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Cell biology of viruses that assemble along the biosynthetic pathway

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In this review we discuss five groups of viruses that bud into, or assemble from, different compartments along the biosynthetic pathway. These are herpes-, rota-, corona-, bunya- and pox-viruses. Our main emphasis will be on the virally-encoded membrane glycoproteins that are responsible for determining the site of virus assembly. In a number of cases these proteins have been well characterized and appear to serve as resident markers of the budding compartments. The assembly and dissemination of these viruses raises many questions of cell biological interest.

Key words: biosynthetic pathway / intracellular budding viruses / membrane glycoproteins / virus assembly

VIRAL MEMBRANE proteins have been extensively used as tools to follow many fundamental processes of intracellular membrane traffic.[1-5] The use of three enveloped viruses, namely Semliki forest virus (SFV; as well as the closely related sindbis virus), vesicular stomatitis virus (VSV) and influenza virus, has been particularly instrumental in elucidating many details of both mechanisms and pathways involved in the ER biosynthetic pathway and endocytosis.

There are many reasons for the widespread use of these viruses as model systems in cell biology. First, they are surrounded by a lipid bilayer, referred to as the envelope, which has a simple protein composition. Second, after infection protein synthesis of the host cell is usually blocked and mainly viral proteins are made. Third, the infected cells synthesize very large amounts of a relatively small number of proteins that are essential for the virus. For example, a BHK cell infected with SFV synthesizes about 10^5 molecules of each viral protein per minute.[6] These three points facilitate both biochemical and morphological analyses. Fourth, the ease with which mutants can be made and expressed make these viruses ideal candidates for genetic studies. Fifth, viral proteins are usually highly antigenic which facilitates the production of high titre antibodies. The final and crucial point is that since the genetic capacity of these viruses is so limited they must follow the basic cellular mechanisms for cell entry (usually via endocytosis), protein synthesis (using the ribosome/ER synthetic and translocation machinery), glycosylation and other post-translational modifications, as well as for intracellular transport from the ER to the plasma membrane. In addition, since these viruses fuse with cell membranes during the infection process they are powerful tools to study membrane fusion: in this respect the haemagglutinin (HA) of influenza virus has now become the best characterized of all fusion proteins.[7]

In the case of viruses such as SFV, VSV or influenza, the viral membrane glycoproteins are synthesized and transported to the plasma membrane in a manner indistinguishable from cellular plasma membrane proteins. It is the accumulation of these proteins at the cell surface which is responsible for the viral budding. This budding event is a very accurate sorting phenomenon since essentially only viral proteins are included in the bud, and all host proteins are excluded. Budding is the result of a protein-protein interaction between the cytoplasmic domain of a key viral membrane ('spike') glycoprotein and the cytoplasmically localized nucleocapsid. The latter is usually composed of a single copy of the genomic RNA or DNA and, in the simplest cases, multiple copies of a single capsid protein. Since the cytoplasmic domains of the viral membrane proteins are, in principle, available for this interaction throughout the biosynthetic pathway from the rough ER onwards, it is not immediately obvious what restricts the budding to the plasma membrane. The answer, in part, appears to lie in the fact that a critical density of viral spike proteins is required for the interaction. A demonstration of this fact came from experiments showing that monensin inhibited transport of SFV spikes out of

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the Golgi complex. These proteins continued to be synthesized and accumulated as a result in vesicles that corresponded to monensin-induced alterations of medial Golgi cisternae. Under this condition the nucleocapsids could bind in high concentrations and even bud into the Golgi complex.[8] However, for other viruses such as VSV the situation is more complex. When the G glycoprotein of this virus was allowed to accumulate in the *trans*-Golgi network (TGN) as a result of a transport block at 20°C there was no evidence for either the matrix (M) or the nucleocapsid (N) protein on the membranes of this compartment.[9]

Although the majority of enveloped viruses normally bud through the plasma membrane, there are a number of viral families whose members bud naturally into intracellular compartments of the biosynthetic pathway.[10,11] This behaviour reflects the fact that the viral proteins which mediate these budding events are endowed with targeting information that retains them after synthesis in a subcompartment of the ER or Golgi complex. Since these proteins essentially behave as resident markers of the compartments into which the viruses bud it follows that they are of interest for cell biologists.

In this review we shall focus on the assembly of five different types of viruses that are made intracellularly at different sites and in different ways, namely herpes, rota, corona, bunya and pox (Figure 1). Our goal will be to highlight some features of these viruses, and in particular their membrane glycoproteins. Aside from the fact that many of these viruses bud into intracellular compartments, the assembly of some of these viruses involves unusual and unprecedented phenomena, many of which have not been extensively studied, let alone explained at the molecular level. These events include the loss of a membrane in the lumen of the ER (rota), the passage of the virions from the nucleus to the cytoplasm by traversing the nuclear envelope (herpes) and the successive envelopment by several membranes giving rise to a four-membraned form of the virus (poxvirus). It is a reasonable guess that a mechanistic explanation of these phenomena may give new insights into fundamental cell processes. A summary of the major features of these viruses is given in Table 1.

Rotavirus

Rotaviruses bud into the endoplasmic reticulum. Although the data shows that this budding occurs through ribosome-free regions of the ER, the fact that it also occurs into the nuclear envelope argues strongly that the site of budding of this virus is the rough ER.[12] The structure of rotavirus, a non-enveloped virus, is unusual in that it consists of two distinct icosahedral outer protein shells that surround the nucleocapsid core; this organization is also seen in other viruses of the Reoviridae family. When the virus buds into the rough ER, it acquires a lipid bilayer enriched in two viral glycoproteins, a structural glycoprotein VP7 and a non-structural protein NS 28. Subsequently, the lipid bilayer is somehow lost leaving the VP7, but not NS28, embedded in the outer capsid layer of the mature, infectious virus particle (for reviews see refs 13-15).

