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VIRUS ENTRY INTO ANIMAL CELLS

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I. INTRODUCTION

In addition to its many other functions, the plasma membrane of eukaryotic cells serves as a barrier against invading parasites and viruses. It is not permeable to ions and to low molecular weight solutes, let alone to proteins and polynucleotides. Yet it is clear that viruses are capable of transporting their genome and accessory pro-

teins into the cytosol or into the nucleus, and thus infect the cell. While the detailed mechanisms remain unclear for most animal viruses, a general theme is apparent: like other stages in the replication cycle, entry depends intimately on the activities of the host cell. In order to take up nutrients, to communicate with other cells, to control the intracellular ion balance, and to secrete substances, cells have devised a variety of mechanisms for bypassing and modifying the barrier properties imposed by their plasma membrane. It is these mechanisms, and the molecules involved in them, that most viruses seem to exploit. The host cell provides surface receptors, endocytic activities, triggers for penetration, intracellular transport, and so on, and the viruses take advantage of these during infection. It follows that our understanding of early virus-cell interactions depends on progress in cell biology, physiology, and aspects of molecular biology which at first glance may appear to be only remotely pertinent.

The early phases of viral infection have remained a relatively neglected field in spite of their obvious importance for understanding viral replication, cell tropism, and pathogenesis. The reason may be that they are relatively intractable experimentally. Identification of viral receptors is often hampered by the weak affinity between individual viral proteins and receptor molecules, and receptors often occur in low numbers on the cell surface. The efficiency of productive entry is usually low (the particle-infective unit ratios can be as high as 100-1000). This means that biochemical and morphological studies are easily confused by virions following side pathways. It is, moreover, difficult to obtain a good signal at reasonable multiplicities of infection because the early events take place, by definition, before the amplification in signal due to replication.

In spite of these obstacles, considerable progress has been made. Model virus systems have been characterized approaching a plaque-forming unit (pfu) to particle ratio of 1. It is possible to incorporate sufficient radioactive label into virus particles to follow their fate even at low multiplicity. Modern immunochemical methods have proved powerful in identifying receptors. Relatively well-characterized inhibitors that block the productive infection pathway of large groups of viruses have been identified. *In vivo* and *in vitro* systems have been developed for studying penetration by membrane fusion. As a result, the step-by-step itinerary of the entry pathway of some viruses, such as the Semliki Forest virus (SFV) (Helenius *et al.*, 1988; Kielian and Helenius, 1986), are now known.

In this review we will focus on some of the principles in virus entry with emphasis on recent work. Individual viruses and virus families will be discussed only briefly to illustrate some of the generalizations

made. For early work the reader should consult reviews by Dales (1973, 1978), Lonberg-Holm and Philipson (1974), and Meager and Hughes (1977). More recent reviews include those of Helenius *et al.* (1980a), Dimmock (1982), Marsh (1984), Sommerfelt and Marsh (1988), White *et al.* (1983), Doms *et al.* (1988), and Stegmann *et al.* (1988). There are three recent meeting reports which contain relevant information on virus entry (Compans *et al.*, 1988; Lonberg-Holm and Crowell, 1986) and on membrane fusion (Hoekstra and Wilschut, 1989; Ohki *et al.*, 1988).

II. EARLY VIRUS-CELL INTERACTIONS: A GENERAL VIEW

The spread of viral infection depends on the transfer of the viral genome and accessory proteins from the cytosol (or the nucleus) of infected cells to the corresponding compartments of uninfected cells. When infection occurs via a virus particle—and not, as is sometimes the case, by direct fusion of the two cells—a sequence of discrete events take place. Depending on the virus, these include virus assembly in the infected cell, release of infective virions from the infected cell into the extracellular space, attachment of the viruses to receptor structures on the host cell surface, internalization by endocytosis, penetration through a host cell membrane, uncoating of the genome, and intracytoplasmic relocation to the nucleus or some other site in the cell.

Virtually all types of viruses can be seen by electron microscopy to be internalized by endocytosis. Endocytic uptake is probably not mandatory, however, for infectivity in all cases; many viruses have been shown to penetrate directly through the plasma membrane. It is now well established that viruses which depend on acidic pH (or some other property unique to endocytic organelles) require internalization to be infective.

Regardless of whether it is the plasma membrane or the membrane of an endocytic vacuole that serves as portal of entry, the genome and accessory proteins are usually first delivered to the cytosolic compartment. For successful entry the complete nucleocapsid may not need to enter; for some viruses only the genome need be made accessible to the cytosol. A dramatic example of such a mechanism is found in T-even bacteriophages, where the bulk of the viral particle actually remains extracellular.

Replication may occur in cytosolic complexes, in association with specific cytosolic membranes, or the genome may have to be transported in one form or other to the nucleus for replication. Although topologically continuous with the cytoplasm, the nucleoplasm can only

be reached through the nuclear membrane or the nuclear pores. Due to a low size cutoff for passage through the pores (see Dingwall and Laskey, 1986), the nuclear membrane constitutes another barrier for those viruses destined for replication in the nucleus. Proteins smaller than about 70 kDa can pass freely through the pores, while larger particles can only enter by energy-dependent, selective uptake mechanisms which depend on specific signal determinants (see Dingwall and Laskey, 1986). Studies with gold particles coated with the appropriate signal peptides have shown that the pore structures can expand to allow particles with a diameter of 20 nm (i.e., close to the size of viral nucleocapsids) to pass (Feldherr *et al.*, 1984).

There is evidence to support the notion that some viruses possess, and make use of, nuclear targeting signals (Davey *et al.*, 1985; Kalderson *et al.*, 1986), and capitalize on existing cellular mechanisms for nuclear transport. Electron-microscopic evidence has, on the other hand, been presented suggesting that adenovirus particles, presumably released into the cytosol after lysis of endosomal membranes, may actually uncoat at nuclear pores and inject their genome into the nucleus (Dales, 1978). It cannot be ruled out that some (such as polyoma and SV40) may be delivered to the nucleus by membrane-bound vesicles (Mackay and Consigli, 1976; Maul *et al.*, 1978; Nishimura *et al.*, 1986) or enter the nucleus during mitosis when the nuclear membrane is dissociated.

III. TRANSPORT OF MACROMOLECULES THROUGH MEMBRANE BARRIERS

While similar in their overall pathways of entry, enveloped and nonenveloped viruses differ in the mechanisms of release and penetration. Enveloped animal viruses make elegant use of membrane fission and membrane fusion. Most nonenveloped viruses rely on lysis to escape from the infected host cell followed by poorly understood membrane translocation mechanisms during entry.

The membrane fission-fusion strategy would seem to have several advantages. It allows the viral genome to egress from and enter into cells without membrane lysis. Perhaps more importantly, at no stage during virus release from the infected cell, or entry, do the nucleocapsids need to be physically translocated through a membrane bilayer. This is a major advantage because major conformational alterations and a complex machinery is usually required for translocation of macromolecules through cellular membranes. Furthermore, packaging of

large and segmented viral genomes need not be as rigorously controlled in terms of genome and capsid size as is the case for nonenveloped viruses. Many enveloped viruses are, in fact, polymorphic in size and shape. In normal cell life vesicle-mediated transport is clearly a preferred mode of cellular transport; it is common within the cytoplasm for flexible and efficient intercompartmental transport of cellular macromolecules (Palade, 1975).

