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REVIEW ARTICLE
The entry of enveloped viruses into cells by endocytosis

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Introduction

Enveloped viruses introduce their genomes into host cells by membrane fusion. With paramyxoviruses, such as Sendai virus, fusion is independent of pH and can occur at the cell surface (see Poste & Pasternak, 1978). However, in other groups of enveloped viruses, including toga-, orthomyxo- and rhabdoviruses, the fusion activity is triggered only in mildly acidic (pH 5–6) conditions (see White *et al.*, 1983). For these viruses fusion occurs, after endocytosis of the intact viruses, with the membranes of acidic endocytic vacuoles (for reviews see Dales, 1973; Lonberg-Holm & Philipson, 1974; Helenius *et al.*, 1980*b*; Howe *et al.*, 1980; Dimmock, 1982; Lenard & Miller, 1982; Simons *et al.*, 1982; Marsh *et al.*, 1982*a*). Here recent work concerning the endocytosis of enveloped viruses and the role of the endocytic pathway in enveloped virus infection will be reviewed. The mechanism of viral membrane fusion will be discussed only briefly; for a full review see White *et al.* (1983).

Enveloped virus entry has been studied using several well-characterized viruses: SFV and Sindbis virus (togaviruses), FPV and influenza virus (orthomyxoviruses) and VSV (a rhabdovirus). The structures of these viruses have been described in detail (see Wagner, 1975; Garoff *et al.*, 1982; Compans & Choppin, 1975). Briefly, the alpha- and orthomyxoviruses are spherical particles approximately 75 and 100 nm in diameter respectively, and VSV is a 150 nm long bullet-shaped particle. All of these viruses contain a nucleocapsid surrounded by a membrane. The nucleocapsid contains the viral nucleic acid complexed with one or more accessory proteins. The viral membrane, which originates from a membrane of the host cell, is a lipid bilayer, containing multiple copies of the viral membrane glycoproteins. The glycoproteins, which span the bilayer and project from the virion surface as spikes, are essential for viral infectivity and during entry have two principal tasks, (1) binding virions to the host cell surface, and (2) membrane fusion. Viral spike glycoproteins are among the best-characterized membrane proteins. The complete amino acid sequences for several have been deduced (see White *et al.*, 1983) and the three-dimensional structures of both of the influenza virus spike glycoproteins, the haemagglutinin and the neuraminidase, have been resolved (Wilson *et al.*, 1981; Varghese *et al.*, 1983).

Abbreviations used: SFV, Semliki forest virus; FPV, fowl plague virus; VSV, vesicular stomatatis virus; BHK, baby hamster kidney; MDCK, Madin–Darby canine kidney.

Mechanisms of endocytosis

Most cells can internalize, or endocytose, extracellular ligands in plasma membrane-derived vesicles. Usually endocytosis occurs by two mechanisms. The first, phagocytosis, is an inducible process which mediates the uptake of large particles (> 200 nm diameter) and is often a property of specialized cells such as macrophages. The second process, pinocytosis, is a constitutive property of virtually all cell types through which the medium, including solutes, macromolecules and small particulate ligands (< 150 nm diameter), is internalized (see Silverstein *et al.*, 1977; Steinman *et al.*, 1983; Besterman & Low, 1983).

Pinocytosis includes both fluid phase and receptor-mediated (or adsorptive) endocytosis. Cells continually internalize the medium that surrounds them (fluid phase endocytosis). In fibroblasts an equivalent of 5–10% of the cell volume is taken up each hour (Steinman *et al.*, 1976). In addition, Steinman *et al.* (1976) estimated that pinocytic activity in L-cell fibroblasts and macrophages results in the internalization, and recycling to the cell surface, of the equivalent of 54% and 180% respectively of the cell surface areas/h. Despite the magnitude of fluid phase pinocytosis the uptake of ligands present in low concentrations is inefficient. The efficiency is increased when receptors that enable a cell to select and concentrate specific ligands are used; this process is termed receptor-mediated or adsorptive endocytosis (see Goldstein *et al.*, 1979; Steinman *et al.*, 1983).

Many physiologically important macromolecules enter cells by receptor-mediated endocytosis. Examples include nutrients (low density lipoprotein, transferrin), polypeptide hormones and growth factors (insulin, epidermal growth factor)

lysosomal hydrolases, asialoglycoproteins and serum proteinase inhibitors (α_2-macroglobulin). Ligands are taken up through coated pits, in coated vesicles. The coated vesicles deliver the internalized ligands to a system of membrane-bound organelles termed the vacuolar apparatus. This system comprises the prelysosomal vacuoles (endosomes), multivesicular bodies, autophagic vacuoles and secondary lysosomes (Fig. 1). In addition, the endoplasmic reticulum and Golgi apparatus are involved in the synthesis of lysosomal hydrolases and vacuolar membrane, and a population of vesicles, as yet uncharacterized, recycles membrane and some content to the cell surface (see Besterman *et al.*, 1981; Steinman *et al.*, 1976, 1983; Brown *et al.*, 1983).

