR. Therefore the bottom cluster is located at the porosome base facing the cytosol where mucin-containing vesicles dock and fuse, and the top cluster at the porosome opening to the outside. Therefore in these studies, the porosome proteome in human airways epithelia has been determined. The interaction between CFTR and the porosome complex in the human airways epithelia is further demonstrated. The possible regulation by CFTR on the quality of mucus secretion via the porosome complex at the cell plasma membrane is hypothesized. These new findings will facilitate understanding of CFTR-porosome interactions influencing mucus secretion, and provide critical insights into the etiology of CF disease.

In view of this, an integrated approach is being used to characterize the molecular architecture of the mucin-secreting porosome complex of the human airways epithelia cell line Calu-3; determine the distribution of CFTR and its interaction with proteins and lipids within the mucin-secreting porosome complex; characterize the molecular architecture of the mucin-secreting porosome; and build and test a functional architectural model to determine how SNAREs, lipids, and calcium, establish continuity between the secretory vesicle membrane and the porosome. These studies will allow a molecular understanding of the porosome function in mucin secretion, and the role of CFTR in the process.

## 3. Ongoing studies

Among the 34 core porosome proteins in mucin-secreting Calu-3 cells, are included CFTR, actin, vimentin, annexin, filamin, Gαi3, tubulin, syntaxin-1A, profiling, ezrin, spectrin, chloride channels CLC-1 and CLC-3, rab1A and rab3A, myosin, SNAP-25, and the ADP-ribosylation factor ARF3[11]. It is anticipated that due to the nature of proteome studies and the dynamics of porosomes[12] that this initial analysis includes most of the core porosome proteins, but not all the peripheral proteins associated with the complex. The STRING 9.1[77] database search utilizing known physical and functional associations between proteins suggests additional candidates likely involved in protein-protein interactions within the Calu-3 porosome complex (Figure 4). Similarly, preliminary lipidomic studies using lipid overlay assays on isolated Calu-3 porosome complex, demonstrate the enriched presence of PA and PIP2. Furthermore, interactions of PA and other polyphosphoinositides with syntaxin-1A, and their involvement in cell secretion have previously been reported[78]. These