Nonenveloped viruses generally exit from the infected cells by inducing cell lysis. During subsequent entry, they must penetrate at least one host cell membrane by a translocation or a lytic mechanism. This may occur either at the level of the plasma membrane or the limiting membranes of endocytic vacuoles. The membrane translocation probably involves dramatic changes in the structure of the outer protein shell of the virus. These changes, which in many instances seem to be acid-activated, result in increased hydrophobicity of the virus surface and hydrophobic membrane attachment. Little detailed molecular data are yet available. The structural and functional studies on picornaviruses are, however, progressing rapidly (Hogle *et al.*, 1985; Rossmann *et al.*, 1985; Rossmann and Palmenberg, 1988) and the mechanism of penetration may soon be understood.

IV. VIRAL RECEPTORS

In order to cause infection, a virus must be able to bind to a cell. Binding occurs through interactions between the surface proteins of the virion and structures on the target cell, "viral receptors." Enveloped and nonenveloped viruses have multiple identical proteins on their surface, thereby possessing the inherent capacity to bind to receptors through multivalent interactions. This provides them with considerable versatility in binding properties. For example, high-avidity binding may occur through a few high-affinity interactions; the human immunodeficiency viruses (HIV-1) gp120 protein binds with high affinity ($K_d = 4 \times 10^{-9} M^{-1}$) to the HIV receptor CD4 (Lasky *et al.*, 1987). Alternatively, high avidity can result from multiple low-affinity interactions (Fries and Helenius, 1979).

Virus binding has, for the most part, been studied in tissue culture systems. A combination of factors including temperature, ionic strength, pH, composition of the medium, and the presence of serum has major effects on binding (Lonberg-Holm, 1981). A variety of techniques including affinity isolation with whole virus or viral components, antireceptor antibodies or antiidiotypic antibodies to the viral

binding sites, chemical cross-linking, DNA transfection, and somatic cell hybrids have been used in attempts to identify specific binding components (reviewed in Sommerfelt and Marsh, 1988). However, few specific virus receptors have been unambiguously identified and still fewer demonstrated to play a functional role in virus entry *in vivo*. A list of molecules reported to bind viruses (or viral components) that infect humans and other vertebrates is given in Table I. The evidence is not clear-cut in all the cases listed; hence the information should be viewed with some caution. Taken together the data indicate, however, that viruses have developed a large variety of strategies by which they recognize and bind to their host cells. They can use proteins, lipids, or oligosaccharides as receptors. It is not surprising that many of the receptors are proteins involved in ligand binding, endocytosis, and cell recognition.

A. High-Specificity Binding/Narrow Host Range

Viruses such as HIV-1 and 2 and Epstein-Barr virus (EBV) bind to components expressed only on a limited number of cell types. In the case of HIV, the virions bind to CD4 molecules expressed on helper/inducer T lymphocytes, cells of monocyte-macrophage lineage, and certain cells transfected with the *CD4* gene (Dalglish *et al.*, 1984; Klatzmann *et al.*, 1984; McDougal *et al.*, 1986; Maddon *et al.*, 1986). The specificity of binding determines viral tropism. Cells that lack receptors for a given virus, or where the binding sites are blocked, are resistant to infection. Replication may occur in such cells if the viral genome is introduced artificially.*

The CD4 antigen is presently the best characterized virus receptor protein. The molecule has been cloned, sequenced and, by expression in receptor-negative cells, confirmed as the HIV receptor (Maddon *et al.*, 1986). Soluble forms of the molecule, which lack the cytoplasmic and transmembrane domains, have been generated and shown to inhibit virus infection *in vitro* (Lasky *et al.*, 1987) and the epitopes involved in virus binding are being mapped with increasing resolution (Sattentau and Weiss, 1988).

B. High-Specificity Binding/Broad Host Range

Orthomyxoviruses and paramyxoviruses bind with considerable specificity to sialic acid, often in distinct linkage configurations. The

* Receptor expression is not, however, the only factor that determines cell tropism. Postpenetration events, such as cell-specific expression of transactivating factors, also determine whether a virus is replicated in a particular cell (Weiss, 1984).

binding site for sialic acid in influenza virus HA has been identified in the X-ray structure as a highly conserved depression in the HA1 subunit at the tip of the spike protein (Wilson *et al.*, 1981). Sialic acid is frequently the terminal saccharide residue on cell surface glycoproteins and glycolipids. Consequently, myxoviruses can infect a wide range of cells through a variety of glycoprotein or glycolipid receptors. Sialic acid has also been implicated in the binding of picornaviruses, papovaviruses, reoviruses, and adenoviruses (Burness, 1981). Competition experiments with the lectin concanavalin A point to a role for other saccharide moieties in human rhinovirus-14 (HRV-14) binding (see Rossmann *et al.*, 1985), and complex carbohydrates such as those present heparan sulfate proteoglycan may be involved in herpes simplex virus infection (WuDunn and Spear, 1987).

C. Broad Host Range

A number of viruses, such as the alphavirus SFV, rhabdo virus, and vesicular stomatitis virus (VSV), exhibit a broad host range in culture but do not appear to utilize carbohydrates as binding components. Semliki Forest virus (SFV) is probably able to utilize several cell surface proteins as receptors. Thus SFV binds to major histocompatibility (MHC) class I antigens on human and murine lymphoblastoid cells (Helenius *et al.*, 1978) but it can also infect cells that do not express MHC molecules (Oldstone *et al.*, 1980). With SFV the affinity of individual spike glycoprotein-receptor interactions appears to be low, but binding of the intact multivalent virus is virtually irreversible (Fries and Helenius, 1979; Marsh *et al.*, 1983b). Vesicular stomatitis virus has been reported to bind preferentially to negatively charged phospholipids (Mastromarino *et al.*, 1987; Schlegel *et al.*, 1983).

D. Different Viruses Using the Same Binding Sites

Orthomyxoviruses and paramyxoviruses, which bind to sialic acid, can use the same binding sites. Competition experiments show that receptors may be shared among other viruses as well. On HeLa cells, four distinct binding sites are used by a number of nonenveloped viruses: group 1 is shared by the HRV types 2, 1A, and 1B; group 2 by coxsackie type A21 and HRV types 3, 5, 14, 15, 39, 41, 51; group 3 by all three serotypes of poliovirus; and group 4 by coxsackie B3, other coxsackie B viruses, and adenoviruses types 2 and 5 (Lonberg-Holm *et al.*, 1976). Similarly, 18 retroviruses that infect cultured human cells utilize eight distinct receptors, although it remains unclear whether they utilize the same epitopes (Sommerfelt and Marsh, 1988).