In addition to physiological ligands, opportunistic ligands such as viruses and toxins also exploit the endocytic pathway to enter cells. For enveloped viruses, receptor-mediated endocytosis is best characterized for SFV uptake in BHK cells. The viruses bind to cell surface sites located preferentially on the microvilli. The bound viruses are translocated to coated pits at the base of the microvilli and are internalized in coated vesicles. The internalized viruses are collected in prelysosomal vesicles, endosomes, and eventually some viral components pass to, and are degraded in, the lysosomes (Fig. 1).

Binding to the cell surface

To infect a cell a virus must first bind to the cell surface (see Lonberg-Holm & Philipson, 1974; Dimmock, 1982). Binding is best studied at low temperatures which inhibit pinocytosis (Steinman *et al.*, 1974; Brown & Goldstein, 1979). At 0–4°C SFV, Sindbis virus, VSV and influenza viruses bind to various cell types, but are not internalized (Fries & Helenius, 1979; Helenius *et al.*, 1980*a*; Marsh & Helenius, 1980; Matlin *et al.*, 1981, 1982; Yoshimura *et al.*, 1982; Miller & Lenard, 1980; Talbot & Vance, 1982; Schlegel *et al.*, 1982). With SFV a binding constant of $3 \times 10^{10} M^{-1}$ has been measured on BHK cells (Fries & Helenius, 1979).

Binding is mediated by the viral spike glycoproteins (see Lonberg-Holm & Philipson, 1974; Dimmock, 1982). Subviral particles containing isolated SFV spike glycoproteins have been used to study binding. SFV spike glycoprotein rosettes and reconstituted lipid vesicles (virosomes) have binding properties on BHK cells similar to those of the intact virus. The particles bind preferentially to the microvilli, the binding is pH-dependent and the particles compete for binding sites with intact viruses (Fries & Helenius, 1979; Marsh *et al.*, 1983*a*). The efficiency with which the particles bind to cells is, however, reduced when compared with intact SFV. The reduced binding correlates

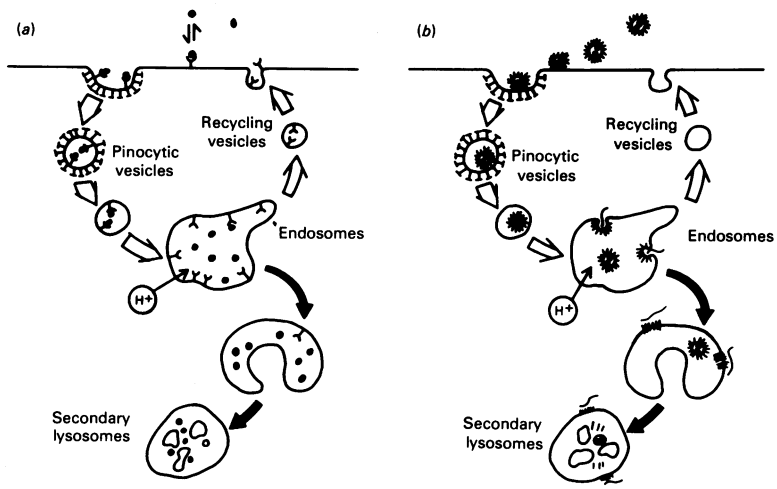

Fig. 1. *The basic pathway of pinocytosis and the entry pathway of Semliki Forest virus*
(*a*) A schematic representation of the pinocytic pathway showing an example of receptor-mediated endocytosis where the ligand binds to receptors on the cell surface, is internalized through coated pits and coated vesicles and enters the endosome compartment. The endosome is acidified by a proton ATPase and, in the acid environment, the receptor–ligand complex dissociates (see Helenius *et al.*, 1983). The receptor is recycled to the cell surface for reutilization, while the ligand passes to the lysosome compartment. (*b*) A schematic representation of SFV entry into BHK cells. Acidification of the endosome in this case results in fusion of the viral membrane with the endosome membrane and the extrusion of the viral RNA into the cytoplasm. The viral spike glycoproteins, which are now components of the endosome membrane, and any unfused viruses, are subsequently degraded in the lysosomes.

with the lower valency of the respective particles, that is, the potential of each particle to make multiple contacts with the cell surface (Marsh *et al.*, 1983*a*). Thus, the high avidity of SFV for BHK cells probably results from multiple low-affinity spike glycoprotein–receptor interactions. The low affinity of the individual spike glycoprotein–receptor complexes may enable a virus to use different cell-surface components as receptors, and may explain why specific receptors for enveloped viruses have proven difficult to identify.