The data at present suggest the following model for the assembly process (Figure 2). First, the nucleocapsid harbouring the 11 double-stranded RNA segments is assembled in specialized areas of the cytoplasm that are referred to as viroplasm. Although morphologically distinct, the molecular organization of these regions in this, as in most, viruses is not known, but presumably involves a reorganization of the cytoskeleton. The outer surface of the nucleocapsid (the inner 'shell' of the virus) consists predominantly of VP6, apparently a myristylated protein that exists as trimers and which represents approximately 50% of the total protein in the mature virus. This protein by itself is able to assemble *in vitro* to form spherical particles closely resembling the 'single shelled' nucleocapsid particles seen in the cytoplasm of infected cells;[16] however in that study this process only occured at pH4.

In vitro binding studies now argue strongly that the crucial event for the budding into the ER is an interaction between the VP6 and the cytoplasmic domain of NS28.[17,18] Somewhat mysterious in this process is the role of the viral haemagglutinin protein VP4 (in the earlier literature referred to as VP3). VP4 is believed to be synthesized in the cytoplasm and to assemble with the nucleocapsid at a relatively late stage in the formation of the capsids, even perhaps on the cytoplasmic surface of the ER.[18] This protein is nevertheless exposed on the surface of the mature virions.

VP4 appears to form an oligomeric complex with the two ER membrane glycoproteins VP7 and NS28,[19] a process that is Ca^{2+} and carbohydrate-dependent.[20] After budding into the ER, the immature (single-shelled) virus is enclosed by a lipid bilayer in which the two glycoproteins NS28 and VP7 are embedded. Isolation of these membrane-encapsulated

intermediates has demonstrated that VP7 is translocated across the membrane during maturation.[21] The lipid bilayer as well as the NS28 is somehow 'discarded' from the virus surface giving rise to the mature, non-enveloped virus particles in the lumen of the ER. Electron micrographs of sections of infected cells reveal the presence of membrane fragments in the lumen of the ER adjacent to mature virions during this process.[20] However, the mechanism of this remarkable event is still a mystery despite the fact that the key proteins involved have been extensively studied. One suggestion is that a calcium-dependent phospholipase activity might be involved.[20] Also enigmatic is the question how cytoplasmic VP4 enters the ER and ends up as a fibre on the outside of the mature virus particle.[22] A recent study using the baculovirus expression system, where both VP6 and VP7 were co-expressed in insect cells, showed that 'double-shelled' particles, very similar to the mature (non-enveloped) rotavirus particles, could be assembled.[23] This suggests that the VP4, is not critical for this assembly process.

Both NS28 and VP7 behave as typical ER resident membrane glycoproteins (Figure 3). NS28 has a membrane spanning domain and a non-cleaved hydrophobic domain at the NH_2 terminus that contains the N-linked glycosylation sites.[24-27] The available evidence suggests that the second of the three hydrophobic domains of this protein is responsible for its retention in the ER.[28] It has a 131 amino acid COOH terminus that, as mentioned, interacts with the inner shell protein VP6. VP7 is a highly unusual protein. It is translocated across the membrane of the ER as a typical membrane glycoprotein that contains a cleavable signal sequence. However, the cleaved signal sequence must somehow interact with the rest of the protein since, when the signal sequence of influenza HA is substituted for the authentic one, the expressed protein is secreted into the medium.[29] It seems likely that the signal sequence is also somehow involved in maintaining the VP7 in a membrane-bound form. Recent unpublished data from Maass and Atkinson shows that three animo acids, Ile, Thr and, Gly, in positions 9-11 from the NH2 terminus of the mature protein, are essential for retaining VP7 in the ER membrane; any conservative alteration or deletion of these three residues results in the protein being secreted (D. Maass, P. Atkinson, personal communication).

Although its signal sequence is cleaved, the mature VP7 in the ER membrane still behaves as a typical integral membrane protein that is not affected by high salt or high pH treatments.[14,24] While the mechanism is not understood, it is clear that this protein must undergo a major conformational change as it switches from being a membrane-associated protein into being the major, non-membrane protein exposed on the surface of the mature virus in the lumen of the ER. This is reflected in the difference in reactivity of the two forms of the protein to different anti-VP7 antibodies. These different reactivities correlate with the maturation of the virus from the enveloped to the non-enveloped form.[30] It should be noted that the VP7 is the only glycoprotein present in the mature virus.

That VP7 and NS28 are typical ER resident proteins is also shown by the nature of the N-linked oligosaccharides.[24,30,31] For VP7 the predominant structure is the N-acetyl glucosamine 2, Man6 or Man8 form. Mutants of VP7 that are transported to the cell surface undergo typical Golgi modifications.[32] The NS28 is predominantly in the Man9 form, with lesser amounts of Man8. These data suggest that NS28 has not been significantly exposed to the ER mannosidase that trims the oligosaccharides down to Man6. This may imply that this protein is retained in a domain of the rough ER proximal to the site where the bulk of the ER mannosidase (and mature VP7) is localized. An alternative explanation is that the oligosaccharides of NS28 are poor substrates for the ER α mannosidases.

