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Vesicular Traffic, Secretion, and Endocytosis

Review the Concepts

1. Palade and colleagues chose the pancreatic acinar cell because it is a specialized secretory cell that packages trypsinogen, chymotrypsinogen, and other digestive enzymes into secretory granules that then are released in response to signaling. Most protein synthesis in these cells is devoted to secretion. The vast majority of ribosomes are found in association with the rough endoplasmic reticulum. The cells are also decidedly polarized with a definite gradient in organelle distribution. In his seminal experiments Palade took advantage of radioactive amino acids to label proteins, the majority of which were incorporated into molecules associated with membrane-bound ribosomes and, subsequently, with secretory organelles. After pulse-chase labeling and sectioning the cells for electron microscopy, the sections were covered with photographic emulsion and exposed. In this autoradiographic method, silver grains exposed to the radioactive decay from the newly labeled protein were reduced and, when the emulsion was developed, the appearance of the silver grains was compared under the electron microscope to their subcellular distribution. In a more contemporary method cells can be transfected with a hybrid gene encoding two proteins. In this manner the transcript encodes a membrane glycoprotein from vesicular stomatitis virus (VSV G) that is fused to green fluorescent protein (GFP), which is the tag for detection. Cells expressing this gene rapidly synthesize VSVG-GFP in the ER, which is then transported through the secretory pathway to the cell surface. Using fluorescence microscopy to detect GFP, the investigator can monitor the expression of the chimeric protein and its localization in live cells. Thus both methods require that proteins be labeled in an early compartment so that their processing and transport can be followed over time. The second necessary requirement is to have a way to identify the compartment containing the labeled proteins.

In each case a form of microscopy was used that complements the labeling reaction.

2. NSF, through its ATPase activity, catalyzes the dissociation of v-SNARE/t-SNARE complexes. Such complexes are essential in specific membrane fusion at several stages of the secretory and endocytic pathways. Why then does the *Sec18* NSF mutation produce a class C phenotype accumulation of ER-to-Golgi transport vesicles? This can be explained readily if one considers the need for NSF to generate free v-SNAREs and t-SNAREs to support multiple rounds of vesicle membrane fusion. In the absence of NSF activity, vesicles bud from the ER but are unable to fuse with downstream membranes because of the lack of v-SNAREs. Vesicles accumulate at what is the first vesicle organelle fusion step within the secretory pathway.

3. Coat proteins play two roles in vesicle budding: 1) they provide a scaffold that establishes membrane curvature; and 2) they interact with cargo proteins or cargo protein receptors to provide enrichment of certain proteins in the bud. Small GTPases of either the Sar or ARF family recruit coat proteins to membranes. It is the GTP bound form of Sar or ARF that is active. The exact mechanisms by which each acts are unknown. The mechanism is particularly unclear in the case of ARF, which recruits clathrin and different adapter proteins at different sites within the cell. As noted above, vesicles are enriched in cargo proteins. Moreover, newly formed vesicles are programmed for subsequent fusion events by the selective inclusion of v-SNAREs and Rabs in their membrane. Dynamin is the one protein well known to have a role in pinching off vesicles at the cell surface and at the *trans*-Golgi network. In all known cases, these are clathrin-coated vesicles.

4. The observation that decoating Golgi membranes by treatment of cells with the drug brefeldin A

(BFA) results in the redistribution of Golgi proteins to the ER suggests that COPI, the major Golgi associated coat, has a role in stabilizing Golgi structure. To some extent, COPI may be equivalent to the dam that holds back the water in a reservoir. ARF1 is the small GTPase that recruits COPI to Golgi membranes. Mutation of ARF1 to give a GDP-restricted form of the protein would result in a GTPase that is now unable to recruit COPI to membranes. Since the COPI association with membranes is dynamic, this mutation would shortly lead to uncoating of Golgi membranes. Note that mutation of ARF1 to the GTP-restricted form would have the opposite effect; the ARF1 would now be permanently in the on-state and the association of COPI with Golgi membranes would be permanently stabilized. Since GDP-restricted ARF1 produces the same phenotype as BFA, this suggests that the drug evokes a normal physiological possibility.

5. Coat proteins must be able to come on and off membranes to produce a cycle of vesicle production and consumption. An antibody such as EAGE that binds to a "hinge" region of COPI is likely to be a blocking or function inhibitory antibody. Molecular flexibility is likely to be required for either uncoating or coating. In reality, EAGE blocks uncoating of COPI from Golgi membranes and vesicles. Inhibition of COPI function should inhibit anterograde transport from the ER to the plasma membrane. COPI is required both for cisternal progression and for retrieval of proteins from the *cis*-Golgi to the ER. Cisternal progression stops because Golgi enzymes, for example, are not retrieved from *trans*- to *medial*- to *cis*-Golgi. Similarly, v-SNAREs would not be retrieved from *cis*-Golgi to ER. Note here that the effect on anterograde transport is indirect; the direct effect is on retrograde transport. One possible experiment to test whether the effect of EAGE microinjection is initially on anterograde or retrograde transport is to characterize the properties of COPI derived vesicles. If these are retrograde carriers, they should be enriched in Golgi enzymes, for example, rather than anterograde cargo.