The cell-surface binding sites for enveloped viruses are poorly characterized (see Lonberg-Holm & Philipson, 1974; Dimmock, 1982). The myxoviruses bind to sialic acid residues and, therefore, have a broad range of potential glycoprotein and glycolipid receptors. Less is known of the binding sites for other enveloped viruses. Several toga- and retroviruses bind to proteinase-sensitive cell-surface components (Helenius *et al.*, 1980*a*; Huggins *et al.*, 1983; Andersen & Nexø, 1983), and with Sindbis virus and Friend murine leukaemia virus proteins of molecular mass 90kDa and 14kDa respectively have been implicated as receptors on human lymphoblastoid cells and murine leukocytes (Maassen & Terhorst, 1981; Robinson *et al.*, 1980). SFV shows specificity for the major histocompatibility antigens on murine and human lymphoblastoid cells (Helenius *et al.*, 1978), but will also infect cells that do not express these antigens, again suggesting that different cell-surface components can be used as binding sites (Oldstone *et al.*, 1980). SFV, Sindbis and influenza viruses will bind lipid (Mooney *et al.*, 1975; White *et al.*, 1980, 1982*a*), but only under conditions (low pH) which promote membrane fusion (White & Helenius, 1980; Väänänen & Kääriäinen, 1979, 1980; Lenard & Miller, 1981). The situation with VSV is less clear. Binding to BHK cells, MDCK cells and mouse fibroblasts is inefficient, releaseable by both trypsin and EDTA, and shows marked pH-dependence in a range above that required for fusion (Miller & Lenard, 1980; Matlin *et al.*, 1982; Schlegel *et al.*, 1982). Recently, Schlegel *et al.* (1983) suggested that phosphatidylserine is the receptor for VSV.

Whether viruses that enter cells by the endocytic pathway need to bind to cell-surface components that normally mediate the endocytosis of physiological ligands is unclear. However, most cell-surface glycoproteins appear to get internalized (see Steinman *et al.*, 1983). Multivalent ligands, such as viruses, will induce the formation of receptor clusters (see Helenius *et al.*, 1980*a*). It is possible that clustering alone is sufficient for ligands to be trapped in coated pits (see below) and internalized in coated vesicles.

Viruses can also bind to cells through antibodies directed against viral surface antigens. Anti-viral antibodies enhance the infection of cultured mouse macrophages and BHK cells by alpha-, flavi- and bunyaviruses (Peiris & Porterfield, 1979, 1982; Kimura *et al.*, 1981; Millican & Porterfield, 1982). Enhancement does not occur if Fab fragments of these antibodies are used, or if cells are incubated with anti-(Fc receptor) antibodies (Peiris *et al.*, 1981), implying that antibodies bind the viruses to cell surface Fc receptors. The Fc receptors on macrophages are well known to effect receptor-mediated endocytosis (see Steinman *et al.*, 1983; Mellman *et al.*, 1983, 1984). Antibody-enhanced infection may account for increased replication of influenza and dengue viruses in individuals with antiviral antibodies, and for the dengue virus shock syndrome. The antibodies can occur in such individuals by exposure to antigenically related viruses or by injection of specific antibodies (Webster & Askonas, 1980; Halstead, 1980*a*,*b*).

Internalization

Fazekas de St.Groth (1948) proposed that influenza virus infection involves internalization of the virions (viropexis). Numerous morphological studies demonstrating virus particles in endocytic vacuoles have supported this proposal (see Dales, 1973). However, it has remained unclear how the viruses are internalized and whether or not internalization results in infection. Recent biochemical and morphological studies show that a number of enveloped viruses enter cells through receptor-mediated endocytosis. As described with a number of physiological ligands (see Goldstein *et al.*, 1979; Steinman *et al.*, 1983) the internalization occurs through specialized regions of the plasma-membrane, the coated pits (Roth & Porter, 1964; Fawcett, 1965; Goldstein *et al.*, 1979). The coated pits appear to invaginate into the cell, encapsulating any ligand associated with them, to form coated vesicles.

An alternative mechanism for the receptor-mediated internalization of bound ligands has been proposed (Willingham & Pastan, 1980; Wehland *et al.*, 1981; Dickson *et al.*, 1981). By this mechanism, internalization occurs through large smooth surface vesicles (receptosomes) that bleb from the side of coated pits. The images of coated vesicles are suggested to result from sections cut through coated pits which do not contain the connection to the cell surface. Such images undoubtedly exist (see Bretscher *et al.*, 1980; Wall *et al.*, 1980), however, a serial section analysis (Peterson & van Deurs, 1983) clearly shows that coated vesicles are discrete structures, without morphological connections to the cell surface, that are involved in the internalization of adsorbed ligands.