Both the assembly and the stability of rotavirus particles are critically dependent on the presence of a threshold concentration of calcium in the culture medium. When purified, infectious virus particles are treated with chelating agents, the outer, VP7- and VP4-containing shell is lost resulting in a loss of infectivity.[33] Shahrabadi and Lee[34] showed that, with concentrations of calcium in the culture medium below 0.17 mM, the production of infectious particles was blocked *in vivo*. The data of this group suggest that the calcium is essential for the VP7 to attain its correct conformation and that in the absence of calcium this protein is degraded much more rapidly, presumably by the ER degradative pathway.[35] A recent *in vivo* study by Poruchynsky *et al*[20] showed that in the presence of a calcium ionophore the maturation of the membrane bound to the mature particles was blocked. Further, this treatment (as well as tunicamycin) prevented VP7 from interacting with NS28 and VP4 into the hetero-oligomeric complexes that are normally seen. Low calcium clearly affected

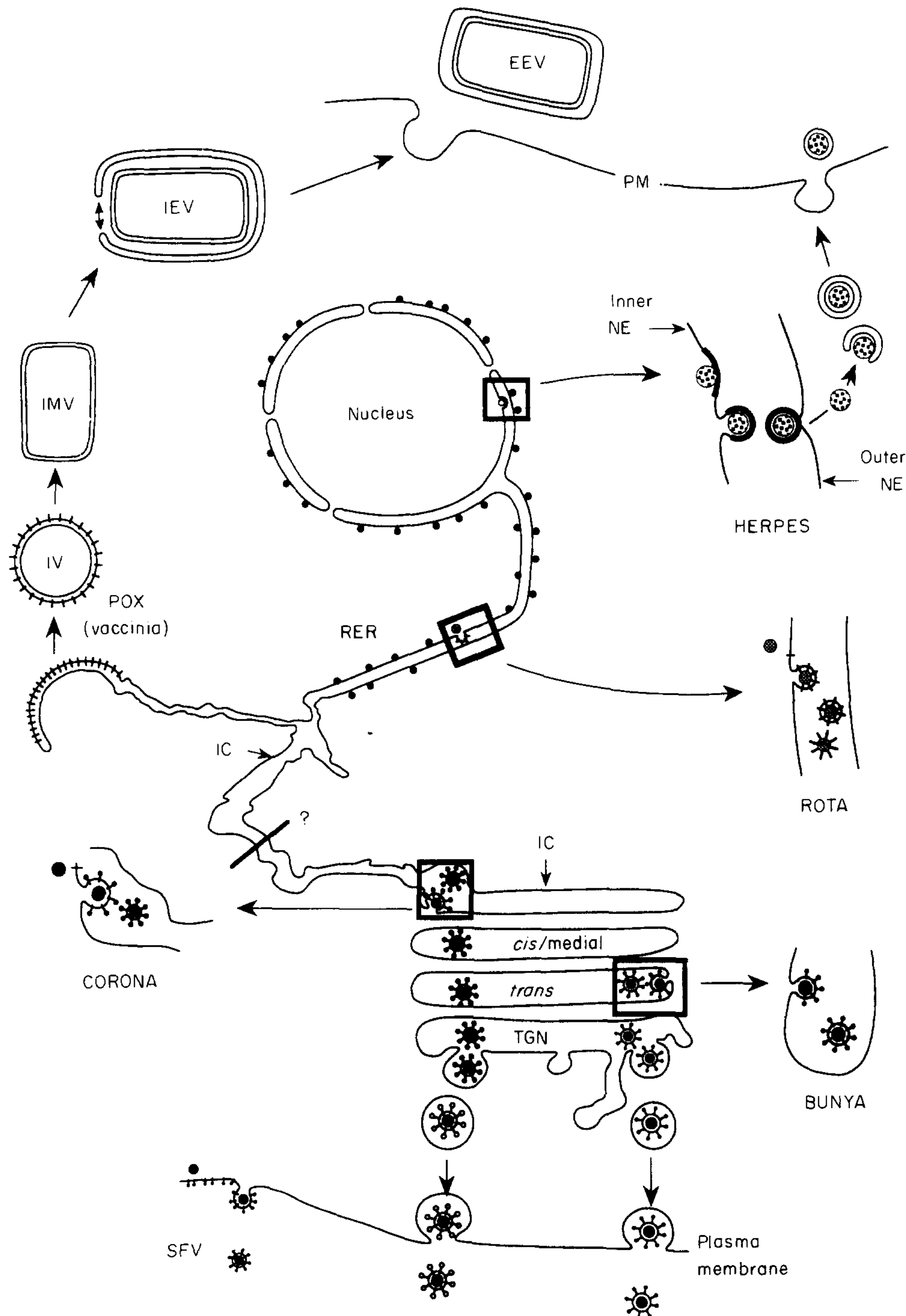

Figure 1. Schematic diagram of the biosynthetic pathway showing the assembly of the five viruses discussed in this review. For herpes the dense plaques on the inner nuclear envelope (NE) are shown as well as the notion that the virus, once budded into the lumen of the nuclear envelope, fuses with the outer nuclear envelope, thereby releasing the capsid into the cytoplasm. The latter is then enveloped by a cisterna that is believed to be of Golgi origin. This form, like the vaccinia IEV is thought to fuse with the plasma membrane releasing enveloped, infectious viruses into the medium. For bunya and corona the viruses are widely believed to exit the cell via the vesicular transport pathway. For reference, the budding of Semliki Forest virus (SFV) at the plasma membrane is also shown. The intermediate compartment (IC) is shown as being continuous from the rough ER to the first morphologically recognizable Golgi cisterna. We emphasize that the available data do not allow an unequivocal distinction between this model, which we prefer, and the alternative idea of a physically separate intermediate compartment that would necessitate an extra vesicular transport step. The line/question mark is meant to indicate this point.

