Revelation through exploitation: the viral model for intracellular traffic

Laura M. Roman and Henrik Garoff

Enveloped viruses exploit the existing routes of membrane traffic to enter and leave the host cell. The similarity between viral envelopes and cellular membranes has allowed the use of animal viruses as probes to examine aspects of intracellular traffic.

Throughout its life cycle, the cell has to generate and maintain, as separate entities, a diverse array of organellar membranes, each having a unique set of protein components. Since the biosynthesis of molecules destined for different subcellular organelles is restricted to a few sites (e.g. cytosol, endoplasmic reticulum, ER), transport systems must exist which ensure the movement of these proteins to their site of function.

The similarity between viral envelopes and cellular membranes has allowed the use of several animal viruses as probes to examine aspects of intracellular traffic. The advantages of using enveloped viruses include: (1) their simple structure; (2) the efficient synthesis of viral proteins in the host cell during infection; (3) the availability of conditionally lethal mutants with defects in viral protein transport; (4) the existence of viruses which mature at different intracellular locations (Fig. 1); (5) the functional activities of various viral spike glycoproteins (e.g. membrane fusion, neuraminidase); and (6) the distinctive morphology of the virus particle (see Refs 2 and 3 for general review).

This article illustrates the use of enveloped viruses as tools in the study of membrane traffic in animal cells.

General features of simple enveloped viruses

Enveloped viruses consist of a lipid bilayer, studded with spike glycoproteins, which surrounds the nucleocapsid. The spike complex spans the bilayer, dividing the spike protein into three topologically distinct domains; a large hydrophilic luminal domain, a hydrophobic intramembranous domain and a hydrophilic cytoplasmic domain. The latter is thought to interact with the nucleocapsid proteins, either directly or

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through an M-protein layer interspaced between the lipid bilayer and the nucleocapsid[2,3]. Some of the biochemical properties of particular viral spike glycoproteins are summarized in Table I. Since only the structural proteins and a few functions required for viral replication are specified by the viral RNA (notably the RNA-dependent polymerase), the virus exploits the existing routes of membrane traffic to enter and leave the host cell.

The endocytic pathway

Vesicular Stomatitus virus (VSV), influenza virus (flu) and Semliki Forest virus (SFV) bind to a variety of cultured cells of mammalian, avian and invertebrate origin, suggesting that surface structures common to many cell types act as receptors (see Ref. 4 for review). Except for the involvement of sialic acid residues in influenza binding, the exact receptors for these viruses have not been identified.

After binding to the cell surface, the virions are internalized. Infection is initiated by the liberation of viral RNA into the cell cytoplasm. The endocytosis of virus particles has a number of features in common with the receptor-mediated uptake of physiological ligands (e.g. low density lipoproteins, asialoglycoproteins, epidermal growth factor) which are usually directed to lysosomes. These common properties include kinetics, temperature dependence, and the involvement of coated pits and vesicles,
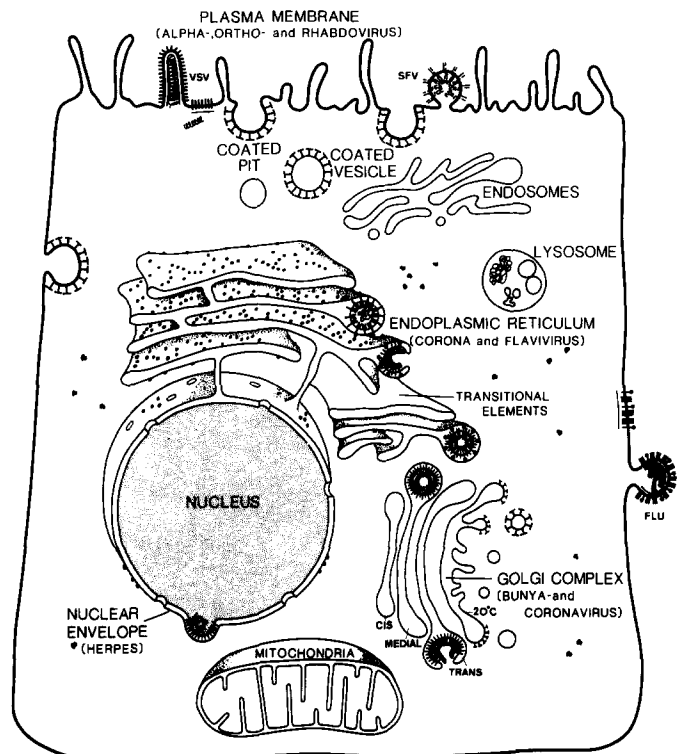

