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Landmark discoveries in intracellular transport and secretion

Kishore M. Paknikar*

Agharkar Research Institute, Pune, India

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Abstract

Cellular protein transport and secretion is fundamental to the very existence of an organism, regulating important physiological functions such as reproduction, digestion, energy production, growth, neurotransmission, hormone release, water and ion transport, *etc.*, all required for the survival and maintenance of homeostasis within an organism. Molecular understanding of transport and secretion of intracellular product has therefore been of paramount importance and aggressively investigated for over six decades. Only in the last 20 years, the general molecular mechanism of the process has come to light, following discovery of key proteins involved in ER-Golgi transport, and discovery of the '*porosome*' – the universal secretion machinery in cells.

Keywords: ER-Golgi transport • cell secretion • porosome/fusion pore • membrane fusion

Introduction

Newly synthesized proteins in the endoplasmic reticulum (ER), are transported via a series of membrane-bound shuttles or vesicles to and between the Golgi apparatus, where their content proteins undergo various post-translational modifications such as glycosylation, to mature. Specialized transport vesicles containing mature cargo originate from the Golgi and destined for secretion, dock and transiently fuse at specialized plasma membrane structures called 'porosomes' to expel intravesicular contents. This unique process of transport and secretion, is universal to living cells. The discovery of proteins involved in the budding and fusion of such transport vesicles [1], the discovery of the 'porosome' [2], and the regulated release of vesicular contents from cells [2], are landmark discoveries. While an elegant mammalian cell-free system was used in the discovery of the various players involved in vesicle budding and fusion, a new and powerful microscope (atomic force microscope or AFM) was brilliantly employed in discovery of the 'porosome' in live cells, in unraveling the interaction, assembly, and disassembly of membrane proteins involved in membrane fusion, and in deciphering the regulated release of intravesicular contents during cell secretion. These seminal and landmark discoveries are summarized.

*Correspondence to: K.M. PAKNIKAR
Agarkar Research Institute G.G. Agarkar Road,
Pune-411004, India.

Tel: 91-20-25 65 36 80 extension 372
Fax: 91-20-25 65 15 42
E-mail: paknikar@vsnl.com

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Discovery of proteins involved in intracellular vesicle budding and fusion

A preparation of isolated Golgi stacks, ATP, and a 100,000 × g cytosolic fraction from mammalian cells, was found to reconstitute transport between Golgi cisternae [3]. For the first time, this assay system provided the tool for discovery of proteins involved in the fission and fusion of transport vesicles within cells [4–6]. In such an *in vitro* system, when a "donor" Golgi population containing VSV-encoded G protein (the cargo) but lacking the glycosylating enzyme, is incubated with an "acceptor" Golgi population containing the enzyme, the transfer of G protein from donor to acceptor is determined as a measure of radiolabled *N*-acetylglucosamine in the oligosaccharide chain of G proteins in the acceptor compartments [5]. Vesicles measuring approximately 70 nm in diameter with an 18 nm "coat" (non-clathrin coat) and containing the VSV G protein, appear only in presence of the 100,000 × g cytosol and ATP [7]. It was further demonstrated that transport was blocked in the presence of *N*-ethylmaleimide (NEM), and in the presence of non-hydrolyzable GTP analogues such as GTPγS [8]. In presence of GTPγS the 70 nm vesicles that accumulated were coated, however, in the presence of NEM, uncoated vesicles accumulated. Since the uncoated vesicles bound to Golgi cisternae, coat had to be removed following budding to allow fusion of vesicles with the target membrane. These results were key to the discovery of the vesicle budding and fusion machinery.

Immediately thereafter, coated vesicles were purified following their accumulation in presence of GTPγS [6]. Eight polypeptides were found to form the coat, which included a previously identified and characterized protein the ADP ribosylation factor (ARF) [9], and seven other proteins termed COPs [10]. In the vesicle budding process, cytosolic GTP-bound ARF binds the Golgi membrane, initiating recruitment of coat proteins from the cytosol, leading to the formation of the bud. Hydrolysis of ARF-bound GTP, releases the coat from vesicles, making them fusion ready with the target membrane. Hence ARF is the master molecule that controls coating and uncoating.