Togaviruses (Pathak *et al.*, 1976; Helenius *et al.*, 1980*a*), rhabdoviruses (Simpson *et al.*, 1969; Dahlberg, 1974; Matlin *et al.*, 1982), paramyxoviruses (Morgan & Howe, 1968), orthomyxoviruses (Patterson *et al.*, 1979; Matlin *et al.*, 1981) and retroviruses (Dales & Hanafusa, 1972) have been observed in coated vesicles. Kinetic studies with SFV, FPV and VSV indicate that this association with coated vesicles is coupled with the initial internalization event (Helenius *et al.*, 1980*a*; Matlin *et al.*, 1981, 1982).

SFV has been used to estimate the magnitude of coated vesicle uptake in BHK cells. At high multiplicities (90000 viruses/cell) about 3000 virus particles/min enter a cell. Electron micrographs of these cells show an average of 1.3 viruses/virus-containing coated vesicle. This means that about 2400 coated vesicles/min leave the cell surface at 37°C (Marsh & Helenius, 1980). Furthermore, SFV does not induce the formation of coated vesicles but uses vesicles which are continually moving into the cell from the cell surface. This was demonstrated by the following experiment: one SFV particle is calculated to occupy 0.25 the internal volume of a coated vesicle; if viruses induce the formation of coated vesicles then, at high multiplicities, an increase in fluid-phase endocytosis equivalent to 0.75 the volume of the virus-containing coated vesicles would be expected. In actuality, a temporary decrease in fluid phase uptake is measured. The decrease is approximately equal to the volume of fluid displaced from coated vesicles by the viruses (Marsh & Helenius, 1980; Marsh *et al.*, 1982*a*).

The experiments with SFV show that endocytosis by coated vesicles has a high capacity and is a constitutive property of cells. We have calculated that 2400 coated vesicles/min leaving the cell surface can account for both the measured fluid-phase endocytosis in BHK cells and for the membrane uptake (assuming values equivalent to those measured by Steinman *et al.*, 1976). This further suggests that in BHK cells fluid-phase endocytosis, receptor-mediated endocytosis and membrane uptake are integrated properties of the same endocytic pathway.

The number of coated vesicles leaving the cell surface is large enough to ensure that bound ligands are taken up with high efficiency. For physiological ligands such as low-density lipoprotein, asialoglycoproteins or α_2-macroglobulin, half times on the surfaces of various cell types of 2–5 min have been measured (see Steinman *et al.*, 1983). With SFV the half-time at the BHK cell surface is 7–10 min (at 37°C) and virtually all bound viruses are cleared, regardless of the multiplicity (Marsh & Helenius, 1980). FPV and influenza virus also have similar half-times on the surface of MDCK cells (Matlin *et al.*, 1981; Yoshimura *et al.*, 1982). However, with VSV on these same MDCK cells the half-time is about 30 min (Matlin *et al.*, 1982) and on both BHK and MDCK cells only half of the bound virus is cleared (Miller & Lenard, 1980; Matlin *et al.*, 1982). The variation in the efficiency of clearance may be explained by the viruses using receptors which internalize at different rates or, alternatively, by the different sizes of the viruses. Orthomyxoviruses and SFV are relatively uniform particles which are easily contained within endocytic coated vesicles (average diameter 80–100 nm). The bullet shaped VSV particle, on the other hand, is larger than the average coated vesicle and its inclusion in most, though clearly not all, coated vesicles may be limited.

After leaving the cell surface, coated vesicles rapidly (< 2 min) lose their coats (Anderson *et al.*, 1977). When cells, with bound SFV, are warmed to 37°C the virus particles are seen in coated vesicles within 15 s and, by 1 min, in large 200–500 nm electron-lucent vacuoles termed endosomes (Helenius *et al.*, 1980*a*). The endosomes are components of a prelysosomal compartment which, cytochemically, is devoid of lysosmal markers (acid phosphatase and aryl sulphatase: Tycko & Maxfield, 1982; Wall *et al.*, 1980), and which can be fractionated away from most lysosomal activities by density gradient centrifugation (Tolleshaug *et al.*, 1979; van Renswoude *et al.*, 1981; Marsh *et al.*, 1983*b*; Merion & Sly, 1983; Galloway *et al.*, 1983). After a delay of 20 min virus components reach the lysosomes. Entry into the lysosome compartment is indicated both morphologically (see Dales, 1973; Helenius *et al.*, 1980*a*; Matlin *et al.*, 1981, 1982) and by the appearance of the products of virus degradation in the medium (Marsh & Helenius, 1980; Matlin *et al.*, 1981, 1982; Yoshimura *et al.*, 1982). The lag in the appearance of degradation products results from the time taken for the viral components to reach the lysosomes and not from delayed activity of the lysosomal hydrolases (Marsh *et al.*, 1983*b*). Fractionation of cells labelled with Sindbis virus and VSV indicates a similar association with endosomes and lysosomes as described for SFV (Talbot & Vance, 1982; Brooks *et al.*, 1982).