Fig. 1. Sites of maturation for different enveloped viruses in a eukaryotic cell. The spike proteins of viruses that mature at the plasma membrane (alpha-, orthomyxo-, and rhabdovirus) are synthesized in the endoplasmic reticulum (ER), transported through the Golgi complex, and externalized at the cell surface where their interaction with capsid or M protein is thought to drive the budding process. Similar interactions appear to be involved in the maturation of viruses that bud into internal compartments. Herpes virus (an enveloped DNA virus) buds into the nuclear envelope[1] and flavivirus into the ER. The site of coronavirus maturation (ER or Golgi) depends on the cell type examined. Bunyavirus buds into the Golgi complex. Following release into these compartments, these viruses follow the ER→Golgi→PM pathway where they are released into the extracellular space (adapted from Ref. 2).

and of endosomes and lysosomes[4]. The analysis of the entry mechanism of enveloped viruses has revealed some of the properties of these endocytic compartments. Studies using SFV *in vitro* showed that a low pH is required for membrane fusion and the release of the nucleocapsid[5]. It was initially thought that the release of viral RNA was a result of the exposure of the virus to the acid environment in lysosomes. However, subsequent kinetic, temperature-shift and inhibitor studies demonstrated that the virus releases its genome in an acidic compartment before reaching the lysosome, i.e. in endosomes[4]. Further experiments with SFV and other viruses have defined some of the biochemical characteristics of the endosomal compartment, such as pH and cholesterol content[4-6].

Detailed examination of the pH-mediated fusion step has defined some of the parameters important in virus fusion, and, perhaps, membrane fusion in general. In transfected cells the expression of the spike proteins from cloned complementary DNA (cDNA copied from the viral genome), in the absence of other viral proteins, results in pH-dependent cell–cell fusion[7-9]. This shows that the fusion event is mediated by the spike proteins. Detailed information on the role of a fusion protein in this process has been obtained with hemagglutinin (HA) of flu since its three-dimensional structure is known[10]. At pH 5–6, HA undergoes a specific, irreversible conformational change resulting in the exposure of previously hidden protease sites and an increased ability to bind detergent. The latter is thought to be due to the exposure of the previously hidden hydrophobic N-terminal peptide of HA2. From these and other observations it is postulated that the pH-induced changes enable the HA to interact with the target membrane, leading to local perturbations, coalescence and separation[6].

The fusion activity of bindin, a protein of sea urchin sperm responsible for the adhesion of the sperm acrosomal process to the vitelline layer covering the egg plasma membrane, was recently shown to have striking structural and mechanistic similarities to HA[11]. This suggests that the function of viral fusion proteins may well mimic cellular activities. To elucidate the parameters involved in the specificity and direction of these endocytic processes, J. Davey *et al.* (*Cell*, in press) have established a cell-free endocytosis system. They have

Table I. Properties of the enveloped virus glycoproteins (adapted from Refs 2 and 3)

Virus	Components	Molecular Weight	Precursor	Function
Semliki Forest virus	E1	51 000	} P62, 62 000	fusion?
	E2	52 000		?
	E3	11 500		?
influenza	HA1	44 000	} HA$_o$, 74 000	attachment
	HA2	30 150		fusion
	NA	48 000–63 000		neuraminidase
vesicular stomatitis virus	G	62 000–60 000		attachment and fusion

mixed a membrane fraction containing internalized SFV (labeled in its sialic acid moieties) with a membrane fraction from other cells, which contains endocytosed influenza. The fusion between the two endocytic fractions was monitored by the release of labeled sialic acid from SFV as a result of its interaction with the neuraminidase of influenza.

Spike protein synthesis and post-translational modifications

Using an *in vitro* system, Blobel and Dobberstein[12] first described the mechanism, summarized in the signal hypothesis, by which secretory proteins are translocated into the lumen of vesicles derived from the ER and processed in a co-translational manner. This hypothesis has since been expanded to include two receptor molecules, signal recognition protein (SRP) and docking protein which mediate the selection of proteins incorporated into the ER membrane[13]. Studies of the biosynthesis and asymmetric insertion of integral membrane proteins were first carried out with the viral spike proteins, because pure mRNA was available and the topography of these proteins was known. Using mRNA for the spike glycoprotein G of VSV, it was shown that: (1) membrane proteins use the same mechanism of translocation as secretory proteins; and (2) insertion into the membrane and glycosylation are co-translational events, with carbohydrate being added within the lumen of the ER[14].