TABLE I
VIRUS-BINDING CELL SURFACE COMPONENTS AND RECEPTORS

Virus group	Virus	Binding component	References
Enveloped viruses	Sendai	Sialic acid (glycoprotein/glycolipid)	Markwell <i>et al.</i> (1984)
	Orthomyxo	Influenza A, B, and C	Sialic acid (glycoprotein/glycolipid)
Toga	Semliki Forest virus	Class 1 MHC	Helenius <i>et al.</i> (1978)
	Sindbis virus	90 kDa Protein/catecholinergetic receptor	Massen and Terhorst (1981), Tignor <i>et al.</i> (1984)
Rhabdo	Lactate dehydrogenase virus	Class 2 MHC	Inada and Mims (1984)
	West Nile virus	Fc receptor (via bound IgG)	Peiris and Porterfield (1979)
	Rabies virus	Acetylcholine receptor	Lentz <i>et al.</i> (1982)
Retro	Vesicular stomatitis virus	Phosphatidylserine and other negatively charged lipids	Mastromarino <i>et al.</i> (1987), Schlegel <i>et al.</i> (1983)
	Human immunodeficiency virus	CD4 (T4)	Dalgleish <i>et al.</i> (1984), Klatzmann <i>et al.</i> (1984)
	Moloney murine leukemia virus	110 kDa Glycoprotein	Johnson and Rosner (1986)
	Radiation leukemia virus	L3T4/T-Cell receptor complex	O'Neill <i>et al.</i> (1987)
	Visna virus	30-33 kDa Protein	Dalziel <i>et al.</i> (1988)

Herpes	Epstein-Barr virus	CR2	Fingeroth <i>et al.</i> (1984), Frade <i>et al.</i> (1985), Reisert <i>et al.</i> (1985), Tanner <i>et al.</i> (1987)
	Cytomegalovirus	β_2 -Microglobulin/MHC	Grundy <i>et al.</i> (1987), McKeating <i>et al.</i> (1987)
	Herpes simplex virus	Heparan sulfate proteoglycan	WuDunn and Spear (1988)
	Varicella-zoster virus	Mannose 6-phosphate receptor	Gabel <i>et al.</i> (1988)
Hepadna	Hepatitis B	Polyalbumin/polyalbumin receptor	Thung and Gerber (1984)
Pox	Vaccinia	Epidermal growth factor receptor	Eppstein <i>et al.</i> (1985)
Corona	Mouse hepatitis virus	110 kDa Glycoprotein	Holmes <i>et al.</i> (1988)
Nonenveloped viruses			
Picorna	Coxsackie B	49 kDa Glycoprotein	Mapoles <i>et al.</i> (1985)
	Rhinovirus	90 kDa Glycoprotein	Tomassini and Colonno (1986)
	Encephalomyocarditis virus	Glycophorin A	Burness and Pardoe (1983)
Reo	Reovirus type 3	67 kDa Glycoprotein, 54 kDa protein	Burness (1981), Co <i>et al.</i> (1985a,b), Gaulton <i>et al.</i> (1985), Maratos-Flier <i>et al.</i> (1988)
	Reovirus type 1	54 kDa Protein	Maratos-Flier <i>et al.</i> (1988)
Papova	Polyomavirus	24, 30-40, 50, 95 kDa Glycoproteins	Griffiths and Consigli (1986), Marriott <i>et al.</i> (1987)
Adeno	Adenovirus type 2	78, 42, 34 kDa Glycoproteins	Hennache and Boulanger (1977), Svensson <i>et al.</i> (1981)

E. Viral Proteins as Virus Receptors

Membrane proteins of enveloped viruses may themselves function as virus receptors. Normally, Madin–Darby canine kidney (MDCK) cells—a cell line that grows as a polarized monolayer in culture—can only be infected with VSV through the basolateral domain. The receptors for these viruses are not expressed on the apical domain. When MDCK cells are infected with influenza virus, HA is expressed apically and the cells become susceptible to VSV through the apical domain. The HA binds to the sialic acid in the envelope protein (G) of VSV and functions as a VSV receptor (Fuller *et al.*, 1985). Similarly, baby hamster kidney (BHK-21) cells do not express a receptor for murine hepatitis virus (MHV-A59, a coronavirus). The cells are therefore resistant to MHV. However, they become susceptible when preinfected with influenza. Thus, virus infection can facilitate secondary infections by other virus *in vitro* (Fuller *et al.*, 1985; Khélifa and Meneses, 1983).* A similar phenomenon may occur *in vivo*.

F. Indirect Binding

The examples just discussed all involve direct interaction of the virus attachment sites with cell surface-binding components. Viruses may also bind to cell surface components indirectly through mediation by an intermediate molecule. The clearest example of this type of interaction occurs in antibody-enhanced viral infection. Antiviral antibodies facilitate flavivirus, myxovirus, and lentivirus infection of macrophages and other cells both in culture and *in vivo* by cross-linking virions to cell surface Fc receptors (McGuire *et al.*, 1986; Peiris and Porterfield, 1979; Peiris *et al.*, 1981; Webster and Askonas, 1980). Cytomegalovirus is believed to bind β_2 -microglobulin, displace MHC-bound β_2 -microglobulin, and use MHC antigens to bind to the cell surface (Grundy *et al.*, 1987; McKeating *et al.*, 1987). Hepatitis B virus appears to bind to hepatocytes through polyalbumin and polymeric albumin receptors (Thung and Gerber, 1984).

A curious example where an related receptor can rescue a virus is supplied by mutant Sendai viruses which lack functional receptor binding proteins: since the glycoproteins contain terminal sialic acid, they

* Infection by one virus may also interfere with subsequent infection by a second. Cells chronically producing retroviruses, and expressing viral envelope glycoproteins on their surface, down-regulate, shield, or otherwise hide the binding site. Thereby the cells become resistant to superinfection by any retroviruses that use the same cell surface-binding sites (see Sommerfelt and Marsh, 1988).

can be internalized through the asialoglycoprotein receptor and infect cells (Markwell *et al.*, 1982).

G. Role of Cell Surface Virus Receptors

Binding facilitates viral entry by providing the initial physical association between a cell surface and the virion. Whether virus receptors play any further role in entry depends on the case. Influenza virus, for instance, does not require sialic acid containing receptors for penetration. The receptors are needed for binding and endocytosis. In the case of alphaviruses and rhabdoviruses it is also known from *in vitro* studies that the fusion activity which underlies penetration in endosomes is not dependent on the presence of specific surface receptors. Experiments in which avian retroviruses bind nonspecifically to receptor-deficient cells but fail to infect suggest, however, that additional essential roles for receptors may exist in other cases (Notter *et al.*, 1982). The receptor for poliovirus may, for instance, be needed for the correct acid-induced conformational change. While binding and endocytosis is required for penetration by pH-dependent viruses (Section V), no evidence for a more direct role for the receptor in penetration is available.

V. RECEPTOR-MEDIATED AND OTHER MODES OF ENDOCYTOSIS

A. Background on Endocytosis

All nucleated interphase cells express continuous, high-capacity endocytic activity whereby components of the medium are internalized in membrane-bound vesicles. Constitutive endocytosis occurs primarily through clathrin-coated vesicles (~100 nm diameter), which form by the invagination of specialized coated-pit domains of the plasma membrane. Coated vesicles mediate efficient uptake of nutrient carriers, growth factors, peptide hormones, immune complexes, and other physiological ligands that bind to specific receptors expressed on the cell surface (see Brown *et al.*, 1983; Goldstein *et al.*, 1985; Helenius *et al.*, 1983). In addition, solutes and small particles (<100 nm diameter) are internalized nonspecifically and less efficiently in the fluid content of the vesicles (fluid-phase endocytosis) (see Steinman *et al.*, 1983).

Following internalization, the clathrin coat is removed and the vesicles fuse with organelles of the endosome compartment. Endosomes are the station for sorting ligands and receptors internalized by coated vesicles and for regulating endocytic membrane traffic (see Helenius

et al., 1983). They are responsible for dispatching molecules to lysosomes and for recycling others to the plasma membrane on the Golgi apparatus (see Goldstein *et al.*, 1985; Helenius *et al.*, 1983; Mellman *et al.*, 1986). Some incoming molecules are proteolytically processed in endosomes (Diment *et al.*, 1988). Others undergo conformational changes.