Intracellular penetration

The fact that a number of enveloped viruses require low pH to trigger the membrane fusion activity, the observation that virus particles are endocytosed and pass to lysosomes, and the known low pH of lysosomes suggested that virus penetration occurs intracellularly in lysosomes (see Helenius *et al.*, 1980*a*; Miller & Lenard, 1980; Marsh *et al.*, 1982*a*). Further, for SFV, VSV, influenza and

Sindbis virus fusion is not seen at the cell surface (Dahlberg, 1974; Dourmashkin & Tyrell, 1974; Helenius *et al.*, 1980*a*; Matlin *et al.*, 1981, 1982), the antigens from infecting viruses are not detected on the cell surface after entry (Fan & Sefton, 1978) and the internalized viruses are infective (Helenius *et al.*, 1982).

Although lysosomes have been implicated in penetration, further examination of the kinetics of entry indicate that SFV penetration occurs early after endocytosis, from the endosomes. Within 5–7 min of the virus leaving the cell surface, uncoated RNA can be detected in the cytoplasm. Whereas degradation, indicating entry into lysosomes, is not detected until 20 min after internalization. In addition, weak bases, which raise the pH of intracellular acidic vesicles (see below), do not inhibit infection if added to cells more than 10 min after the viruses (Helenius *et al.*, 1980*a*; Helenius & Marsh, 1982). To confirm that SFV penetration occurs from endosomes we exploited an observation that asialoglycoprotein taken into hepatocytes at 20°C remains in endosomes and fails to reach the lysosomes (Dunn *et al.*, 1980). We found in BHK cells that SFV is also retained in endosomes at 20°C; the virions do not pass to lysosomes and degradation is not observed (Marsh *et al.*, 1983*b*). Under these conditions virions are, however, uncoated and initiate infection, demonstrating that the fusion reaction occurs in the endosomes. Furthermore, a mutant of SFV which fuses at pH < 5.5 (as compared with pH 6.0 for the wild type) will also uncoat and infect cells at 20°C (Kielian *et al.*, 1984). Recently, intracellular fusion of SFV with the membrane of endosomes has been observed morphologically (A. Helenius & E. Bolzau, unpublished work).

These results demonstrate several important properties of endosomes and the endocytic pathway. Firstly, the endocytic pathway is acidified prior to the lysosomal compartment and, as the fusion mutant infects cells at 20°C, the endosomes are acidic to at least pH 5.5. Secondly, the endocytic pathway is acidified rapidly; internalized ligands encounter pH 6.0 (the pH required for wild type SFV fusion) within 5 min of internalization. Tycko & Maxfield (1982) and van Renswoude *et al.* (1982), using fluorescein-conjugated α_2-macroglobulin and transferrin as pH probes, have estimated the endosome pH in murine fibroblasts and human erythroleukemia cells to be 5.0 ± 0.2 and 5.5 ± 0.4 respectively. Finally the results with SFV show that endosome membranes contain cholesterol, which is required in the target membrane for SFV fusion (White & Hellenius, 1980).

The penetration of SFV from endosomes enables this virus to accomplish the crucial fusion step before entering the hydrolytic lysosomal compartment. Thus the functions of the viral spike glycoproteins are completed prior to their degradation. The intracellular fusion site for other enveloped viruses has not been defined. However, VSV fuses at pH 6.0 (White *et al.*, 1981) and may also be expected to penetrate from endosomes. Similarly, the fact that an SFV fusion mutant penetrates from endosomes indicates that endosomal pH may be low enough to trigger fusion in strains of influenza virus which fuse at pH 5.3 (White *et al.*, 1982*a*).

Acidification

The acidification of the endocytic pathway is necessary not only to activate lysosomal hydrolytic enzymes but may also be involved in the dissociation of some ligand–receptor complexes, in sorting events of the endocytic pathway, and in the control of membrane recycling (Brown *et al.*, 1983; Helenius *et al.*, 1983). Acidification of lysosomes involves an ATP-driven proton pump (see Reeves, 1983). Studies have now demonstrated a similar pump in the membranes of endosomes (Galloway *et al.*, 1983; Maxfield, 1982). This pump has been characterized in endosomes isolated from a mouse macrophage-like cell line (J774) and from BHK cells. Fluorescein-conjugated dextran was used as a pH probe. The emission intensity of fluorescein is a titratable function of pH in the pH range 4–8. Changes in the emission intensity can be used as very sensitive indicators of pH changes (see Ohkuma & Poole, 1978). Endosomes labelled with fluorescein-conjugated dextran acidify rapidly on addition of ATP (Galloway *et al.*, 1983) or GTP (C. Galloway & I. Mellman, unpublished work). The acidification is not affected by inhibitors of the mitochondrial F_1–F_0 ATPase or $(Na^+ + K^+)$-ATPase and does not require permeant anions. At present, *N*-ethylmaleimide is the only reagent demonstrated to inhibit the pump.