Since in presence of NEM, uncoated vesicles accumulate and are bound to acceptor Golgi cisternae without fusing, implied the requirement of a NEM-sensitive factor [5] in membrane fusion. Addition of fresh cytosol to the NEM treated reaction mixture, restored fusion, demonstrating that the NEM-sensitive factor was present in the cytosol, and thereby guided the purification of the NEM-sensitive factor (NSF) [11]. Using NSF as bait, a 20S membrane "fusion particle" was purified from the Golgi membrane [12]. Protein sequencing of the resolved particle demonstrated the presence of both a membrane protein found in synaptic vesicles [13], and two proteins found at the pre-synaptic membrane, Syntaxin [14] and SNAP-25 [15]. VAMP and Syntaxin are both integral membrane proteins, consisting of an amino terminal cytoplasmic domain, a membrane-spanning segment, and polar residues at their carboxy terminus. Although SNAP-25 is intrinsically a water-soluble protein, it associated with Syntaxin.

Discovery of the 'porosome': the cells secretion machinery

In the mid 1990s, an atomic force microscope (AFM) trained on isolated live pancreatic acinar cells in near physiological buffer solution, provided for the first time, the structure, organization, and dynamics of the cell plasma membrane at nanometer resolution and in real time [16]. Organized circular structures measuring on the average 400–500 nm in diameter termed 'pits', containing on the average 3–4, 100–180 nm in diameter and 25–45 nm in depth 'depressions', were observed for the first time at the apical plasma membrane in live pancreatic acinar cells using high-resolution AFM [16]. When cells were stimulated to secrete, the 'depressions' enlarged and returned to their resting size following completion of secretion. Exposure of live pancreatic acinar cells to the fungal toxin cytochalasin, a known inhibitor of actin polymerization and cell secretion, resulted in the collapse of 'depressions' and a concomitant loss in secretion [16]. These results suggested 'depressions' to be the long sought-after fusion pores, however, there was the need for direct evidence of secretory products exiting the pits and depression structures. It took almost 5 years to finally demonstrate using immuno-AFM, that indeed secretory products are discharged through depressions during cell secretion [17]. Subsequent studies using

growth hormone secreting cells of the pituitary gland, also demonstrated the presence of depressions, and the release of growth hormone through them [18]. These findings finally demonstrated depressions to indeed be the 'long-sought fusion pores' in cells. Further studies on chromaffin cells [19], mast cells [2, 20], β-cells of the endocrine pancreas [2, 20], and neurons [2, 21], all demonstrate the presence of depressions and their function as the cells secretion machinery. The universal presence of depressions as the secretory apparatus in cells, probably led to coining of the term 'porosome' [2] for this "new cellular structure" [22]. For decades it had been speculated, that such a structure may exist at the cell plasma membrane where secretory vesicles transiently associate and fuse to release their contents from the cell. Therefore the discovery of the porosome is a landmark in biology, ending both speculations for its presence as well as the race for its discovery. Unlike what had previously been postulated, AFM studies on live cells demonstrated porosomes to be permanent structures at the cell plasma membrane, and not arising following a secretory stimulus. The subsequent imaging of porosomes, and porosome-associated secretory vesicles *in situ* using conventional transmission electron microscopy (TEM) [21, 23, 24], has further confirmed their presence and determined their structure and function. Porosomes are cup-shaped basket-like-designed lipoprotein structures at the cell plasma membrane [21, 23, 24], typically 100–180 nm in exocrine and neuroendocrine cells, and 10–15 nm at the nerve terminal [21]. There appears to be a direct correlation between secretory vesicle size and the size of porosomes in cells.