The mechanisms of these sorting reactions are not well understood. However, acidification of endocytic organelles through membrane-bound H^+ -ATPases is one important element (Mellman *et al.*, 1986; Tycko and Maxfield, 1982). Endosomes are the first acidic compartment in the endocytic pathway (Fuchs *et al.*, 1987). From a pH of 6.2 in early endosomes the pH decrease to approximately 5.0 by the time ligands reach the terminal compartment of the pathway, the lysosomes. Agents, such as acidotropic weak bases (ammonium chloride, chloroquine, amantadine, methylamine) and carboxylic ionophores (monensin and nigericin), which raise the pH of acidic organelles,* disrupt endosomal function, prevent the dissociation of receptor-ligand complexes, and inhibit the recycling of receptors (Brown *et al.*, 1983; Maxfield, 1982; Mellman *et al.*, 1986; Ohkuma and Poole, 1978). Acidification is also important for lysosome function. Lysosomes provide a hydrolytic environment where ligands, and receptors to be down-regulated or turned over, are degraded. The hydrolytic enzymes are active at low pH and at least partially inhibited by reagents that raise the pH of acidic organelles (Ohkuma and Poole, 1978). Some hydrolytic enzymes are already encountered and active in the endosomal compartment, where they modify and degrade certain incoming ligands (Diment *et al.*, 1988).

In addition to constitutive endocytosis, cells can internalize large particles (>200 nm diameter) such as yeast or bacteria. Termed phagocytosis, this form of endocytosis is usually the property of specialized phagocytic cells such as macrophages (see Steinman *et al.*, 1986), but with an appropriately opsonized particle it can occur in most cell types. Phagocytosis is ligand-induced, receptor-dependent, and occurs through an actin-based mechanism that can be inhibited by cytochalasin B. Following invagination, phagocytic vesicles are acidified in a similar way to endosomes and fuse with lysosomes to form degradative phagolysosomes.

* In nonprotonated form, weak bases readily diffuse across membranes. In acid compartments they become protonated, accumulate, and increase the vesicular pH. Carboxylic ionophores have a similar effect but achieve this by exchanging H^+ for Na^+ or K^+ (Maxfield, 1982; Ohkuma and Poole, 1978).

B. Many Viruses Are Endocytosed

Many viruses, including alphaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, retroviruses, herpesviruses, and a number of nonenveloped viruses, have been shown to be internalized by endocytosis (Dales, 1973; Helenius *et al.*, 1980b; Matlin *et al.*, 1982a,b). Morphological evidence for this process, originally termed "viropexis," indicates that uptake usually occurs through clathrin-coated vesicles by receptor-mediated endocytosis, but other modes of endocytosis may also be operational.

Detailed experiments with SFV have shown that the endocytosis is very similar to the receptor-mediated uptake of physiological ligands (see Kielian and Helenius, 1986; Fig. 1). At 0°C virions are bound over the entire cell surface but are not internalized. On warming to 37°C, the virions are relocated to coated pits and internalized in coated vesicles. Uptake is not induced by the virions, is independent of multiplicity, and occurs through the constitutive endocytic activity of the host cell (Marsh and Helenius, 1980; Marsh *et al.*, 1983a). The SFV particles (65 nm diameter) can be contained within endocytic coated vesicles. They are internalized with a time course similar to that of serum low density lipoprotein (LDL) (the half-time of bound viruses on the cell surface is 5–10 minutes) (Helenius *et al.*, 1988; Marsh and Helenius, 1980; Schmid *et al.*, 1989). In BHK cells up to 3000 SFV particles can be internalized in coated vesicles per minute (Marsh and Helenius, 1980). The injection of anticlathrin antibodies into the cytoplasm of cells inhibits the internalization of SFV and blocks infection (Doxsey *et al.*, 1987).

Morphological observations indicate that many other viruses follow essentially the same pathway, although the kinetics of endocytosis can vary apparently as a function of particle size. Influenza virus (100 nm diameter) and VSV (150 nm long, 50 nm diameter) have a half-time of 10–15 and 30 minutes, respectively (Matlin *et al.*, 1982a,b).

Although clathrin-coated vesicles are clearly involved in the uptake of most enveloped and nonenveloped viruses, other endocytic mechanisms have been encountered. Morphological experiments with influenza and Sendai viruses show that, in addition to coated vesicles, virions are occasionally seen in noncoated membrane vacuoles resembling phagocytic vesicles (Dourmashkin and Tyrell, 1974; Hayward, 1987; Matlin *et al.*, 1982a). Although herpesviruses are often observed close to coated pits, EBV (250 nm diameter) is reported to enter B lymphocytes in noncoated vesicles (Nemerow *et al.*, 1985). The endocytic uptake of herpesvirus may at least in part, occur by a phagocytic

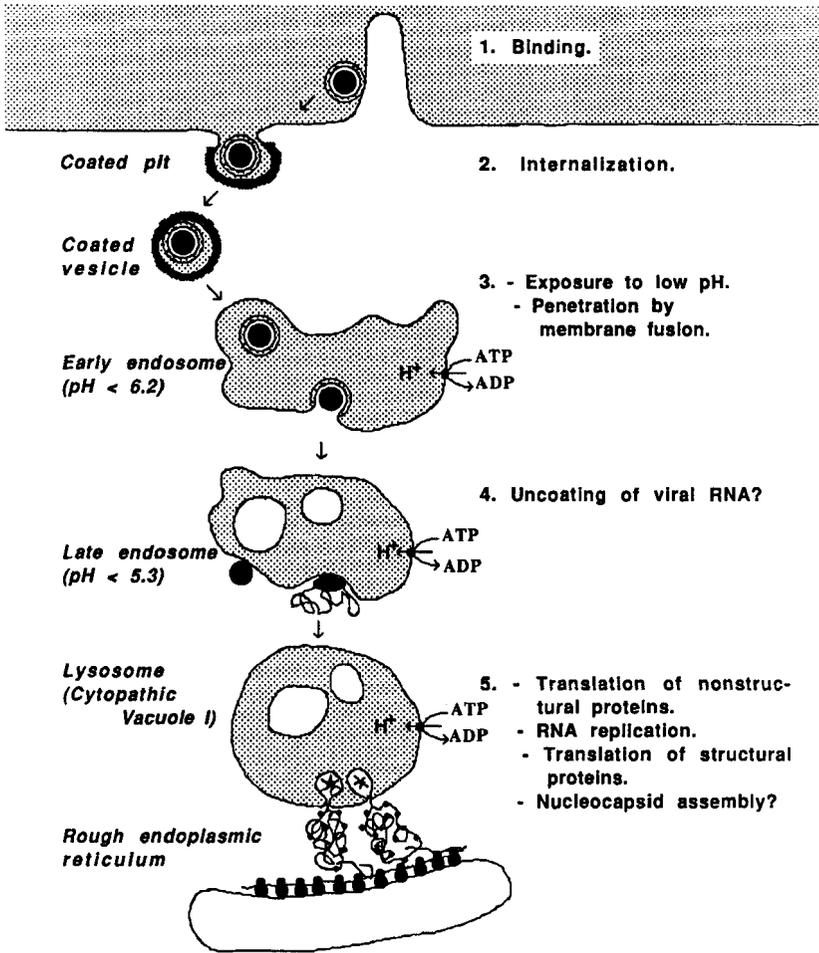


FIG. 1. The entry pathway of SFV into tissue culture cells. Semliki Forest virus enters by receptor-mediated endocytosis, and penetration by membrane fusion is triggered in the early endosome. Attachment occurs preferentially to microvilli followed by lateral movement of the virus to coated pits. Exposure to a pH of < 6.2 and penetration of virus occurs almost immediately after internalization. If mutant viruses with a lower pH threshold of fusion (Kielian *et al.*, 1986) or cell mutants with an endosomal acidification defect are used, penetration occurs from late endosomes (Helenius *et al.*, 1988; Kielian *et al.*, 1986; Schmid *et al.*, 1988). Recent studies on the site of replication of the viral RNA suggest that it occurs on the cytoplasmic surface of the lysosomal membrane (Froshauer *et al.*, 1988). Frequently, a connection between the lysosome and the rough endoplasmic reticulum is seen, suggesting that viral RNA synthesis, the synthesis of nonstructural and structural protein, as well as nucleocapsid assembly may occur in the same extensive structure suspended over the space between the cytopathic vacuoles and the lysosomes (Froshauer *et al.*, 1988).