6. Vesicle fusion involves two stages, first a docking stage mediated by long coiled-coil proteins such as

EEA1 and then a specific membrane fusion step mediated by SNAREs. The docking or tethering protein, EEA1, is recruited to vesicles by Rab proteins in their GTP-activated state. Rab5 plays a role in prompting vesicle fusion with early endosomes. Overexpression of the GTP-restricted form, the activated form, of Rab5 would have the effect of prompting such fusions and lead to enlarged early endosomes.

7. Lys-Asp-Glu-Leu (KDEL) and Lys-Lys-X-X (KKXX) are both retrieval sequences for ER proteins. KDEL is a sequence feature of soluble ER luminal proteins; KKXX is found on the cytosolic domain of ER membrane proteins. Retrieval of a normally ER luminal protein from the *cis*-Golgi is a COPI-dependent process. COPI is found on the cytosolic face of the *cis*-Golgi membrane. The KDEL containing protein is within the lumen of the *cis*-Golgi. The two interact through a bridging membrane protein, the KDEL receptor. It is the KDEL receptor/KDEL-containing protein complex that is retrieved to the ER. In the cisternal progression model, *trans*-Golgi proteins, for example, must be retrieved to the *medial*-Golgi to generate a new *trans*-Golgi cisterna. This is a COPI-mediated process. There must be interactions between COPI and Golgi proteins to promote such retrieval.

8. There are four known clathrin adapter protein complexes: AP1 (*trans*-Golgi to endosome), GCA (*trans*-Golgi to endosome), AP2 (plasma membrane to endosomes), and AP3 (Golgi to lysosome, melanosome, or platelet vesicles). Each contains one copy of four different adapter protein subunits. The clathrin coat, unlike the COPI or COPII coat, is a double layered coat with a core coat of adapter proteins and an external clathrin coat. Each adapter complex is different, but all are related. Presently, it is not known if the coat of AP3 vesicles contains clathrin. This is consistent with the possibility that evolutionarily the adapter complex may well be the core coat with clathrin an accessory layer added later.

9. I-cell disease is a particularly severe form of lysosomal storage disease. Multiple enzymes are lacking in the lysosome and the organelle becomes stuffed with nondegraded material and therefore

generates a so-called inclusion body. I-cell disease is inherited; it is caused by a single gene defect in the *N*-acetylglucosamine phosphotransferase that is required for the formation of mannose 6-phosphate (M6P) residues on lysosomal enzymes in the *cis*-Golgi. This enzyme recognizes soluble lysosomal enzymes as a class and hence a defect in this protein affects the targeting of a large number of proteins. A defect in the phosphodiesterase that removes the GlcNAc group that initially covers the phosphate group on mannose 6-phosphate would also produce an I-cell disease phenotype. Similarly, defects in mannose 6-phosphate receptors would affect the targeting of lysosomal enzymes as a class.

10. The *trans*-Golgi network (TGN) is the site of multiple sorting processes as proteins exit the Golgi complex. The sorting of soluble lysosomal enzymes occurs via binding to mannose 6-phosphate (M6P) receptors. Binding is pH dependent and occurs at the TGN pH of 6.5 but dissociates at the late endosomal pH of 5.0–5.5. Hence lysosomal enzymes reversibly associate with M6P receptors. Clathrin/AP1 vesicles containing M6P receptors and bound lysosomal enzymes bud from the TGN, lose their coats, and subsequently fuse with late endosomes. Vesicles budding from late endosomes recycle the M6P receptors back to the TGN. Packaging of proteins such as insulin into regulated secretory granules is a very different process. This sorting is thought to be due to selective aggregation followed by budding. The TGN also may be the site of protein sorting to the apical and basolateral cell surfaces in polarized cells. This is the case in MDCK cells, a line of cultured epithelial cells, where there is direct basolateral-apical sorting at the TGN cells. In contrast, hepatocytes use different mechanisms for sorting to basolateral versus apical surfaces. Here newly made apical and basolateral proteins are first transported in vesicles from the TGN to the basolateral surface and incorporated into the plasma membrane by exocytosis. From there, both basolateral and apical proteins are endocytosed in the same vesicles, but then their paths diverge. The endocytosed basolateral proteins are recycled back to the basolateral membrane. In contrast, the apically destined endocytosed proteins are sorted into transport vesicles that move across the cell and fuse with the apical membrane in a process termed transcytosis.