How does the endosome acquire the proton ATPase? A proton ATPase, with properties very similar to those of the endosome ATPase, has been demonstrated in isolated coated vesicles (Forgac *et al.*, 1983; Stone *et al.*, 1983). It is therefore possible that the pump enters the endocytic pathway from the cell surface and that incoming coated vesicles contain the components necessary to form an endosome.

The interest in acidification as a control mechanism in the endocytic pathway has prompted attempts to find mutant cells with deficiencies in functions of the endocytic pathway which depend on acidification. The selection of such mutants has been facilitated using toxins, such as diphtheria toxin, which also require low pH to enter the cell (Draper & Simon, 1980; Sandvig & Olsnes, 1980). Several groups of these toxin-resistant mutants

show marked resistance to enveloped viruses including SFV, VSV and Sindbis virus (Moehring & Moehring, 1972; Robbins *et al.*, 1983). The lesion does not affect binding or endocytosis but appears to be at the level of intracellular penetration and can be overcome by introducing either the toxin or the viruses through the plasma membrane by brief low pH treatment. The implication is that these mutants do indeed have a defect in acidification; however, it remains unclear whether the proton ATPase is defective or whether an alternative defect exists, such as altered permeability properties of the endosome membrane.

Viral membrane fusion

For orthomyxo-, toga- and rhabdoviruses, low pH triggers fusion between the viral membrane and the membrane of an endocytic vacuole and is mediated by the viral spike glycoproteins (see Poste & Pasternak, 1978; White *et al.*, 1983). The fusion activity of the SFV spike glycoprotein and the influenza virus haemagglutinin have been studied extensively. Virosomes, containing only the SFV spike glycoproteins, fuse at low pH (pH5.5) with the plasma membrane of BHK cells and are haemolytic. Fusion occurs with about 25% the efficiency of the intact virus (Marsh *et al.*, 1983*a*). The SFV spike glycoprotein contains two transmembrane glycopolypeptides (E1 and E2). The cDNA coding for these glycoproteins has been cloned and inserted into eukaryote expression vectors. BHK cells expressing the spike glycoproteins on their cell surface will fuse when the pH is lowered and the pH-dependence reflects that of viral fusion (Kondor-Koch *et al.*, 1983). Fusion occurs only if both E1 and E2 are expressed; with vector constructs which result in the expression of only E2 at the cell surface, fusion does not occur (Kondor-Koch *et al.*, 1982). Similarly, cells expressing the cloned influenza virus haemagglutinin gene will, after trypsin activation, fuse at low pH (White *et al.*, 1982*b*). It appears that the spike glycoproteins must be inserted in a lipid bilayer for fusion to occur. Thus SFV spike glycoprotein rosettes, bromelain fragments of influenza virus haemagglutinin (the water-soluble ectodomain) and a secreted form of haemagglutinin, which lacks the *C*-terminal membrane-associated domain, all fail to induce cell-to-cell fusion at low pH (Väänänen & Kääriäinen, 1980; White *et al.*, 1982*b*; Gething & Sambrook, 1982; Marsh *et al.*, 1983*a*).

The molecular mechanism of low pH-induced membrane fusion is best understood for the orthomyxoviruses. These viruses can have two types of spike glycoprotein, the haemagglutinin and the neuraminidase, of which the haemagglutinin is required for membrane fusion (see White *et al.*, 1983). The complete amino acid sequence of the haemagglutinin has been determined for a number of strains of influenza virus (see White *et al.*, 1983). The haemagglutinin is a trimer: each monomer consists of two disulphide-linked glycopolypeptides (HA1 and HA2) which are derived from a precursor (HA0) by proteolytic cleavage. This proteolytic cleavage is required to render the virus infective (Lazarowitz & Choppin, 1975; Klenk *et al.*, 1975) and fusogenic (Maeda *et al.*, 1981; White *et al.*, 1982*a*). The *N*-terminus of HA2, revealed after the activating cleavage, contains a sequence of 10 uncharged amino acids followed by a further 14 amino acids in which only three residues are charged. The three-dimensional structure of the bromelain cleavage fragment, resolved to 0.3 nm, indicates that at neutral pH the HA2 *N*-termini encircle the stem of the trimer and are partially hidden within the stem region (Wilson *et al.*, 1981). When the fusion activity is triggered at low pH (Maeda & Ohnishi, 1980; Huang *et al.*, 1981; White *et al.*, 1982*a*) an irreversible conformational change occurs in the bromelain fragment such that it becomes hydrophobic and assumes the properties of an amphipathic molecule (Skehel *et al.*, 1982). These changes are consistent with the notion that the hydrophobic *N*-terminus of HA2, hidden at neutral pH, is uncovered at low pH. How the conformational change brings about the fusion of two lipid bilayers remains unclear; current ideas are discussed by White *et al.* (1983).