While secretory and membrane proteins use the same mechanisms for translocation across the ER membrane integral proteins must contain additional information signalling their anchorage in the bilayer. The availability of cDNA encoding for the spike proteins of VSV, flu and SFV has permitted a more detailed examination of the features

required to attach integral proteins to the membrane. The observation that deletion mutants lacking the region encoding the hydrophobic transmembrane domain result in secreted forms of the spike proteins[15,16] implicates this region as a stop transfer sequence. The construction of chimeric molecules in which the transmembrane domain of VSV-G has been fused to a secretory protein[17] or the ectodomain of another membrane protein (Ref. 18 and M. J. Gething, pers. commun.) has indicated that the anchoring function of integral membrane proteins resides in this stretch of hydrophobic amino acids. Directed mutagenesis of the anchor region is in progress in several laboratories and will in the future define the parameters which are important in membrane binding.

Although the present data are sufficient to explain the insertion of G- or HA-type glycoproteins (Group 1, Fig. 2) across the lipid bilayer, the mode of insertion of proteins with the opposite orientation in the membrane (Group 2) and proteins that span the membrane several times (Group 3) remains an enigma. Other viral proteins which have these alternative orientations include, respectively, the neuraminidase of influenza and the E1 glycoprotein of the mouse hepatitis virus, a coronavirus. Since the cDNA encoding these proteins have been characterized[17,18], experiments similar to those used for VSV-G and the SFV spike proteins should provide insight into how they achieve their asymmetric distribution in the bilayer.

The pathway to the plasma membrane: involvement of the Golgi apparatus

In pioneering autoradiographic and biochemical studies, Palade and his colleagues (Palade, G. (1975) *Science* 189, 347–358) showed that secretory proteins

move from the ER to the Golgi complex where they undergo post-translational modifications *en route* to the plasma membrane. The details of the involvement of the Golgi in the routing of plasma membrane proteins were first shown with the glycoproteins of enveloped viruses[21,22]. Bergmann and Singer[21] used a temperature-sensitive mutant of VSV which accumulated G-protein in the ER at the non-permissive temperature. When shifted to the permissive temperature, the accumulated VSV-G passed as a synchronous wave into and through the Golgi complex, allowing the precise details of transport to be mapped.

While passing through the Golgi complex, the viral glycoproteins undergo an ordered sequence of structural modifications including carbohydrate processing[23], fatty acylation and sulfation. Using the spike proteins of SFV as markers and monensin (a carboxylic ionophore which blocks transport within the Golgi complex), it has been shown that the Golgi complex is comprised of a series of discrete, biochemically defined compartments (*cis*, medial, *trans*[24]), through which the viral problems pass sequentially. The transport of one viral protein, VSV-G, between these compartments has been reconstituted in a cell-free system[25]. In this *in vitro* assay, a clone of VSV-infected CHO (Chinese hamster ovary) cells deficient in the (medial) Golgi enzyme *N*-acetylglucosaminyl transferase is used as a source of a 'donor' Golgi membrane fraction containing newly synthesized VSV-G protein lacking terminal *N*-acetylglucosamine. This fraction is mixed with 'acceptor' Golgi membranes obtained from uninfected wild-type cells containing the transferase. Fusion between the donor and acceptor fractions indicates transport between successive Golgi compartments and is monitored by the incorporation of labeled *N*-acetylglucosamine into VSV-G. In this system, transport is dissociative (i.e. carried out by small vesicles) and vectorial, such that there is only a defined period of time in which the donor membranes can transfer VSV-G to the acceptor compartment. This interaction requires ATP, cytosol and exposed proteins on both membrane fractions. These studies provide information about some of the factors and intermediates involved in intra-Golgi transport and illustrate the advantages of an *in vitro* approach; however,
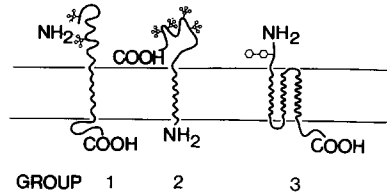