11. Within the endocytic pathway, there is a progressive acidification (increased hydrogen ion concentration) in compartments going from early to late endosomes to lysosomes. The pH drops from almost neutral to pH 4.5. The binding of LDL to LDL receptor is pH sensitive. At the cell surface, neutral pH, LDL binds to LDL receptor. At an acidic pH, pH 5.5, LDL dissociates from its receptor. LDL is then transported to lysosomes and LDL receptor is sorted and recycled back to the cell surface. Mannose 6-phosphate bearing lysosomal enzymes dissociate from mannose 6-phophate receptors in the acidic pH late endosomal compartment. Elevating pH prevents this. Receptors become saturated with lysosomal enzymes and the cell no longer has the capacity to direct newly synthesized lysosomal enzymes to lysosomes. Instead, the enzymes are secreted from the *trans*-Golgi network by constitutive secretion.

12. In terms of membrane topology, both the formation of multivesicular endosomes by budding into the interior of the endosome and the outward budding of HIV virus at the cell surface are equivalent. Important mechanistic features are shared. Both processes involve an ubiquitination step. In multivesicular endosome formation, cargo proteins to be included in the budding endosome and the Hrs protein are ubiquitinated. For HIV budding, it is the HIV Gag protein that is ubiquitinated. In closing off the budding endosome or the budding HIV a cellular ESCRT protein complex recognizes the ubiquitin, and cellular Vps4 is used later to dissociate the ESCRT complex. The viral Gag protein mimics the function of cellular Hrs, redirecting ESCRT complexes to the plasma membrane. ESCRT binds to the C-terminal portion of HIV Gag protein. One logical peptide inhibitor/competitor of HIV budding is a synthetic peptide corresponding to the portion of Gag protein that binds to ESCRT. Such a peptide might well compete or interfere with normal cellular proteins such as ESCRT binding to ubiquitnated Hrs.

13. Phagocytosis is the actin-mediated process used by some cells to engulf whole bacteria and other

large particules. During the process, extensions of the plasma membrane envelop the ingested material, forming vesicles called *phagosomes* that are transported to the lysosome for degradation. Autophagy is the process whereby a double membrane organelle or autophagosome envelopes soluble cytosolic proteins, peroxisomes, or mitochondria and delivers them to the lumen of the lysosome for degradation. The three steps in the formation and fusion of autophagic vesicles are: 1) nucleation, whereby either a fragment of a membrane bound organelle, probably the ER, forms a vesicle to randomly envelop a portion of the cytoplasm, or purposely to form around a particular organelle; 2) growth and completion, involving new membrane contributed to the autophagosome membrane, thereby facilitating its growth into a cup-shaped organelle; and 3) targeting and fusion of the intact sealed and double membrane autophagosome and its contents to the lysosome.

Analyze the Data

a. Only specific v-SNARE and t-SNARE combinations result in fusion. In this case, v-SNARE 1 can induce fusion only with membranes containing t-SNAREs of the plasma membrane whereas v-SNARE 2 induces fusion only with membranes containing those of the Golgi. v-SNARE 3 appears to be a bit more promiscuous, permitting fusion with membranes containing either plasma membrane or vacuolar t-SNAREs, though it induces more rapid fusion with those of the vacuole.

b. Because v-SNARE 1 induces fusion with the plasma membrane, this v-SNARE might be expected to be on vesicles that emerge from the *trans*-Golgi network. Alternatively, it might be present on endosomal membranes that cycle in and out of the plasma membrane. v-SNARE 2 fuses with the Golgi and thus might be expected to be on vesicles that mediate transport between the ER and Golgi or within the Golgi. Alternatively, v-SNARE 2 might be on vesicles moving retrogradely from the plasma membrane back to the Golgi. v-SNARE 3 might be on vesicles that move between the Golgi and the

vacuole or between the vacuole and the plasma membrane.

c. In yeast, mutations in a specific v-SNARE gene would be useful for determining the specific function of that SNARE. Temperature-sensitive mutations could be quite helpful, as incubation at the restrictive temperature may result in an increase in the number of unfused vesicles carrying cargo and these might be assessed to determine where in the secretory pathway these vesicles likely reside.

d. The data show that the cytoplasmic domain of v-SNARE 2 competitively interferes with the ability of liposomes containing v-SNARE 2 to fuse with liposomes containing Golgi t-SNARES. Competition occurs only if the cytoplasmic domain is mixed with the liposomes containing the Golgi t-SNARES and not if it is incubated with liposomes containing v-SNARE 2. These findings indicate that the cytoplasmic domain of v-SNARE 2 binds to the Golgi t-SNARES and thereby interferes with the t-SNARES' ability to bind to intact v-SNARE 2. Yeast that overexpress the cytoplasmic domain of v-SNARE 2 would be predicted to exhibit a defect in the secretory pathway (similar to a dominant negative mutation in v-SNARE 2) at the fusion step in which this SNARE is needed.