Inhibitors of penetration

Inhibitors which block specific steps in infection have been used to confirm that penetration occurs intracellularly and that internalized viruses are infective (Matlin *et al.*, 1981, 1982; Helenius *et al.*, 1982; Marsh *et al.*, 1982*a*). Two types of inhibitors, which raise the pH of endosomes and lysosomes (Ohkuma & Poole, 1978; Poole & Ohkuma, 1981; Geisow *et al.*, 1981; Maxfield, 1982) and collapse ATP-induced proton gradients in isolated endosomes (Galloway *et al.*, 1983), have been used. Firstly, weak bases dissipate proton gradients by virtue of being able to diffuse across a lipid bilayer in the uncharged form but not (or much more slowly) in the protonated form. Secondly, carboxylic ionophores, monensin and nigericin, dissipate proton gradients by the transmembrane exchange of sodium or potassium ions for protons.

Weak bases

NH_4Cl, amantadine, chloroquine, methylamine and tributylamine can inhibit virus infection in culture and in some cases *in vivo* (see Oxford & Gal-

braith, 1980; Helenius *et al.*, 1982). These weak bases inhibit SFV, VSV, Sindbis virus and influenza virus infection at an early step (see Helenius *et al.*, 1980*a*, 1982; Miller & Lenard, 1980, 1981; Talbot & Vance, 1980, Matlin *et al.*, 1981, 1982; Schlegel *et al.*, 1982). For SFV, binding and endocytosis occur as normal in BHK cells (Marsh & Helenius, 1980; Helenius *et al.*, 1982; Marsh *et al.*, 1982*a*) and, even though several weak bases cause extensive swelling of some cytoplasmic organelles (Ohkuma & Poole, 1981), the routing to lysosomes appears normal as the degradation of SFV proteins is only slightly inhibited by the agents (Marsh *et al.*, 1982*a*). Furthermore, free-flow electrophoretic fractionation of cells infected with radioactive SFV, in the presence or absence of NH_4Cl, shows that similar amounts of radioactivity co-migrate with lysosomes (Marsh *et al.*, 1982*b*).

For both SFV and influenza virus weak bases inhibit penetration, that is the release of viral RNA to a cytosolic, ribonuclease-sensitive form (Koff & Knight, 1979; Helenius *et al.*, 1982). The viral low-pH fusion activity as such is unaffected by the agents as infection can occur by low-pH-induced fusion at the plasma membrane when the medium pH is taken below the pH optimum required for fusion (White & Helenius, 1980; White *et al.*, 1980; Helenius *et al.*, 1982; Robbins *et al.*, 1983). Significantly, infection is not inhibited if the weak base is added to the cells more than 8–10 min after the virus, which is consistent with the time required for viruses to reach a pH 6.0 environment (Helenius *et al.*, 1980*a*; Helenius & Marsh, 1982; Miller & Lenard, 1982).

Together the results indicate that inhibition is caused by elevation of the endosomal and lysosomal pH above that required to trigger fusion. This interpretation is strongly supported by the fact that FPV, which fuses at pH 5.4, and SFV fusion mutants which fuse at pH < 5.5, are more sensitive to weak bases than is SFV which fuses at pH 6.0 (Matlin *et al.*, 1981; Kielian *et al.*, 1984; see Marsh & Helenius, 1983).

Although inhibition with several weak bases is consistent with the elevation of endosome and lysosome pH, the mode of action of dansylcadaverine, another weak base, has been disputed. Schlegel *et al.* (1982) have reported that this agent inhibits the internalization of VSV and other ligands by affecting their interaction with coated pits. In support of this, Maxfield (1982) finds that, at concentrations used to inhibit virus infection, dansylcadaverine does not raise the pH of endosomes above that required to inhibit virus fusion. We have found, however, that dansylcadaverine does not differ significantly from other weak bases in its effect on SFV infection in BHK cells (Marsh *et al.*, 1982*a*).