mechanism, since it is inhibited by cytochalasins (Rosenthal *et al.*, 1985). Whereas poliovirus seems to enter mainly by coated pits, HRV-2 may infect cells via a coated vesicle-independent endocytic mechanism (Madshus *et al.*, 1987). Papovaviruses are known to be internalized mainly in noncoated vesicles (Maul *et al.*, 1978; Mackay and Consigli, 1976). They are so small and tight fitting that it almost looks like the viral particles would be budding from the extracellular space into the cell. All these mechanisms require further study, as they may provide new insights into the versatility of cellular endocytosis.

C. Penetration from Endosomes

Like other ligands internalized by receptor-mediated endocytosis, internalized virions are delivered to endosomes. Kinetic, morphological, biochemical, and cell fractionation experiments have demonstrated that SFV, Sindbis, and influenza virions penetrate into the cytoplasm by fusing with the limiting membrane of endosomes (Kielian *et al.*, 1986; Marsh *et al.*, 1983a; Richman *et al.*, 1986; Talbot and Vance, 1982; Yoshimura and Ohnishi, 1984). Internalized viruses have been shown to be infectious (Helenius *et al.*, 1982), and fusion of SFV with endosome membranes has been observed morphologically (Helenius, 1984). Particularly convincing images of viruses fusing out of endosomes have been obtained for frog 3 viruses (Braunwald *et al.*, 1985).

For alphaviruses, myxoviruses, rhabdoviruses, and many other viruses, endocytosis is essential for productive entry. The acidic conditions in endosomes trigger the reactions which lead to fusion of the viral and endosomal membranes. The precise time and location of penetration is determined by the pH dependence of the fusion activity. Viruses in which fusion is triggered at a pH close to neutral (e.g., SFV, pH 6.2) fuse in so-called early endosomes (Helenius *et al.*, 1988; Schmid *et al.*, 1988). Viruses that require more acidic conditions, such as SFV_{fus-1} (pH 5.3) and influenza X-31 (pH 5.3), fuse in so-called late endosomes ($t_{1/2} = 20-35$ minutes) (Helenius *et al.*, 1988; Kielian *et al.*, 1986; Schmid *et al.*, 1988; Stegmann *et al.*, 1987b). The differences reflect the gradual increase in acidity within the endocytic pathway.

In all cases studied, fusion appears to occur in endosomes prior to delivery to the lysosomes (Kielian *et al.*, 1986; Marsh *et al.*, 1983a; Nussbaum and Loyter, 1987; Stegmann *et al.*, 1987b). Viruses, or viral components that are delivered to lysosomes, are rapidly inactivated and degraded (Marsh *et al.*, 1983a), which may explain why lysosomes

are usually not able to support viral penetration. One exception could be reoviruses, where specific proteolytic cleavages are needed for infectivity (Sturzenbecker *et al.*, 1987).

The dependence on endocytosis and subsequent exposure to acid conditions presents a point at which penetration can be blocked. By raising the pH of endosomes and lysosomes, weak bases and carboxylic ionophores block the acid-induced changes essential for fusion and translocation of many viruses (Gollins and Porterfield, 1986b; Helenius *et al.*, 1982; Matlin *et al.*, 1982a,b; Richman *et al.*, 1986; Yoshimura and Ohnishi, 1984).* As a result, the viral genome is not released into the cytoplasm. The efficacy of weak bases is related to the pH threshold of fusion. Viruses that fuse at pH 6.0 (SFV, VSV) and 6.5 (West Nile) require higher concentrations of weak bases to be effectively neutralized than viruses that fuse at pH 5.3 (influenza X-31) (Kielian and Helenius, 1986).

Weak bases and carboxylic ionophores do not affect binding, endocytosis, and transport of prebound virions to endosomes, nor do they affect the fusion reaction directly. In some cases weak base-induced blocks to infection can be bypassed by briefly incubating cells, with bound virus, in low-pH medium. This treatment induces fusion of virions at the cell surface, and, even in the presence of the inhibitors, results in infection of the cell (Helenius *et al.*, 1980b, 1982; Marsh *et al.*, 1982). The effects of acidotropic weak bases are, moreover, very sensitive to a lowering of the pH in the medium, which has to be controlled throughout the experiment (Kielian and Helenius, 1986; Yoshimura *et al.*, 1987).

D. What Advantages Does Endocytosis Offer a Virus?

Internalization through the endocytic pathway usually results in the delivery of ligands to the hydrolytic lysosome compartment, an environment in which they are rapidly inactivated and degraded. Since entry via the endocytic pathway carries such a risk for viruses, it must offer significant balancing advantages. Some potential advantages

* Note that the effects of weak bases and carboxylic ionophores on virus entry should not be confused with effects on other steps in replication. Weak bases and carboxylic ionophores affect all acidic compartments in the cell and are not specific to endocytic organelles. For example, transport of newly synthesized enveloped virus membrane proteins to the cell surface can be inhibited through the effects of these agents on the exocytic pathway (see Cassell *et al.*, 1984; Mellman *et al.*, 1986; Tartakoff, 1983). When using these agents as diagnostic inhibitors for acid-dependent entry, it is important to assay for early events such as uncoating or RNA replication rather than late events such as the production of progeny virus.

have been discussed previously (Helenius *et al.*, 1988; Sommerfelt and Marsh, 1988). By requiring endocytosis, virions can, for instance, ensure that they enter metabolically active cells capable of supporting replication. For pH-dependent viruses, endocytosis is required to deliver the virions to an acidic environment.

Another likely advantage is based on the fact that viral genome may need to be delivered to a specific region of the cytoplasm, such as the perinuclear region, to initiate infection. Entry through the endocytic pathway provides a natural means for achieving such relocalization. Evidence suggesting that productive infection of some cell types only occurs efficiently from endocytic vesicles comes from experiments with pH-dependent enveloped viruses. As already mentioned, fusion can be induced at the plasma membrane by briefly acidifying the medium (Helenius *et al.*, 1980b). In baby hamster kidney (BHK), fusion at the cell surface results in infection (Helenius *et al.*, 1980b; White *et al.*, 1980). In several other systems, however, including VSV and fowl plague virus (an avian influenza virus) in MDCK cells, West Nile virus on murine macrophagelike cells, and SFV on Chinese hamster ovary (CHO) cells, fusion at the cell surface does not cause infection (Gollins and Porterfield, 1986a; Matlin *et al.*, 1982a,b; M. Marsh, unpublished observations), although it is clear that the nucleocapsids have been delivered into the cytosol (Matlin *et al.*, 1982a). The most likely reason is that the incoming nucleocapsids are trapped in the space between the plasma membrane and the underlying membrane cytoskeleton, and cannot move into the cytoplasm proper. The result implies that endocytosis in many cell types may be essential for transporting viruses through obstacles within the cytoplasmic compartment, and deliver the genome into the central perinuclear area. In neuronal cells, which have long cytoplasmic projections, penetration is likely to occur after transport of viruses to the cell body.