The molecular structure and chemistry of the porosome was determined following its immuno-isolation, and both its structural and functional reconstitution into artificial lipid membrane [21, 23, 24]. The composition of the porosome was further assessed, and the distribution of various porosome proteins determined using porosome dynamics [16, 18], immuno-AFM studies [23], yeast 2-hybrid analysis [25], and cholesterol-depletion experiments [26].

Demonstration that the fungal toxin cytochalasin, a known inhibitor of actin polymerization and cell secretion, resulted in the collapse of porosomes, clearly demonstrated actin to be a major component of the porosome complex [16]. Similarly, since membrane-bound secretory vesicles dock and fuse at porosomes to release vesicular contents, it was demonstrated that plasma membrane-associated t-SNAREs are part of the porosome complex [23]. Immunochemical analysis of isolated porosomes, demonstrated the association of SNAP-23/25, syntaxin, cytoskeletal proteins actin, α-fodrin, and vimentin, and the calcium channels β3 and α1c, the chloride channel CLC-3, synaptotagmin, the alpha subunit of the heterotrimeric GTP-binding protein G_o, together with the SNARE regulatory protein NSF [21, 23, 24]. Additional yeast 2-hybrid analysis [25], further demonstrated the direct interaction between SNAP-23 and the L-type calcium channel in porosomes of the exocrine pancreas. Studies further demonstrate that Syntaxin-1 co-localizes with cholesterol, in a Triton/Lubrol-solubilized synaptosomal membrane preparation. Depletion of cholesterol from the Triton/Lubrol-solubilized synaptosomal membrane preparation using saponin, results in the dissociation of both Syntaxin-1 and N-type calcium channel from neuronal porosomes. These studies demonstrate that cholesterol is an integral component of the porosome complex in neurons, and is required for its stability [26].

The size and shape of the isolated porosome complex has been determined using both negative staining TEM and AFM [21, 23, 24]. Electron micrographs of isolated porosomes are identical to the AFM micrographs of hydrated porosome preparation [24]. Porosomes isolated from exocrine pancreas and reconstituted into artificial liposomes, demonstrate a similar basket-like morphology in TEM micrographs [23]. When purified porosomes were reconstituted into lipid membranes in an electrophysiological bilayer setup (EPC9) and exposed to isolated secretory vesicles, both the electrical activity of the reconstituted membrane as well as the transport of vesicular contents from the *cis* to the *trans* compartment, was observed [21, 24]. Results of these experiments demonstrate that isolated lipid-reconstituted porosomes are functional supramolecular complexes [21, 24]. Isolated secretory vesicles fuse with the porosome-reconstituted bilayer, as demonstrated by an increase in capacitance and conductance, and in a time-dependent release of secretory products from the *cis* to the *trans* compartment of the bilayer chamber. In the exocrine pancreas, the secretory vesicles called zymogen granules contain the starch digesting enzyme amylase. Amylase transport from the *cis* to the *trans* compartment of the bilayer chamber is detected using immunoblot analysis of the buffer in

the *trans* compartment. Furthermore, the chloride channel inhibitor DIDS, was found to inhibit current activity in the porosome-reconstituted bilayer [24], demonstrating its function in the porosome complex. These studies demonstrate that porosomes in the exocrine pancreas and neurons are 100–150 nm and 10–15 nm in diameter, respectively, are supramolecular cup-shaped lipoprotein basket-structures at the cell plasma membrane, where membrane-bounded secretory vesicles dock and fuse to release intravesicular contents. The discovery of the porosome, the universal secretory machinery in cells, has finally provided a molecular understanding of the secretory process in cells.