Table 1. Biological features of the viruses discussed in this review

Virus family/ genus	Rota	Corona	Bunya	Pox	Herpes
Prominent members	Simian virus	Mouse hepatitis virus Infectious bronchitis virus	Uukuniemi virus Punta Toro virus	Vaccinia Variola major virus	Epstein Barr virus Herpes simplex virus Cytomegalovirus
Infectious form genome	Non-enveloped, double-stranded RNA	Enveloped, single-stranded, + RNA	Enveloped, single-stranded or double-stranded, ± RNA	Enveloped, double-stranded DNA	Enveloped, double-stranded DNA
Genome size	18.5 kb	30 kb	13 kb	191 kb	150 kb
Membrane proteins	(NS28), VP7	M, S, (HE)	G1, G2	many	many
Intracellular assembly sites(s)	ER	Intermediate compartment, ER	Golgi	Intermediate compartment, TGN or endosome	Nuclear envelope, Golgi?
Proteins responsible for budding	VP6, NS28, VP7	M, S?	G1, G2	p62, p14, p37	gp110?
Mechanism of virus release	Cell lysis	Vesicle transport/exocytosis	Vesicle transport/exocytosis	INV?IEV fusion with PM	Fusion with PM
Particle diameter (nm)	60-80	80-160	90-120	200 × 400	150-200

the pattern of glycosylation of both VP7 and NS28. This study also showed that the requirement for calcium cannot be substituted by manganese ions. From their primary amino acid sequences both NS28 and VP7 have consensus calcium binding sites.[17] For VP7 this would be consistent with earlier studies showing binding of radioactive calcium to isolated virus particles.[34]

The haemagglutinin protein, VP4, is also a unique protein whose role in the assembly is far from clear. It is apparently made on free ribosomes, lacks a signal sequence and is not glycosylated; it is, however, a relatively hydrophobic protein which possesses a conserved 17 amino acid hydrophobic stretch that shows homology to the putative fusion peptides of the E1 proteins of SFV and Sindbis virus.[36] When expressed by itself this protein apparently accumulates in the cytoplasm of transfected cells.[13,37] Immunocytochemical data suggest that it is absent from the viroplasms, but close to ER membranes,[38] while biochemical analyses of isolated (single shelled) nucleocapsids fail to detect this protein.[30] For these reasons it has been suggested that it may assemble on the surface of the VP6-containing inner shell of the nucleocapsid just prior to the budding event.[30] As mentioned, VP4 appears to form a tight interaction with NS28 and VP7 in the membrane form of the virus. It is clearly exposed on the surface of the mature virus particles since antibodies against it neutralize infectivity.[13] This is also consistent with high resolution EM studies showing that this protein forms the dimeric spikes that protrude from the surface of the virus.[22,39,40] In the mature virus VP4 and VP7 are the only exposed proteins to which neutralizing antibodies can be made.[13] When treated with

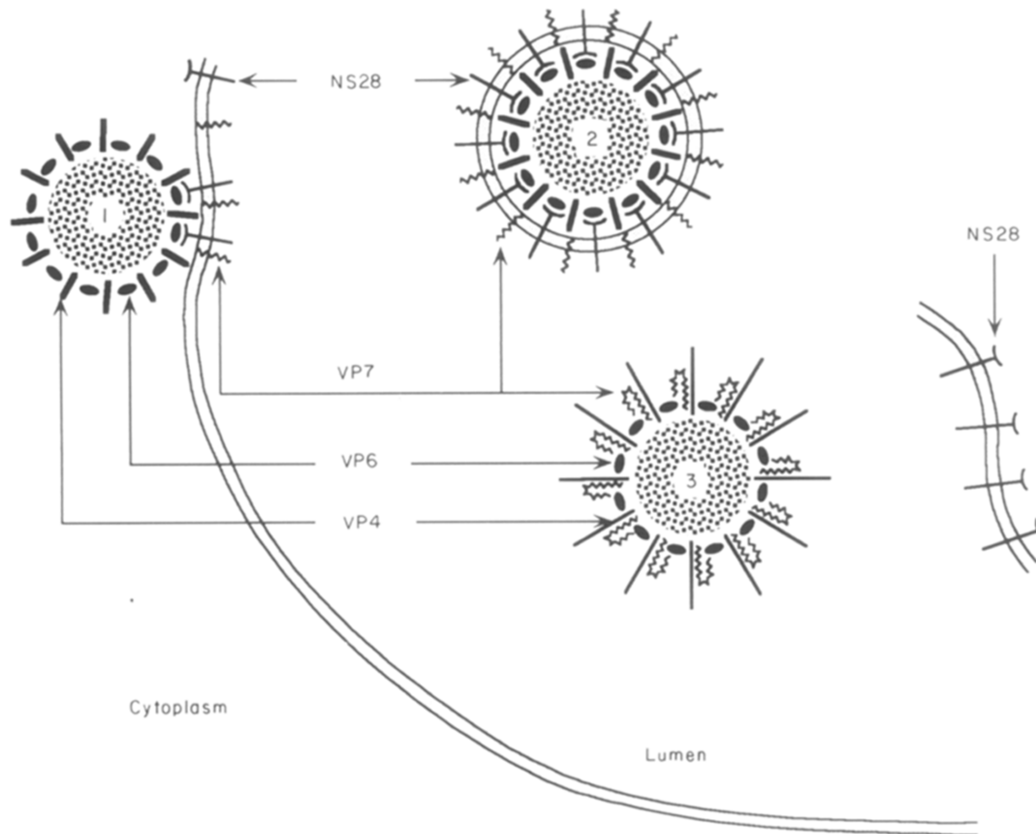

Figure 2. Schematic model that attempts to incorporate the main observations about the assembly of rotavirus in the rough ER. For more details see text.

trypsin, VP4 is cleaved to VP5 and VP8, an effect which increases, and may even be obligatory for, viral infectivity.[41] This cleavage does not occur in the infected cell but rather in the intestine of the target organism prior to infection.[14]

Rotavirus particles do not enter the secretory pathway and are only disseminated following cell lysis. Whether this process is facilitated by viral proteins remains to be determined.