Fig. 2. Three possible dispositions for membrane proteins that span the bilayer. Group 1 proteins span the bilayer once and have their COOH-terminal in the cytoplasm. A representation of E2 of SFV is drawn. A heterodimer of E2 and E1 forms the spike complex of SFV. Group 2 proteins span the bilayer once and have their NH₂-terminal in the cytoplasm. The neuraminidase of influenza is drawn[19]. In the virus, this molecule is found as a disulfide-linked tetramer. Group 3 proteins span the bilayer more than once. E1 of a coronavirus is drawn. From the available DNA sequence data, it has been predicted that the molecule spans the bilayer three times[20]. ✤ =N-linked sugar group, ○-○ = 0–linked sugar group.

the molecular components responsible for initially directing the viral glycoproteins to the Golgi and controlling the specificity of the various fusion events remain elusive.

Protein signals for transport to the plasma membrane

It is unclear whether the movement of a protein from the ER to the plasma membrane (via the Golgi) is an active

(i.e. involving specific transport signals) or a passive process. It has been postulated that structural features of the protein are involved in this sorting process. One current idea is that transmembrane proteins destined for the plasma membrane interact with cytoplasmic elements which direct their transport. The role of the cytoplasmic tails of VSV-G, HA and E2 (of SFV) in intracellular transport has been examined by the expression of cDNAs from which variable amounts of the 3' end (carboxy-terminal) had been removed. Some of these mutants fail to be transported from the ER, probably because of gross conformational changes induced by the mutations. The results with the forms that are transported to the plasma membrane can be summarized as follows: (1) alterations in the cytoplasmic domain often decrease the rate of transport between the ER and the Golgi without affecting the rate from the Golgi to the plasma membrane; and (2) there appears to be no dominant or conserved region of the carboxyterminal domain which dictates the efficiency of transport. Mutants having different tail lengths and charge distributions display phenotypes which are indistinguishable from the native molecule[7,26,27]. These findings suggest that the cytoplasmic tail
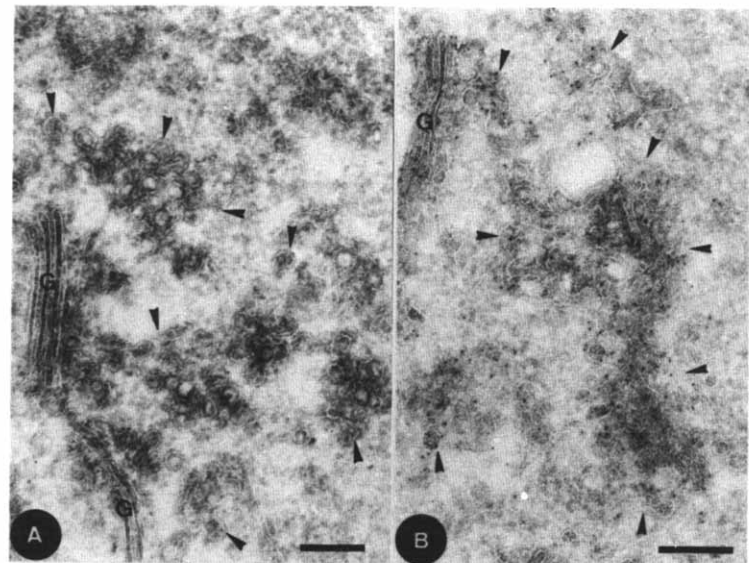

Fig. 3. A. Frozen thin sections of a BHK cell infected with a temperature-sensitive mutant of VSV (ts045) for 4 h at the permissive temperature (39°C) and then switched to 20°C for 2 h. The accumulation of G protein in an extensive reticular structure (arrows) on the trans side of the Golgi (G) alters the morphology of this compartment. Such structures are not identifiable in uninfected cells. B. A similar preparation has been labeled with an antibody to the cytoplasmic tail of G protein followed by protein A gold (gold particles of 5 nm) to demonstrate the presence of this protein in the trans Golgi compartment (courtesy of G. Griffiths). Bars = 0.2 μm.

alone is not the recognition signal for transport to the plasma membrane.