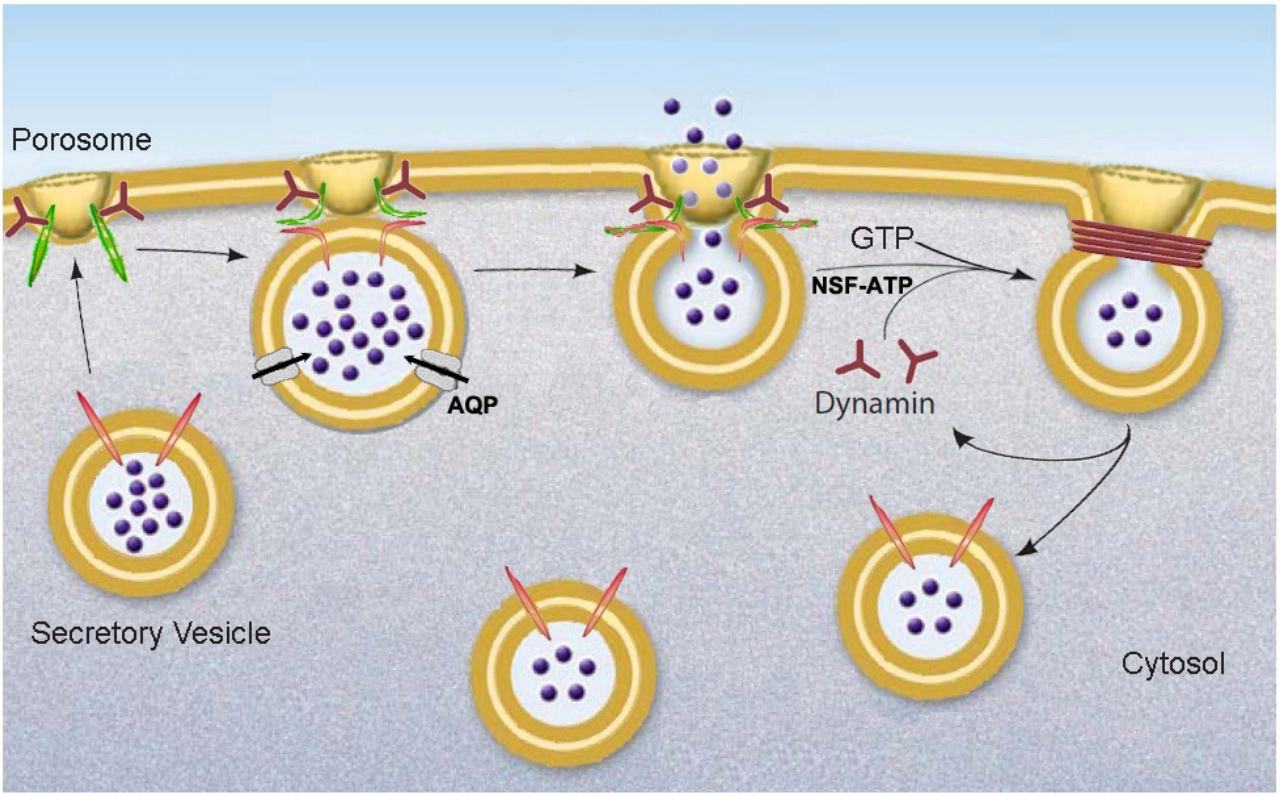


**Figure 6** Schematic drawing depicting the presence and increased association of dynamin with the porosome complex following stimulation of neurotransmitter release[12], which may be similar in the mucin-secreting Calu-3 cell. Following stimulation of secretion, synaptic vesicles would dock at the porosome base, develop intravesicular pressure via active transport of water through water channels or aquaporins (AQP) at the vesicle membrane, transiently fuse at the porosome base via SNAREs and calcium, and expel neurotransmitters. After secretion, NSF an ATPase, and dynamin a GTPase, would work synchronously to disassembly t-/v-SNARE complexes and fission the neck of fused vesicles at the porosome base respectively. By this mechanism, partially empty vesicles could go through multiple rounds of docking-fusion-expulsion-dissociation. Unlike protein and peptide containing vesicles, synaptic vesicles have neurotransmitter transporters at the vesicle membrane to rapidly refill vesicles[12]. In case of the Calu-3 cell, once mucin containing vesicles empty, they may recycle via the endosome or lysosomal pathway. ©Bhanu Jena.

observations indicate the importance of lipid interactions in the structure and function of the porosome. Based on this information, and since mucin containing vesicles dock at the porosome base, and the t-SNARE syntaxin-1A and SNAP-25 associated with CFTR, rab3C, CLC3, and SNAP91 are among other proteins present at the porosome base (Figure 4), it could be surmised that PA and PIP2 together with these proteins are present at the porosome base. The protein cluster composed of cytoskeletal and signaling proteins on the other hand, is likely associated with the porosome opening to the outside of the cell, regulating dilation of the porosome opening during mucin secretion. Since in the presence of the actin depolymerizing agent cytocholasine, there is loss of mucin secretion (unpublished observation) as in the case of the exocrine pancreas[7] or growth hormone secreting cells[1], further supports the presence of signaling and motor proteins at the porosome opening, that regulate dilation of the porosome opening and mucin secretion.

Unlike individual proteins or lipids, determination of the atomic structure of such dynamic macromolecular lipoprotein complexes such as the porosome, poses a difficult challenge, requiring the use of several experimental and computational approaches to maximize resolution and accuracy. Although recently the isolated Calu-3 porosome has been functionally reconstituted as in case of the exocrine pancreas or neurons, further functional reconstitution studies are under way

- **Mucin-secreting porosomes are 100 nm cup-shaped secretory portal at the plasma membrane; the porosome has been isolated from Calu-3 cells and its proteome determined**
- **Porosome structure has been determined using EM and AFM**
- **Distribution of lipids and various proteins especially CFTR within the porosome complex is under way**

using the established lipid bilayer EPC9 system[6]. These experiments further determine if the entire porosome complex has been isolated prior to determination of its composition and molecular structure-function. Electron microscopy (EM) especially single particle cryo electron tomography, small angle X-ray solution scattering (SAXS), supra resolution microscopy (SRM), and AFM analyses are being used and complemented by techniques from structural mass spectrometry and proteomics to obtain molecular details of the mucin-secreting porosome structure. The mass spectrometry studies include subunit stoichiometry, interacting subunits, and site of contact between subunits. Changes to porosome subunit composition and subunit interactions during the secretory process are being studies using these approaches. CXL-MS and multiple quantitative mass spectrometry techniques are being utilized to determination details of the protein-protein interactions within the native mucin-secreting porosome complex, which is central to building a structural model of the complex for a molecular understanding of its structure-function.

New and recently developed crosslinkers[79] combined with tandem mass spectrometry are being carried out, which will provide identities of interacting subunits and provide the identities of specific residues crosslinked both between and within subunits in the porosome complex. Results from these studies will provide information on interaction domains and distance constraints on protein structures. Quantitative mass spectrometry using iTRAQ are also being carried out, which will provide additional information on changes in porosome subunits composition and dynamics, as a function of the secretion status of the organelle. Immuno-AFM[5], immuno–EM, and SAXS[80] (Figure 5) studies on isolated Calu-3 porosomes as in porosomes of the exocrine pancreas and neurons, are being performed to determine the distribution of some of the major proteins within the complex. Similar to studies using SRM on the nuclear pore complex[81], SRM is being employed to obtain additional information on the structure of the mucin-secreting porosome complex. Finally, computational approaches are being employed, such as coarse-grain molecular docking studies[82-97], homology modeled interactions[98-100], and fitting of known atomic structures of protein-protein interactions and complexes[101-108]. It is becoming increasingly clear that the ultrastructural and mass spectrometry methods show promise in providing complementary information and the high degree of cross-validation required to build an accurate structural model of the mucin-secreting porosome complex. Collectively, the outlined studies briefly discussed here will enable an understanding at the molecular level, the elegant mechanism of porosome-mediated secretion (Figure 6) in Calu-3 and other cells.

**Conflict of Interest**

The authors do not declare any conflict of interest.

**Acknowledgements**

The author thanks members of his laboratory for lively discussions on the subject.

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