Coronavirus

Coronaviruses (for reviews see refs 42-44) bud into the intermediate region between the rough ER and the *cis* part of the Golgi complex and, later in infection, also into the rough ER and nuclear envelope.[45,46] For simplicity, we shall refer to this region between the ER and the *cis*-Golgi as the intermediate compartment (IC, see Figure 1; see also review by Saraste and Kuismanen). The budding process involves an interaction between the helical nucleocapsid, which contains one nucleocapsid protein N, and the viral membrane glycoprotein M (or E1).[44] M is a membrane protein with three spanning regions the first of which behaves as an uncleaved signal sequence (Figure 3).[47-49] Recent data shows that each of the three membrane-spanning hydrophobic domains can individually insert and anchor the polypeptide in the membrane.[50,51] Protease protection studies indicate that only about 20 terminal residues of the M protein are exposed to the cytoplasmic side of membranes and are presumably available for interaction with the nucleocapsid.[48] The M protein most likely forms a complex with a second membrane glycoprotein, the S (or E2) protein, a class I membrane protein which has a short cytoplasmic tail and a large luminal domain. The latter forms the morphologically visible spike or peplomer of the virus. It is also the protein responsible for binding to cell surfaces and for the fusion activity required for infection.

The M protein has generally been thought to play the major role in coronavirus budding. In the case of avian infectious bronchitis virus (IBV) this protein,

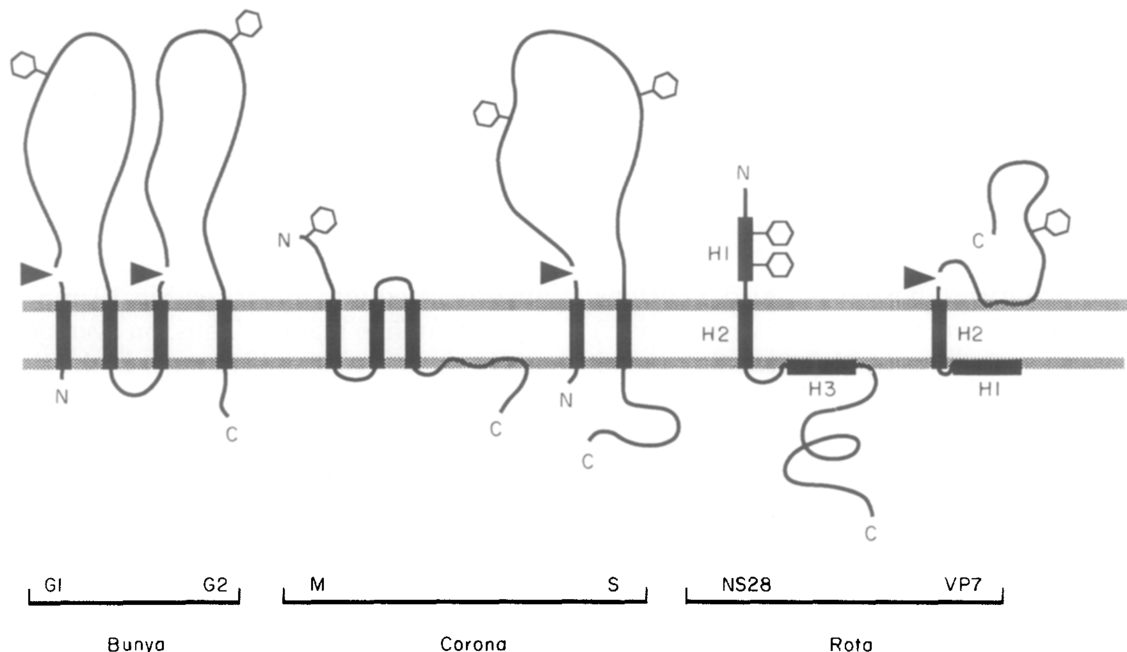

Figure 3. Diagram to show the topological models of the key viral glycoproteins known to be involved in the assembly of bunya, corona and rotaviruses. Note that the bunyavirus polyprotein in many cases also contains the non-structural protein NS_M either preceding or in between G1 and G2, and is co-translationally cleaved (for more details see Matsuoka *et al*[63]). The arrowheads indicate sites of proteolytic cleavages.

expressed by itself, localizes to the IC/*cis* Golgi region,[52] where viral budding has been shown to occur for the mouse hepatitis virus (MHV).[45,46] In the case of MHV, however, the situation is more complex. The M protein of this virus when expressed in culture cells accumulates in the Golgi complex,[53] where it is predominantly retained in the *trans*-Golgi/TGN. The latter inference is based on an analysis of its O-linked oligosaccharides and on immunocytochemical localization data with reference to an established TGN marker, TGN 38.[54] Recent experiments argue that the S protein of MHV may also have information that retains it in the IC. Hence, expression studies show that the N-linked oligosaccharides of this protein acquire endo H resistance only at a very slow rate. This is consistent with preliminary EM immunocytochemical data which suggest that the protein accumulates in pre-Golgi elements (H. Vennema *et al*, unpublished data). We consider it likely that the M and S proteins form a complex which is retained in the site of budding.

Considerable effort has been spent in attempts to determine which domain of the M protein is responsible for retaining it intracellularly. For IBV, the data of Swift and Machamer[55] show clearly that the first spanning membrane domain is responsible for its retention in IC/*cis* Golgi structures. A reporter molecule containing this domain was efficiently retained in this compartment. For MHV the results are different. Both Armstrong *et al*[56] and our unpublished observations (P. Rottier) demonstrate that mutants possessing only the first *trans*-membrane domain are retained in the ER region.