The observations that several weak bases inhibit infection by retro- and Herpes viruses has suggested that these viruses may also infect their host cells through an intracellular route (Wallbank *et al.*, 1966; Banfield & Kisch, 1973; Pazmino *et al.*, 1974; Anderson & Nexø, 1983). In addition, paramyxovirus infection can also be inhibited by weak bases (Skehel *et al.*, 1977; Miller & Lenard, 1981). While it is clear that paramyxoviruses can fuse at the cell surface, it is not clear that this route is infective. The kinetics of paramyxovirus fusion are slow compared with SFV and influenza virus (see White *et al.*, 1983) and, given the high endocytic capacity of many cells, it is possible that virions are internalized before fusion with the plasma membrane occurs. Indeed Sendai virus has been observed to enter cells in coated vesicles (Morgan & Howe, 1968). It is therefore probable that paramyxoviruses can infect cells both through the endocytic pathway and at the cell surface. But why do agents which raise endosome and lysosome pH inhibit paramyxovirus infection? An answer is suggested by several observations made with related orthomyxoviruses. To replicate, the viral nucleocapsid must not only penetrate the cell membrane but must also be uncoated, i.e. converted to a form which can be replicated. In several mutants of influenza virus which are not sensitive to the weak base amantadine the resistance is carried by the M-protein gene (Hay *et al.*, 1979). Further, Bukrinskaya *et al.* (1982*a*,*b*) reported that rimantadine, an amantadine analogue, blocks influenza virus infection by preventing the release of M-protein during uncoating. M-proteins (standing for membrane or matrix) are non-spanning, non-glycosylated proteins associated with the inner aspect of the membrane bilayer in some enveloped viruses, e.g., myxo- and rhabdoviruses. Lenard & Miller (1982) have suggested that a low pH may be required not only to trigger fusion but also for an additional uncoating step. Similarly for paramyxoviruses, exposure to low pH may influence an uncoating step involving the M-protein.

Carboxylic ionophores

Carboxylic ionophores, such as monensin and nigericin, inhibit SFV and VSV infection (Schlegel *et al.*, 1981; Marsh *et al.*, 1982*b*). With SFV, monensin has no effect on binding but inhibits virus internalization into BHK cells by 30%. The inhibition of virus internalization is paralleled by a 50% decrease in the accumulation of fluid-phase markers (Marsh *et al.*, 1982*b*). However, SFV is clearly endocytosed in the presence of monensin, and in electron micrographs viruses can be seen in coated pits, coated vesicles and endosomes (Marsh *et al.*, 1982*b*). As with weak bases the penetration of the nucleocapsids into the cytoplasm is blocked,

but unlike the weak bases, monensin completely inhibits degradation of the viral proteins (Marsh *et al.*, 1982*b*). Again, fusion activity *per se* is unaffected and low-pH fusion at the plasma membrane can by-pass the block (Marsh *et al.*, 1982*b*). Double inhibition studies with monensin and NH_4Cl indicate that the major inhibitory activity of both agents occurs at the same step, i.e. the low-pH-triggered membrane fusion in the endosomes. We have no evidence that the primary effect of monensin is to inhibit the internalization of viruses as reported for VSV with Swiss mouse 3T3 cells (Schlegel *et al.*, 1981).

Conclusion

It is now clear that a number of enveloped animal viruses enter cells by endocytosis and, with the possible exception of the paramyxoviruses, the endocytic route leads to productive infection. To summarize: (1) virtually all viruses are endocytosed; (2) internalized viruses are capable of infecting the cell; (3) fusion at the plasma membrane is not observed morphologically, and antigens from infecting viruses are not detected on the cell surface after entry; (4) the fusion reaction requires a pH lower than that normally found in the extracellular medium; (5) weak bases that increase the pH in acidic intracellular vesicles inhibit penetration, and the efficiency of these agents correlates with measured elevation in lysosomal pH; and (6) fusion can be induced at the plasma membrane by lowering the pH of the medium and under these conditions infection is not inhibited by weak bases.

Why when the most direct entry route for a fusogenic virus is through the plasma membrane do most enveloped viruses use the endocytic route? Firstly, by being dependent on endocytosis, viruses which bind to sialic acid residues or other ubiquitous binding sites can avoid fusing with, for example, red blood cells which cannot support infection. Secondly, by fusing intracellularly, virally infected cells escape immediate recognition by the immune system as the viral spike glycoproteins are not inserted into the plasma membrane. Thirdly, low pH may be required to effect not only membrane fusion but also other conformational changes involved in uncoating.

The fact that enveloped viruses do use the endocytic pathway has been extremely useful. Studies of enveloped virus interactions with tissue culture cells have provided, and will continue to provide, not only a means to elucidate the pathway for the productive infection of these viruses and possible ways to prevent infection, but also information on the properties of the endocytic pathway and its interaction with various physiological ligands.

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