VI. MEMBRANE FUSION

A. Fusion Proteins

The membrane fusion activity of enveloped animal viruses is catalyzed by the spike glycoproteins (Table II). While different in their detailed structure, the fusion proteins so far identified in different virus families have several properties in common. They are, as a rule, homooligomeric or heterooligomeric transmembrane glycoproteins with a combined molecular weight in the 200K–400K range. The N termini are located in the external (ecto) domain. Since the trans-

TABLE II
SOME VIRAL FUSION FACTORS

Virus	Protein	Oligomeric structure	Protomers	pH Dependence	References
Influenza	HA	Homotrimer	HA1-HA2	5.1-5.8	see Wiley and Skehel (1987)
Sindbis	E1,2	Heterohexamer	E1, E2	<6.2	see Fuller (1987)
SFV	E1,2,3	Heterononamer	E1, E2, E3	<6.2	see Vogel <i>et al.</i> (1986)
VSV	G	Homotrimer	G	<6.2	Dubovi and Wagner (1977), Doms <i>et al.</i> (1987), White <i>et al.</i> (1981)
Rous sarcoma	Env	Homotrimer	gp85, gp37	ND	Hunter <i>et al.</i> (1983), Hunter and Einfeld (1988)
MMTV	Env	ND ^a	gp71, gp36	<5.4	Redmond <i>et al.</i> (1984)
HIV	Env	ND	gp120, gp41	pH-Independent	Kowalski <i>et al.</i> (1987)
Sendai	F	Homotetramer	F1-F2	pH-Independent	Schoy <i>et al.</i> (1987)
West Nile	E	Homotrimer	E	<6.5	Wengler <i>et al.</i> (1987)
Vaccinia	14 kDa	Homotrimer	14 kDa	ND	Janeczko <i>et al.</i> (1987)
Mouse hepatitis	E2	Heterodimer	90A, 90B	pH-Independent	Sturman <i>et al.</i> (1985)

^a ND, Not determined.

membrane domains and the C-terminal, cytoplasmic domains are relatively small, the bulk of the polypeptide is external and visible by high-resolution electron microscopy as spikelike protrusions on the surface of the virus envelope. Most viruses only need a single type of spike glycoproteins for fusion, but it has been suggested that others, such as paramyxoviruses, may need the cooperative action of two (see later). Each virus particle contains 80 or more spike glycoproteins. Whether more than one individual spike is required in each fusion event is unclear.

The fusion factors are invariably glycosylated, and many of them contain covalently bound palmitic acid. While addition of the N-linked sugars is often required for correct folding of the nascent fusion proteins and transport to the surface (see Rose and Doms, 1988; Schlesinger and Schlesinger, 1986), the carbohydrate moiety has no direct role in fusion. The role of fatty acid acylation is less clear. Mutants and naturally occurring strains of VSV, which lack fatty acylation in their G protein, are not impaired in their fusion activity, showing that the fatty acid is dispensable (Rose *et al.*, 1984). In the case of influenza HA it has been reported, however, that removal of palmitic acid using hydrolysis with hydroxylamine inhibits fusion activity, suggesting that it may play a role (Lambrecht and Schmidt, 1986). It is not clear in this case whether the treatment modified the spike trimers in other subtle ways. In VSV G protein the fatty acid is coupled by a thioester bond to a cysteine residue in the cytoplasmic tail (Rose *et al.*, 1984). A similar location for the palmitic acid is likely for other acylated spike glycoproteins.

Activating proteolytic cleavages catalyzed by cellular proteases late in the secretory pathway are critical for the fusion activity of at least orthomyxoviruses, paramyxoviruses, coronaviruses, and retroviruses (see Choppin and Compans, 1975; Sturman *et al.*, 1985; White *et al.*, 1983). The new N termini are frequently hydrophobic, and some have been shown to be important in fusion (Boulay *et al.*, 1987a; Gething *et al.*, 1978, 1986; Harter *et al.*, 1988). In influenza HA strains the "fusion sequence" at the newly created N terminus of HA2 is highly conserved (Wiley and Skehel, 1987). While the uncleaved precursor hemagglutinin, called HAO, can undergo an acid-activated conformational change similar to that observed for the mature HA, it does not expose a hydrophobic moiety with which it can interact with membranes (Boulay *et al.*, 1987a). It is thus apparent that the hydrophobic "fusion sequence" must be present as a free N-terminal for fusion activity.

Proteolytic activation is not required for all viral fusion proteins. A precursor subunit in the spike glycoprotein of SFV (p62) undergoes a

late cleavage to the mature glycopolypeptides E2 and E3, very similar to those listed earlier, but this cleavage is not needed for fusion activity (Cutler and Garoff, 1986; Edwards *et al.*, 1983). Rhabdovirus G proteins do not undergo any posttranslational cleavages, but they are still very efficient fusogens.

Aside from the general similarities, fusion factors constitute quite a heterogeneous group of proteins. Sequence homologies are seldom observed, and not all have N-terminal hydrophobic fusion sequences. Some fusion proteins have receptor-binding activities, others do not. While fusion proteins are generally oligomeric, some contain a single type of subunits; others are heterooligomeric. The mechanisms of fusion also display distinct differences. It seems clear that, as a group, viral fusion factors do not have their origin in a single ancestral gene. Perhaps they evolved from different cellular surface receptors or cell recognition proteins, and their initial functions may have been to provide efficient and specific attachment to cells. The fusion function may have evolved later. Since many different proteins display latent fusion activities, particularly when denatured by extremes of pH, the structural requirements for this activity may not be very stringent (Stegmann *et al.*, 1988).

B. Influenza HA and the Mechanism of Fusion

Most biological membranes have the capacity to fuse with other membranes. During cell life, membrane fusion is a frequent activity in endocytosis, exocytosis, and numerous other cellular functions. Fusion events in biological systems occur between specific membranes, at specific times, and at defined locations. Since they are strictly regulated, it is usually assumed that proteins are involved.

So far, the best-studied fusion proteins are the viral spikes from influenza virus, Sendai virus, SFV, HIV-1, and VSV. Their properties and activities have been described in numerous papers, and have been the topic of recent reviews, by White *et al.* (1983), Wiley and Skehel (1987), Stegmann *et al.* (1989), and Wharton (1987), and by several other authors in books on membrane fusion (Hoekstra and Wilschut, 1988; Ohki *et al.*, 1989). Here we will restrict the discussion to some general principles, focusing mainly on influenza HA.

Influenza virus HA constitutes one of the most efficient fusogens known. The X-ray structure of the HA ectodomain in its inactive neutral pH form is known (Wilson *et al.*, 1981), and its biosynthesis and assembly have been extensively analyzed. It is a homotrimer (3×84 kDa) in which every protomer consists of two disulfide-linked glyco-

polypeptides, HA1 and HA2, derived by proteolytic cleavage from a common precursor HA0. HA2 constitutes the transmembrane subunit and plays a key role in fusion (Gething *et al.*, 1986; Harter *et al.*, 1988; Wharton, 1987). HA1, which is entirely outside of the membrane, is responsible for binding the virus to sialic acid residues on the surface of the host cell.

