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VESICULAR TRAFFIC, SECRETION, AND ENDOCYTOSIS

REVIEW THE CONCEPTS

1. The two modern methods for following intercompartamental transport are microscopy of GFP-labeled VSV G protein and detection of compartment-specific oligosaccharide modifications.
   These two methods share the following two basic features: 1) It must be possible to label a cohort of proteins in an early compartment so that their subsequent transfer to later compartments can be followed over time. 2) It must be possible to identify the compartment in which a labeled protein resides.

2. 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 mem-branes. It is the GTP-bound form of Sar or ARF that is active. The exact mecha-nisms 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 pro-teins. 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.

3. 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. ARF is the small GTPase that recruits COPI to Golgi membranes. Mutation of ARF 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 ARF to the GTP-restricted form would have the opposite effect; the ARF would now be permanently in the "on" state and the association of COPI with Golgi mem-branes would be permanently stabilized. Since GDP-restricted ARF produces the same phenotype as BFA, this suggests that the drug evokes a normal physio-logical possibility.

4. Coat proteins must assemble onto a membrane to form a vesicle and must disas-semble to allow the vesicle to fuse with a target membrane. Accumulation of Golgi enzymes in transport vesicle indicates that EAGE allows COPI assembly and formation of vesicles but blocks the uncoating of COPI necessary for retro-grade transport of Golgi enzymes for cisternal maturation. This inhibits cisternal maturation and anterograde transport of newly synthesized proteins from the ER to the plasma membrane.

5. Vesicle fusion involves two stages: first,adockingstagemediatedbylongcoiled-coil proteins such as EEA1, and then a specificmembranefusionstepmediated 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.

6. NSF, through its ATPase activity, catalyzes the dissociation of v-SNARE/t-SNARE complexes. Such complexes are essential in specificmembranefusionat

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.

7. Procollagen subunits, synthesized by ER-associated ribosomes and assembled in the ER, associate laterally into bundles in the Golgi. The procollagen bundles are too large to fit into the vesicles that go between Golgi compartments and have never been found in those vesicles. This observation supports the Golgi cisternal maturation model in which vesicles transport Golgi enzymes in the retrograde direction rather than transporting newly synthesized protein in the anterograde direction through the Golgi.

8. 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.

9. 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.

10. 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.

11. 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.

12. In infected MDCK cells influenza viruses bud only from the apical membrane, whereas vesicular stomatitis (VSV) viruses bud only from the basolateral membrane. Likewise, in the TGN, newly synthesized influenza HA coat protein is sorted into vesicles that fuse with the apical membrane and VSV G protein is sorted into different vesicles that fuse with the basolateral membrane. The virus coat proteins use the same processes by which cell plasma membrane proteins are sorted to the apical and basolateral membrane domains in polarized cells. Inhibition by a mimetic peptide but not by the mutant peptide indicates that the VSV G protein cytoplasmic domain contains a basolateral membrane targeting signal that most likely interacts with a component of the basolateral sorting and targeting mechanism, and that the tyrosine is part of that signal.

13. 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 dissociates from mannose 6-phosphate 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.

14. 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 ubiquitinated Hrs.

15. 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 it 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.

16. Plasma membrane LDL receptors (LDLRs) bind LDL from the extracellular environment at neutral pH. Once LDL has been internalized by receptor-mediated endocytosis, the low pH of the late endosome dissociates the LDL ligand from the LDLR, and the empty LDLR is recycled back to the plasma membrane while the LDL is digested in the lysosome.

17. The mutant LDLRs bind LDL normally but only randomly get internalized into clathrin-coated vesicles because the mutated cytoplasmic domains fail to interact with the AP2 complex. Inefficient receptor-mediated endocytosis (RME) of full-length LDLRs with a mutation in any one of the residues of the NPXY sequence in the LDLR cytoplasmic domain revealed that sequence functions as a RME sorting signal.