Neurotransmission, and the release of neuroendocrine and exocrine substances require certain specialized cells to secrete. Altered or loss in cellular secretions is causal to numerous diseases [2, 20], and has therefore been the focus of continued scientific investigations for over six decades. It was previously believed that during cell secretion, secretory vesicles merge with the cell plasma membrane, leading to passive diffusion of vesicle contents and subsequent retrieval of excess membrane (vesicle membrane) through endocytosis [27, 28]. This view however, was in stark contrast to the universal findings that during cell secretion, empty and partially empty vesicles accumulate with no appreciable loss in vesicle number. Unable to account for this discrepancy was therefore frustrating, as previously echoed by Professor Erwin Neher in a 1993 article in the Journal *Nature* [29]: 'It seems terribly wasteful that, during the release of hormones and neurotransmitters from a cell, the membrane of a vesicle should merge with the plasma membrane to be retrieved for recycling only seconds or minutes later.' This cloud of frustration has finally lifted with the discovery of the 'porosome', a universal new cellular structure at the cell plasma membrane, where secretory vesicles transiently dock and fuse without complete merger, to expel intravesicular contents from cells [2]. Since this discovery, a large body of evidence has accumulated demonstrating transient fusion of secretory vesicles at the cell plasma membrane instead of the dogmatic view of complete merger of secretory vesicles at the cell plasma membrane during cell secretion. Studies now demonstrate that "secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells" [30], that "single synaptic vesicles fuse transiently and successively without the loss of identity" [31], and that 'Zymogen granule exocytosis is characterized by long fusion pore openings and preservation of vesicle lipid identity' [32], and similarly the 'number of secretory vesicles in growth hormone cells of the pituitary remain unchanged after secretion' [33]. Numerous articles have been written on these landmark discoveries, three of which [34–36] would complement and provide a further in-depth view on the subject.

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References

1. **Rothman JE.** The protein machinery of vesicle budding and fusion. *Prot Sci.* 1996; 5: 185–94.
2. **Jena BP.** Molecular machinery and mechanism of cell secretion. *Exp Biol Med.* 2005; 230: 307–19.
3. **Balch WE, Dunpy WG, Braell WA, Rothman JE.** Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N -acetylglucosamine. *Cell.* 1984; 39: 405–16.
4. **Balch WE, Glick BS, Rothman JE.** Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell.* 1984; 39: 525–36.
5. **Malhotra V, Orci L, Glick BS, Block MR, Rothman JE.** Role of an N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack. *Cell.* 1988; 54: 221–7.
6. **Malhotra V, Serafini T, Orci L, Shepherd JC, Rothman JE.** Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell.* 1989; 58: 329–36.
7. **Orci L, Palmer DJ, Ravazzola M, Perrelet A, Amherdt M, Rothman JE.** Budding from Golgi membrane requires coatomer complex of non-clathrin coat proteins. *Nature.* 1993; 362: 648–52.
8. **Melancon P, Glick BS, Malhotra V, Weldman PJ, Serafini T, Gleason ML, Orci L, Rothman JE.**

Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell.* 1987; 51: 1053–62.
9. **Kahan RA, Gilman AG.** The protein cofactor necessary for ADP-ribosylation of Gs by choleratoxin is itself a GTP binding protein. *J Biol Chem*. 1986; 261: 7906–11.
10. **Waters MG, Serafini T, Rothman JE.** "Coatomar": A cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature.* 1991; 349: 248–51.
11. **Block MR, Glick BS, Wilcox CA, Wieland FT, Rothman JE.** Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc Natl Acad Sci USA.* 1988; 85: 7852–6.
12. **Wilson DW, Whiteheart SW, Wiedmann M, Brunner M, Rothman JE.** A multisubunit particle implicated in membrane fusion. *J Cell Biol.* 1992; 117: 531–8.
13. **Trimble WS, Cowam DM, Scheller RH.** VAMP-1: A synaptic vesicle-associated integral membrane protein. *Proc Natl Acad Sci USA*. 1988; 85: 4538–42.
14. **Bennett MK, Calakos N, Scheller RH.** Syntaxin: A synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science.* 1992; 257: 255–9.
15. **Oyler GA, Higgins GA, Hart RA, Battenbarg M, Bloom FE, Wilson MC.** The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. *J Cell Biol*. 1989; 109: 3039–52.
16. **Schneider SW, Sritharan KC, Geibel JP, Oberleithner H, Jena BP.** Surface dynamics in living acinar cells imaged by atomic force microscopy: Identification of plasma membrane structures involved in exocytosis. *Proc Natl Acad Sci USA.* 1997; 94: 316–21.
17. **Cho SJ, Quinn AS, Stromer MH, Dash S, Cho J, Taatjes DJ, Jena BP.** Structure and dynamics of the fusion pore in live cells. *Cell Biol Int.* 2002; 26: 35–42.
18. **Cho SJ, Jeftinija K, Glavaski A, Jeftinija S, Jena BP, Anderson LL.** Structure and dynamics of the fusion pores in live GH-secreting cells revealed using atomic force microscopy. *Endocrinology.* 2002; 143: 1144–8.
19. **Cho SJ, Wakade A, Pappas GD, Jena BP.** New structure involved in transient membrane fusion and exocytosis. *Ann New York Acad Sci.* 2002; 971: 254–6.
20. **Jena BP.** Discovery of the porosome: revealing the molecular mechanism of secretion and membrane fusion in cells. *J Cell Mol Med.* 2004; 8: 1–21.
21. **Cho WJ, Jeremic A, Rognlien KT, Zhvania MG, Lazrishvili I, Tamar B, Jena BP.** Structure, isolation, composition and reconstitution of the neuronal fusion pore. *Cell Biol Int.* 2004; 28: 699–708.
22. **Horber JKH, Miles MJ.** Scanning probe evolution in biology. *Science.* 2003; 302: 1002–5.
23. **Jena BP, Cho SJ, Jeremic A, Stromer MH, Abu-Hamdah R.** Structure and composition of the fusion pore. *Biophys J.* 2003; 84: 1337–43.
24. **Jeremic A, Kelly M, Cho SJ, Stromer MH, Jena BP.** Reconstituted fusion pore. *Biophys J.* 2003; 85: 2035–43.
25. **Cho WJ, Jeremic A, Jena BP.** Direct interaction between SNAP-23 and L-type calcium channel. *J Cell Mol Med.* 2005; 9: 380–6.
26. **Jeremic A, Cho WJ, Jena BP.** Cholesterol is critical to the integrity of neuronal porosome/fusion pore. *Ultramicroscopy.* 2006; 106: 674–7.
27. **Dreifuss JJ.** A review on neurosecretory granules: Their contents and mechanisms of release. *Ann New York Acad Sci*. 1975; 248: 184–201.
28. **Almers W.** Exocytosis. *Annu Rev Physiol.* 1990; 52: 607–24.
29. **Neher E.** Secretion without full fusion. *Nature.* 1993; 363: 497–8.
30. **Taraska JW, Perrais D, Ohara-Imaizumi M, Nagamatsu S, Almers W.** Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. *Proc Natl Acad Sci USA*. 2003; 100: 2070–5.
31. **Aravanis AM, Pyle JL, Tsien RW.** Single synaptic vesicles fusing transiently and successively without loss of identity. *Nature.* 2003; 423: 643–7.
32. **Thorn P, Fogarty KE, Parker I.** Zymogen granule exocytosis is characterized by long fusion pore openings and preservation of vesicle lipid identity. *Proc Natl Acad Sci USA.* 2004; 101: 6774–9.
33. **Lee JS, Mayes MS, Stromer MH, Scanes CG, Jeftinija S, Anderson LL.** Number of secretory vesicles in growth hormone cells of the pituitary remains unchanged after secretion. *Exp Biol Med.* 2004; 229: 291–302.
34. **Anderson LL.** Cell secretion – finally sees the light. *J Cell Mol Med.* 2006; 10: 271–2.
35. **Jeftinija S.** The story of cell secretion: events leading to the discovery of the "porosome" – the universal secretory machinery in cells. *J Cell Mol Med.* 2006; 10: 273–9.
36. **Leabu M.** Membrane fusion in cells: molecular machinery and mechanisms. *J Cell Mol Med.* 2006; 10: 423–8.