HA is the only protein component required for fusion activity (White *et al.*, 1982a). To be active it must be an integral component of one of the fusing membranes (White *et al.*, 1982a) and (in exceptional cases) when present as a polyvalent, water-soluble rosette (Wharton, 1987). The lipid compositions of the HA-containing membrane and the target membrane are not very critical (Maeda *et al.*, 1981; Nussbaum *et al.*, 1987; Stegmann *et al.*, 1987a; White and Helenius, 1980). Thus, HA can be reconstituted in functional form into artificial membranes without loss of activity, and it mediates fusion with virtually any type of biological or artificial membrane (Kawasaki *et al.*, 1983; Maeda *et al.*, 1981; Nussbaum *et al.*, 1987; Stegmann *et al.*, 1987a; White and Helenius, 1980). Only certain liposomes with nonbiological lipid compositions have been found to be poor targets (Stegmann, 1987), and drastic enzymatic modification of the viral lipids has been shown to be inhibitory (Huang and Uslu, 1986). The target membranes do not need to contain any sialic acid-carrying molecules or proteins (Maeda *et al.*, 1981; White and Helenius, 1980). The fact that sialic acid-containing receptors such as gangliosides sometimes increase the efficiency of fusion may reflect improved attachment of the two membranes to each other rather than receptor involvement in fusion (Stegmann *et al.*, 1988).

The fusion activity of HA is acid-activated and involves a major conformational change in the HA trimer. The conformational change, studied in several laboratories using a variety of techniques (see Doms *et al.*, 1985; Stegmann *et al.*, 1988; Wharton, 1987; White and Wilson, 1987; White *et al.*, 1983), involves a partial dissociation of the trimer. The top domains of the HA1 chains dissociate from each other. A previously hidden hydrophobic moiety, which includes the N termini of the HA2, is exposed, and additional changes occur in the interface between the three HA2 subunits (Doms and Helenius, 1986). Figure 2 depicts schematically our interpretation of the data. The half-life of the irreversible change is about 15 seconds at pH 5 and 37°C; at lower temperatures the change is slower and less complete. There are indications that the change occurs in two steps. The first involves a change at the tip of the molecule and the second a change further down. However, studies with isolated bromelain fragments, which comprise

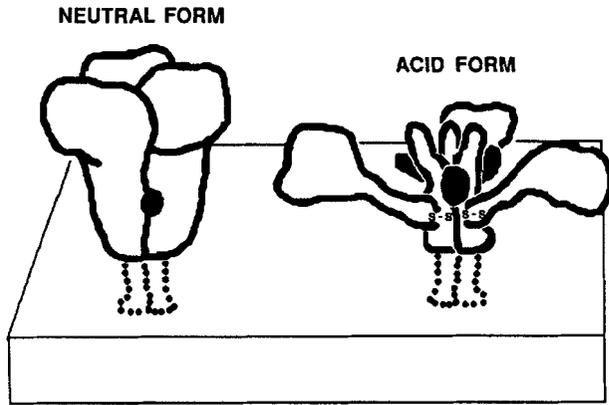


FIG. 2. The acid-induced conformational change in influenza HA. It is generally agreed that acid exposure leads to a partial dissociation of the ectodomain of the HA spike trimer, that a hydrophobic moiety involving the N termini of HA2 (here shown in black) is exposed. Though based on a variety of biochemical, genetic, immunochemical, and morphological data, the proposed structure of the acid HA shown is, however, still highly speculative.

the trimeric ectodomain (and approximate the intact HA in their acid conversion), have indicated that exposure of the hydrophobic fusion peptide may also be an early event (White and Wilson, 1987). The conformational change in the HA trimer is cooperative (Boulay *et al.*, 1987b), and we have indirect evidence that fusion may involve more than one spike trimer (Doms and Helenius, 1986). It has been suggested on the basis of radiation inactivation analysis that the fusion-active unit in influenza virus fusion with red blood cells is no larger than about 60 kDa (Bundu-Morita *et al.*, 1987). The dose-response curve is complex, however, and other interpretations are possible.

Despite extensive work in analyzing the conformational change in HA, a single generally accepted model does not exist. For alternative interpretations of its nature and its relevance for fusion, see Doms and Helenius (1988), Landsberger and Schegal (1986), and Ruigrok *et al.* (1986). Reports suggesting that influenza can fuse at neutral pH have appeared in the literature (Haywood and Boyer, 1982; Stegmann *et al.*, 1985, 1986). One of them was shown to be an artifact due to the use of cardiolipin vesicles as targets (Stegmann *et al.*, 1986). The highly charged cardiolipin membranes are known to serve as efficient fusion vesicles for a large number of proteins including many with no known physiological fusion activity (see Stegmann *et al.*, 1989). The ac-

cumulation of positively charged ions (including protons) close to the membrane, according to the electrical double-layer theory of Gouy and Chapman, may at least in part explain the effect. Too little quantitative data are available to evaluate properly another report of neutral pH fusion between influenza virus and ganglioside-containing vesicles (Haywood and Boyer, 1982).

The detailed mechanism of HA-mediated fusion remains elusive. The view that we favor is as follows: When exposed to a pH of 5.0–5.6 (depending on the virus strain) in the late endosomes, the HA undergoes the conformational change. A hydrophobic interaction between the spikes and the target membrane is established through insertion of the exposed hydrophobic moieties into the target membrane. This results in attachment of the virus to the target membrane and close apposition between the two membranes. The close proximity of the two membranes results in local dehydration of the membrane surface, which in turn leads to a lowering of the hydration force, which normally prevents close apposition of polar surfaces in aqueous solution (Rand, 1981). As a consequence of local dehydration and a change in the local lipid configurations induced by the inserted polypeptide, fusion of the outer bilayer leaflet of the viral membrane and the inner bilayer leaflet of the endosome occurs. The fusion with the outer leaflet of the endosome and the inner leaflet of the viral envelope membrane follows, resulting in the release of the nucleocapsid into the cytosolic compartment. Fusion between two membranes obviously needs to occur only at a single focal site. This model predicts that the HA molecules are associated—at least transiently—as integral proteins in both fusing membranes and that the insertion of protein into the target membrane and the effect on surface hydration brings about a change in local lipid orientation.

It should be emphasized that other mechanisms are conceivable and that it is unclear what exactly is needed to induce fusion of any type of biological membrane (Blumenthal *et al.*, 1987; Rand, 1981). Other viruses and viral fusion factors seem to differ in their fusion properties in significant ways. Some depend on the presence of cholesterol in the target membrane (Kielian and Helenius, 1984; White and Helenius, 1980), some do not have distinct hydrophobic fusion sequences (Woodgett and Rose, 1986), and some may, unlike HA, undergo reversible conformational changes at acid pH (Doms *et al.*, 1987). As already mentioned, several viral proteins differ from HA in that they do not require acid pH for fusion (Tables II and III). Additional information on these matters will be presented next, when a selection of individual virus families are described.