The M glycoprotein of MHV contains only O-linked oligosaccharides, which is quite unusual among viral membrane glycoproteins. They are formed by the sequential, post-translational addition of N-acetyl galactosamine, galactose and sialic acid to a cluster of four Ser/Thr residues which are directly adjacent to the N-terminal initiating Met. This Ser-Ser-Thr-Thr motif is identical to the O-glycosylated amino terminus of glycophorin A. While the N-acetyl galactosamine residue is most likely added in the IC,[46] recent data using brefeldin A (BFA) argues that the subsequent additions of galactose and sialic acid occur in a Golgi compartment(s) proximal to the TGN.[54] Thus after treatment with BFA newly synthesized M acquires all three

sugars whereas, under identical conditions, newly synthesized G protein of VSV acquired endo H resistance (in agreement with a previous study[57]), and fucose, but did not acquire sialic acid on its N-linked oligosaccharides. These data argue that the addition of sialic acid to O-linked oligosaccharides occurs in a BFA-'sensitive', pre-TGN compartment while sialylation of N-linked oligosaccharides occurs in the BFA-'resistant' TGN.

Many coronaviruses possess a third envelope glycoprotein, the haemagglutinin esterase (HE) which correlates with the presence of small granular projections on the virus surface in addition to the normal spikes.[58-60] HE has acetylesterase activity and may inactivate receptors for the virus on the target cells. It is synthesized as an N-glycosylated, class I membrane protein that dimerizes rapidly and forms interchain disulfide bonds. Though its transport properties have not been studied in detail, some HE reaches the cell surface.[61] As this protein is only present in some coronaviruses it is clearly not essential for virus assembly. Presumably the protein is incorporated into virions through an interaction with M and/or S.

Once budded into the intermediate compartment, coronavirus particles are transported, presumably via vesicular transport, through the Golgi complex to the plasma membrane.

Bunyaviruses

Bunyaviruses bud into the Golgi complex. Uukuniemi virus (UUK) and Punta Toro virus (PTV) have been most extensively studied, especially with respect to the viral assembly.[11,62,63] The site of budding is determined solely by the glycoproteins G1 and G2 of the virus. These are believed to interact on their cytoplasmic tails with the helical nucleocapsids, which consist of a single nucleocapsid protein N, the RNA polymerase and the RNA genome itself. After budding into the Golgi complex the viruses are transported to the extracellular medium along the secretory pathway.

Bunyavirus glycoproteins G1 and G2 are class I membrane glycoproteins which are generated from a common polypeptide precursor (Figure 3). They are successively and co-translationally inserted by cleavable signal sequences which precede both mature protein moieties. The two mature proteins are anchored by a hydrophobic domain in their carboxy-terminal region. Cleavage of the G2 signal sequence processes the precursor into G1 and G2. It is unknown whether this signal sequence remains part of G1 or is removed. During synthesis both proteins are N-glycosylated and undergo intramolecular disulfide bond formation.

The data of Persson and Pettersson[64] showed that the time required for UUK virus G1 and G2 to fold and dimerize was significantly different: whereas G1 appeared to be fully disulfide-bonded and incorporated into dimers within 10 min, it took G2 up to 60 min to mature. Thus, on average, molecules of G2 spent considerably longer in the ER than G1. During the folding in the ER, G2 could be co-precipitated with Bip while both proteins formed a complex with protein disulfide isomerase (PDI). In contrast to these observations Chen and Compans[65] found the majority of both G1 and G2 of PTV engaged in heterodimer formation between newly synthesized molecules. Already after a 3 min pulse labelling both glycoproteins could be demonstrated in oligomeric complexes. In addition, a small fraction of G2 also assembled into homodimers. This may be related to the observation that G2 is produced in excess over G1, as has been reported also for Rift Valley fever virus.[66] The precise mechanism of this independent synthesis of G2 is not firmly established but may involve internal translation initiation.[66]

Recent expression studies using vaccinia virus showed that the two glycoproteins of another bunyavirus, the Hantaan virus, can only leave the ER when they are co-expressed,[67] possibly because the newly-synthesized proteins have to interact in order to acquire their correct conformation. In contrast, the G2 glycoprotein of PTV was found to be able to leave the ER and reach the plasma membrane when expressed by itself.[65,68]

Both after bunyavirus-infection and after co-expression of G1 and G2, the G1/G2 heterodimer clearly possesses information that is essential and sufficient for Golgi targeting and retention.[62,69-71] This complex cannot be chased out of the Golgi complex, even after about 6 h of cycloheximide treatment,[70] unless released from the cell within virions. Morphological studies[72] as well as the analysis of the N-linked oligosaccharides of mature G1 and G2 are consistent with their localization to a Golgi compartment prior to the TGN.[65,73] Thus, the mature proteins have negligible sialic acid.[65,70] However, the small amount of these glycoproteins that are found on the cell surface (perhaps in fully budded virions) do contain sialic acid.[74] This argues that the lack of sialic acid on these proteins when they

are resident in the Golgi complex does indeed reflect their pre-TGN localization.

The signal for the Golgi retention of G1/G2 heterodimers has not yet been identified and could either reside specifically in the complex as such or be carried separately by one or both of its constituents. Since G2 alone reaches the cell surface[68] and since this protein is retained in the Golgi only when associated with G1 it seems likely that the G2 has no retention signal. Significantly, an anchor minus mutant of G2 which, when expressed by itself is secreted, could form heterodimers with the G1 in double expression studies. These heterodimers were now retained in the Golgi complex.[68] Collectively, these data argue that the information for Golgi retention resides in the G1 protein. This is supported by recent unpublished data from Compans's group arguing that the retention information is contained in the combined transmembrane and cytoplasmic domain of G1 (R.W. Compans, personal communication). Similar data are now also available for UUK (R.F. Pettersson, personal communication).