The asymmetric distribution of surface proteins, characteristic of polarized epithelial cells, implies the existence of sorting mechanisms which target molecules to their site of function in the plasma membrane. When MDCK cells (Madin-Darby canine kidney, a polarized epithelial cell line) are infected with enveloped viruses, a striking asymmetry of virus budding is observed[28,29]. Influenza virus is selectively assembled at the apical surface whereas VSV and SFV mature from the basolateral plasma membrane. Thus, viral envelope proteins, like endogenous epithelial cell membrane proteins, are segregated into specific surface domains. Since it has been established that the carbohydrate moieties are not involved in their segregation[28], some other feature located in at least one of the three topological domains of a transmembrane protein must be recognized by the sorting machinery. The polar expression from engineered cDNA of truncated forms of HA and SFV-E2, lacking either the cytoplasmic tail or both the tail and the transmembrane region, suggests that these domains are not needed for directed transport in AGMK (African Green Monkey kidney) and MDCK cells, respectively (M. Roth et al., pers. commun.; L. Roman and H. Garoff, in preparation). Presumably, the putative sorting signals must reside in the extracytoplasmic domain of these membrane proteins.

In which cellular compartment are apical and basolateral proteins segregated from one another? In doubly infected cells, it appears that VSV-G and flu HA travel through the Golgi in tandem, implying that the segregation into the basolateral or apical membrane is a post-Golgi event[30]. When BHK (baby hamster kidney) or MDCK cells infected with flu or VSV are incubated at 20°C, the viral proteins, while fully glycosylated, are not transported to the plasma membrane but accumulate in a clathrin-coated tubular compartment near the *trans* Golgi containing acid phosphatase[31,32] (Figs 1 and 3). Whether this compartment is the site in which segregation occurs remains to be determined.

The budding process

Given the multiplicity of fusion:fission events in intracellular transport, how does the cell maintain organellar speci-
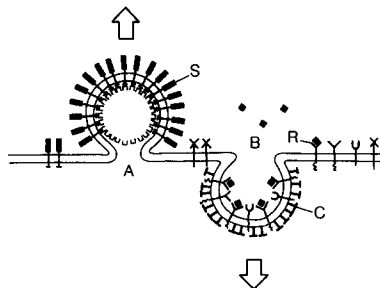

Fig. 4. Representation of the similarities between virus budding and the segregation of proteins into coated pits. The budding of a virion and coated, pit-mediated internalization of plasma membrane components share the ability to concentrate a subset of plasma membrane molecules. For the virus (A) it has been suggested that the affinity between the capsid protein (n) and the spike complex (S) enables the capsid to selectively concentrate the spike glycoproteins. For the coated pit (B) the topology of the vesicle is reversed; the clathrin coat (C) with associated proteins appears to select certain plasma membrane proteins and exclude other membrane molecules (⌽). R = receptor.

ficity? It is clear that vesicle carriers do not randomly transport membrane components. This implies either that the proteins belonging to an organelle are selectively retained while other proteins are passively transported, or that proteins destined for other locations are specifically removed. In either case, a stabilizing force is needed either to retain proteins within an organelle or to collect them for subsequent transport. An outstanding example of such stabilizing infrastructure is provided by the budding envelope virus. The affinity between the capsid protein (SFV) or the matrix protein (VSV) found immediately under the plasma membrane ensures the selection of the viral glycoproteins and the exclusion of host proteins. The analogy between virus budding, and the concentration of receptors and/or ligand–receptor complexes in coated pits can easily be drawn (Fig. 4). Further studies on spike–capsid and spike–matrix protein interactions should shed light on the mechanism by which membrane proteins are recognized and clustered in cellular membranes.

Closing remarks

It is clear that enveloped viruses that interact primarily with the plasma membrane have served as ideal probes to investigate many aspects of intracellular transport. Several viruses which bud into intracellular compartments (Bunyaviruses, and Coronaviruses, ER and

Golgi) have yet to be exploited (see Refs 2 and 3). The high density of the viral proteins in these membranes should facilitate the immunoisolation and subsequent characterization of these organelles. Through the kinds of molecular mapping experiments carried out on the plasma membrane spike proteins, information will be obtained as to the domains of these molecules involved in their restricted distribution. Similarly, examination of the secretory pathway taken by internally budding virions in polarized epithelial cells may provide additional insight on the signals involved in these directed transport processes. In the future, the various biochemical characteristics of these viruses combined with *in vitro* reconstitution systems should further define the molecular mechanisms of intracellular transport.