Although the organization of the Golgi complex is usually significantly altered during infection with bunyaviruses, the available data suggest that this does not severely affect Golgi function. A striking demonstration of this came from the experiments of Gahmberg *et al*[75] who carried out double infection studies with UUK and SFV. Whereas the UUK virus glycoproteins were retained in the Golgi complex, as expected, the SFV glycoproteins were glycosylated and transported (with a slight delay in the ER to Golgi step) to the cell surface. It seems clear that it is the site of accumulation of the G1/G2 heterodimers that is important for the bunyavirus assembly since in the presence of BFA the PTV assembles in the region of the ER.[76]

Although the bunyaviruses normally bud into the Golgi complex, the Rift Valley fever (RFV) virus appears to bud both into the Golgi complex and out through the basolateral membranes of primary cultures of rat hepatocytes.[77] In the hepatocyte, recent data argue strongly that the pathway of newly synthesized plasma membrane proteins from the TGN to the apical membrane of hepatocytes obligatorily passes via the basolateral membrane.[78] In the case of RFV a significant fraction of the viral glycoproteins seems to escape the Golgi retention mechanism and presumably follows the same route to the basolateral membrane as the VSV-G protein in MDCK cells.[79]

Vaccinia virus

Vaccinia is the best studied member of the Poxviridae, which represents the largest and most complex of all viruses. These DNA-containing viruses are unique in that they encode in their genome the machinery required for their replication and transcription which occurs in large cytoplasmic structures referred to as viral factories.[80-82] The biogenesis of these viruses is especially complex since they become engulfed by four membranes. Four morphological forms can be identified (see Figure 1). The first of these, the spherical immature virus (IV) was widely thought to acquire its membrane by *de novo* membrane biogenesis.[82] Our recent morphological data using a spectrum of different cellular markers suggests, rather, that the membranes of this form originate from cisternae that are continuous with the intermediate compartment (IC) between the ER and the Golgi complex.[83] According to this model the IV acquires two membranes simultaneously. Although the two membranes are often difficult to visualize in the IV itself, they become quite distinct after maturation of the IV into the brick-shaped intracellular mature virus (IMV; previously referred to as intracellular 'naked' virus, INV[83]).

The formation of the IV is efficiently and reversibly blocked by the drug rifampicin, which directly or indirectly affects a 65 kDa cytosolic, virally encoded protein.[84,85] Since this protein is predominantly localized to the inner membrane of the forming IV, it might behave as a linker protein between the nucleocapsid and the cytoplasmic domain of a vaccinia-coded spanning membrane protein.[83] To date, however, the latter has not been identified. The conversion of the spherical IV to the infectious IMV involves the acquisition of p14, a 14 kDa protein on the surface of the particle. The latter protein, which behaves as a neutral pH fusogen,[86] is probably responsible for facilitating infection of the IMV.[87]

A variable proportion of the IMV becomes engulfed by a second cisterna,[82,88,89] giving rise to a four-membrane IEV form (Figure 2). This cisterna contains high concentrations of a number of viral membrane proteins, such as the 85 kDa haemagglutinin, a type I glycoprotein[90] and p37, a 37 kDa protein that evidently lacks an obvious signal sequence but contains putative *trans*-membrane domains.[91] Possibly, the latter binds post-translationally to another viral protein in the enveloping cisterna.

A number of experiments argue that the envelopment to form the IEV might involve an interaction between the p14 protein on the surface of the IMV and the p37 on the cytoplasmic surface of the enveloping cisterna. First, deletion of either protein blocks IEV and EEV formation.[92,93] Second, the formation of the four-membraned form is blocked by the drug IMCBH:[94] recent data show that IMCBH-resistant vaccinia virus strains carry a single point mutation in the *p37* gene.[95]

The identity of the cellular compartment from which the second enveloping cisterna is derived is still unclear. Since it labels strongly with both ricin and WGA it appears to be either a late Golgi or a post-Golgi compartment. Surprisingly, however, it can also be labelled with endocytic markers (M. Schmelz, B. Sodeik and G. Griffiths, unpublished data). Whether the compartment is a modified endocytic organelle or a *bona fide* TGN compartment which receives a higher amount of traffic from the endocytic pathway as a result of the vaccinia infection remains to be established.

The available evidence suggests that the four-membraned IEV is transported to the plasma membrane where it fuses, thereby releasing the second infectious form, the three-membrane EEV into the extracellular medium.[80,82]

Herpesvirus

The assembly of this DNA-containing class of virus, second only to the pox viruses in size, is probably the most enigmatic of all viruses. Unlike the pox virus family, the herpesviruses are fully dependent on the nuclear machinery of the host for their replication and transcription and it is in the nucleus that the first morphological form of the virus, the nucleocapsid, is observed.[96,97]

There is now convincing evidence that the first maturation step in the life cycle of herpesviruses is a budding of the nucleocapsid through the inner membrane of the nuclear envelope.[97] Morphologically, the sites of budding show distinct thickenings of the membrane and immunoelectron microscopy has indicated that in Epstein Barr virus infected cells these regions are highly enriched in a viral glycoprotein gp110.[98] The oligosaccharides of this glycoprotein are in the high mannose form, as expected for an ER species.

An intriguing problem with respect to this nuclear envelope budding is the question of how the nucleocapsid traverses the nuclear lamina prior to making contact with the inner membrane. A recent report by Radsak *et al.*[99] has shown that two of the three lamin molecules, lamins A and C, become dephosphorylated following infection with a human cytomegalovirus, a herpes virus. It is far from clear at present whether, or how, this relates to the budding process.

Enveloped virions are clearly seen in the lumen of the nuclear envelope in herpes infected cells. In some cases the virions are also observed in the lumen of the rough ER. Although the next step is not unequivocally established, the most likely scenario is that the enveloped form in the lumen of the nuclear envelope fuses with the outer nuclear envelope leaving the naked capsid free in the cytoplasm.[100,101] If this pathway is correct, it would argue that it simply functions to transport the nuclearly synthesized, DNA-containing capsids into the cytoplasm; it seems reasonable to expect that the capsid, which is about 100 nm in diameter, is too large to traverse through the nuclear pore, whose size limit is considered to be in the 10 nm range.[102]

In the next step, which appears similar to the second envelopment of vaccinia virus to form the IEV (see above), the nucleocapsid appears to become engulfed by two cisternal membranes in the region of the Golgi complex.[103] The latter are enriched in viral glycoproteins such as gp350/220 (Epstein Barr virus), whose oligosaccharides are endo H resistant and rich in fucosyl residues,[98,104] consistent with their Golgi localization. This cisterna stains with the cytochemical marker acid phosphatase which, within the Golgi complex, is a marker of the *trans*-cisternae.[105] Together, these data suggest a model whereby herpes-encoded envelope proteins would behave as *trans*-Golgi markers which could interact on their cytoplasmic domains with the nucleocapsids. The resulting enveloped form would be covered by two membranes. This envelopment step appears to be crucial for infectivity. It is blocked by BFA which also leads to a large accumulation of viral particles in the lumen of the nuclear envelope.[103] As for the vaccinia EEV, it is currently believed that the latter fuses with the plasma membrane releasing the mature virus with one envelope into the extracellular medium.[97]

Concluding remarks

In this review we have focused on the assembly of five different viruses that bud into, or assemble from,

different compartments of the biosynthetic pathway. Collectively, the data argue that the key step in this assembly is an interaction between the nucleocapsid and the cytoplasmic domain of one or two virally-encoded membrane glycoproteins. Of those glycoproteins that have been well characterized all appear to behave as resident proteins of the compartments where the viruses assemble and are therefore attractive tools for many cell biological experiments.

It seems that for most budding viruses a protein-protein interaction between the nucleocapsid and the tail of the membrane 'receptor' must exist, as first proposed by Garoff and Simons.[106] For VSV this idea has recently been given strong support by the genetic data of Whitt *et al.*[107] However, the precise molecular details of this interaction remain to be elucidated. For those viruses in which the nucleocapsid is fully assembled before the interaction with the membrane occurs it is still not clear, for example, whether the capsids bind a preformed patch of membrane receptors or whether they initially bind a few molecules and that this process then recruits and traps further copies of the membrane protein.[2]

It is also far from clear why or how the viruses we have discussed have evolved the capacity to bud into intracellular organelles rather than out through the plasma membrane, the route taken by the majority of budding viruses. We can suggest two possible advantages of the intracellular budding route. First, at least some of the viral membrane proteins that, for most viruses, are exposed on the plasma membrane, are kept within the cell in the viruses discussed in this review. This could be an advantage in reducing the chances that the infected cell will be recognized by antibodies and killed by complement lysis before infectious viruses are produced. The second possibility to consider is that the intracellular budding route avoids the need for the nucleocapsids, or nucleocapsid precursors, to be assembled on the cytoplasmic side of the plasma membrane. The zone beneath the plasma membrane is usually the site of a dense cortical network of cytoskeletal elements. These could, conceivably, provide a barrier for the nucleocapsids of some, but clearly not all, viruses to pass through. In other words, the viruses take advantage of the transport system that the secretory pathway provides from the perinuclear region to the plasma membrane.

The different mechanisms by which these intracellular assembling viruses get out of the infected cell also raise many questions. Bunya- and coronaviruses are able to exit via the secretory route. It is widely thought that this occurs via vesicular transport. While definitive evidence for this is not available it would appear that one of these viral systems could be used to isolate and characterize these putative transport vesicles: such an approach combined, for example, with a perforated cell method[108] might give new insights into this fundamental process of membrane traffic. For rotaviruses it is an intriguing question why the viral particles are unable to enter the secretory pathway and must await cell lysis for dissemination. Although not studied we speculate that this is mediated by viral proteins. Finally, for herpes and pox viruses one has the unsolved problem how these relatively large particles are able to find their way to, and fuse with, the plasma membrane. For vaccinia virus there is evidence that actin filaments may play a role in this process since the release of EEV is blocked by cytochalasin D (ref 89; M. Schmelz and G. Griffiths, unpublished data).

What direction should the study of intracellular budding viruses now take? During the past decade there has been an increasing emphasis on the use of both cell-free assays and perforated cell systems to follow basic mechanisms of many cell biological events.[108-112] The former system has the advantage of being completely accessible to classical biochemical analysis; such an approach is, however, limited to those processes that still function in the absence of a more normal cell organization. The last few years has seen an increasing use of the powerful perforated systems that enable easy access of membrane impermeable reagents into the cell while still maintaining a high degree of structural organization. Examples of this approach are the filter disruption method,[113] the use of mechanically broken cells[114] and the use of streptolysin O.[115] It is our belief that the time is now ripe for the use of both *in vitro* and perforated cell systems to study the assembly of the intracellular viruses discussed in this review.

In the case of Rotaviruses, the cell-free approach has already been initiated by the pioneering studies of Au *et al*[17,116] and Meyer *et al*[18] who have followed the binding of radiolabelled, purified nucleocapsids to crude membranes or membrane fractions from cells expressing the NS28 protein of this virus. These data show clearly that a high affinity interaction exists between VP6 on the surface of the nucleocapsid and the cytoplasmic domain of the NS28. For coronavirus an attempt is now being made (PR) to add purified nucleocapsids to membranes from cells double-expressing

the S and M proteins. In the case of vaccinia virus (GG) we have started to develop a system to study the *in vitro* assembly of the IMV which takes advantage of the fact that rifampicin can be used to reversibly block the assembly of the IV. Thus, cells can be broken by gentle homogenization, the drug can be washed out and conditions are sought that enable, first the IV and subsequently the IMV to be assembled. For herpesvirus one could envisage setting up an *in vitro* system with isolated nuclei in order to follow the proposed pathway across the nuclear envelope. The attractive feature of these *in vitro* approaches with respect to the assembly of intracellular viruses is that a straightforward functional assay is available. The ultimate test and goal of such studies must be the production of infectious viral particles.

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