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Intracellular transport mutants of yeast

Peter Novick

Temperature-sensitive yeast secretory mutants are blocked in the transport of all major cell surface proteins. The early portion of the export pathway is also used for transport of newly synthesized vacuolar proteins, while the late portion of the export pathway shares its gene product requirements with the endocytic pathway. Actin may play a role in the polarized transport of vesicles.

Eukaryotic cells possess a variety of membrane-bound organelles. The assembly of these specialized structures requires the transport of specific sets of proteins to particular cellular locations. Transport events include: the insertion of newly synthesized proteins into the nucleus, the mitochondria, the plasma membrane, and the lysosome (in yeast, the vacuole); secretion of proteins into the extracellular space; and the endocytic retrieval of surface proteins and external compounds. While cells in the specialized organs of higher eukaryotes may use variations of the basic transport schemes, the transport of specific proteins to their target organelles is probably accomplished by similar mechanisms in all eukaryotes. Yeast offers an ease of genetic manipulation unequalled by other eukaryotes. This article will summarize what has been learned through the isolation of yeast mutants defective in various aspects of intracellular transport. Nuclear and mitochondrial transport will not be covered.

Isolation and characterization of secretion mutants

To isolate the first secretory mutants (*sec1–1* and *sec2–1*), the behavior of a *sec* mutant was predicted and colonies derived from mutagen-treated cells were screened for strains exhibiting the pre-

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dicted phenotype[1]. Three predictions were made: (1) protein secretion is an essential process; (2) a defect in the secretion machinery would affect the export of a range of proteins; and (3) a block in protein secretion would not prevent general protein synthesis. We began by identifying conditional-lethal strains. We then screened this collection for strains which failed to export the periplasmic proteins invertase and acid phosphatase at the restrictive temperature but did export them at the permissive temperature. Finally, strains which were defective in general protein synthesis were discarded. Only two mutants were identified by this 'brute force' screen. The low overall efficiency stressed the need for an enrichment step, the clue to which was the observation that *sec1* cells failed to increase in volume at the restrictive temperature, but continued to increase in mass[1], a situation similar to that observed upon starvation of an inositol auxotroph[2]. In the latter case, Henry *et al.*[2] demonstrated that the starved cells could be resolved from normal cells on a density gradient. A similar gradient resolved *sec1–1* cells from wild-type cells[3]. By incubating mutagen-treated cells at 37°C for 3 h and using a density gradient to isolate the densest cells, we achieved a 50-fold enrichment for temperature-sensitive secretion mutants[3].

Two classes of *sec* mutants were obtained. Class A mutants accumulate active secretory enzymes in an intra-

cellular pool as a result of the block in export[3]. Class B mutants do not secrete or accumulate active secretory proteins at the restrictive temperature despite the nearly normal levels of general protein synthesis[4]. Only the Class A mutants will be discussed here. (The characterization of the Class B mutants is described in an article by Susan Ferro-Novick in this issue (p. 425)).

In total, 200 Class A *sec* mutants were identified[1,3]. These define 23 complementation groups. Five groups contain only one member, suggesting that new groups remain to be found. These results imply that the secretory machinery of yeast is indeed complex.

In many of the *sec* mutants, invertase secretion can be reversibly blocked; returning the mutants to the permissive temperature allows the accumulated invertase to be exported[1,3]. Inhibition of protein synthesis by cycloheximide does not eliminate the reversibility, indicating that continued protein synthesis is not a prerequisite for secretion and that in these cases the defective gene product can regain activity. In all cases, addition of energy poisons inhibits reversibility[5], implying the existence of one or more energy-requiring steps on the secretory pathway. Experiments designed to order the energy-requiring step(s) with respect to the *sec* blocks suggest that there may be three or more energy-requiring steps[5].

The reversible accumulation of secretory proteins in the *sec* mutants indicates that a functional intermediate of the pathway develops at the restrictive temperature. Thin-section analysis has helped to establish the nature of these intermediates[1,3]. Members of 10 complementation groups accumulate 80–100 nm vesicles. Members of 9 groups develop an exaggerated endoplasmic reticulum (ER) lining the inner surface of the plasma membrane and connected to the lumen of the nuclear envelope. Members of two groups (*sec7* and *sec14*) accumulate structures related to that of