TABLE III
 pH DEPENDENCE OF PENETRATION BY DIFFERENT VIRUSES

Virus family	Species	References
Acid-triggered penetration		
Alpha	SFV, Sindbis	see Kielian and Helenius (1986)
Orthomyxo	Influenza A, B, and C	see White <i>et al.</i> (1983)
Flavi	West Nile virus	Gollins and Porterfield (1986a,b), Kimura <i>et al.</i> (1986)
Rhabdo	VSV, rabies	see Superti <i>et al.</i> (1984, 1987a), Tsing and Superti (1984), White <i>et al.</i> (1983)
Bunya	La Crosse virus, HFRS	Cash (1982), Gonzalez-Scarano (1985), Gonzalez-Scarano <i>et al.</i> (1984)
Retro	MMTV	Redmond <i>et al.</i> (1984)
Irido	African swine fever	Geraldes and Valdeira (1985)
Picornia	Polio, rhino, FMDV	Carrillo <i>et al.</i> (1984, 1985), Madshus <i>et al.</i> (1984a,b,c)
Adeno	Adeno	Pastan <i>et al.</i> (1986)
Reo	Reovirus type 3	Silverstein and Dales (1968), Sturzenbecker <i>et al.</i> (1987)
Possibly acid-triggered penetration		
Corona	MHV-3	Krzystyniak and Dupuy (1984)
Baculo	Nuclear polyhedrosis virus	Volkman <i>et al.</i> (1986)
Retro	Murine retrovirus	Anderson and Nexø (1983)
Pox	Vaccinia	Kohn <i>et al.</i> (1988)
Hepadna	Hepatitis A	Superti <i>et al.</i> (1987b)
pH-Independent penetration		
Paramyxo	Sendai, NDV	see Choppin and Scheid (1980), Citovsky and Loyter (1985)
Retro	HIV	Stein <i>et al.</i> (1987), McClure <i>et al.</i> (1987)
Herpes	Herpes simplex virus I	Spear <i>et al.</i> (1988), Koyama and Uchida (1987)
Papova	SV40, polyomavirus	Shimura <i>et al.</i> (1987)
Rota	Porcine rotavirus	Ludert <i>et al.</i> (1987)

VII. ENTRY OF INDIVIDUAL VIRUS FAMILIES

A. Enveloped Viruses

1. Alphaviruses

The pathway of entry and penetration of toga(alpha)viruses has been studied in considerable detail. The work on SFV now serves as a

general paradigm for viruses with endocytic entry pathways. It was the first virus to be shown to have acid-activated penetration from endosomes and to fuse with liposomes and cell membranes in an acid-triggered fashion (Helenius *et al.*, 1980b; Marsh *et al.*, 1983a; White and Helenius, 1980). The major advantages of this virus as a model system is the extremely high pfu to particle ratio, the ease of radioactive labeling to very high specific activities, and the wide host cell specificity. Since the literature on alphavirus entry has been recently reviewed (Kielian and Helenius, 1986), and some of the material already described here, a relatively short description will suffice.

Although restricted to a few host organisms in the wild, these arthropod-borne, positive-stranded RNA viruses infect a wide range of cells in tissue culture. Some have been shown to bind to MHC antigens and to other surface proteins (Table I), but the true physiological receptors are not known. Endocytic internalization occurs by receptor-mediated endocytosis through clathrin-coated pits and vesicles (Helenius *et al.*, 1980b; Marsh and Helenius, 1980). This has not only been demonstrated at the morphological level, but injection of anticlathrin antibodies into the cytosol has also been shown to inhibit endocytosis and infectivity (Doxsey *et al.*, 1987). Internalized virions are rapidly and efficiently delivered in intact form to early endosomes where the mildly acid conditions (pH <6.2) trigger an irreversible conformational change in the viral fusion proteins, thus inducing membrane fusion and penetration (Edwards *et al.*, 1983; Helenius, 1984; Kielian and Helenius, 1985; Kielian *et al.*, 1986; Schmid *et al.*, 1988; Talbot and Vance, 1982; White and Helenius, 1980). The overall pathway of SFV entry is shown in Fig. 1. It is important to note that up to 60% of cell-associated viruses are actually able to release their RNA into the cytosol (Helenius *et al.*, 1982; Kielian and Helenius, 1984; Marsh *et al.*, 1983a), and that the viral RNA replication appears to take place on the surface of the endosomal and lysosomal membranes (Froshauer *et al.*, 1988). The endosomes and lysosomes are modified in the process to morphologically distinct organelles called cytopathic vacuole I (Froshauer *et al.*, 1988; Grimley *et al.*, 1972).

Alphavirus penetration is blocked by weak bases and carboxylic ionophores (Helenius *et al.*, 1982; Marsh *et al.*, 1982; Miller and Lenard, 1981; Talbot and Vance, 1982). Semliki Forest virus reaches the endosomes in the presence of these acidotropic agents, but fusion is inhibited. The virus-loaded endosomes thus obtained can be isolated, and the SFV nucleocapsid induced to penetrate out of them *in vitro* by adding ATP to drive the endosomal H⁺-ATPase and decrease the endosomal pH (M. Marsh, unpublished observations). This *in vitro*-penetration reaction is inhibited by carboxylic ionophores and other

agents that prevent acidification of the isolated organelles. Like influenza HA, the spike glycoproteins can be extracted from the viral membrane with detergents and reconstituted into lipid vesicles in fusion-active form (Scheule, 1986, 1987).

For successful fusion of SFV with target membranes, three conditions must be fulfilled. i.) A pH of less than 6.2 is required to trigger the conformational changes that render the envelope proteins fusogenic (Edwards *et al.*, 1983; White and Helenius, 1980; Young *et al.*, 1983). ii.) The target membrane must contain cholesterol (Kielian and Helenius, 1984; Scheule, 1987; White and Helenius, 1980), without which fusion does not occur. Cholesterol is needed for the low-pH-induced conformational change in the E1 glycoprotein (Kielian and Helenius, 1985). iii.) Finally, the cell must have a normal membrane potential (Helenius *et al.*, 1985). In depolarized cells, virions are bound and internalized, and undergo acid-induced conformational changes in endosomes, but they do not fuse. This requirement remains somewhat perplexing because no pH gradient, ion gradient, or membrane potential over the target membrane is needed for SFV fusion with liposomes (Kielian and Helenius, 1984).

Since the mode of acid-activated, endocytosis-dependent entry of alphaviruses presented here is not unanimously accepted, it may be important to discuss some of the objections that have been raised. Because infection by Sindbis virus is not inhibited by cytochalasin B, Coombs *et al.* (1981) concluded that endocytosis is not essential for infection. However, it is known that, while this microfilament inhibitor blocks phagocytosis, it leaves coated vesicle-mediated pinocytic processes essentially unaffected (Steinman *et al.*, 1983). Marsh and Helenius (1980) suggested, in fact, that the lack of cytochalasin inhibition was consistent with a pinocytic coated vesicle-dependent pathway of entry. The notion that acid-activated fusion only occurs after pH neutralization (Edwards and Brown, 1986) is also incorrect, as shown by recent studies on cell-cell fusion (Kempf *et al.*, 1987). The confusion may in this case have arisen from the light-microscopic fusion assay used by Edwards and Brown (1986); they did not score fusion as such but subsequent changes in cell morphology, which take time to develop and may be more efficient after returning to neutral pH. Taken together, the overwhelming evidence contradicts the view presented by Edwards *et al.* (1983) that low pH causes the expression of a fusion function which merely "mimics" some intrinsically pH-independent event which occurs during normal infection.

2. *Flaviviruses*

Flavivirus entry has been studied in most detail with West Nile virus. West Nile virus is internalized by coated vesicles and trans-

ported to endosomes (Gollins and Porterfield, 1985). Infection is inhibited by weak bases and is therefore presumed to involve acid-induced fusion (Gollins and Porterfield, 1985, 1986b). The conclusion is supported by experiments in which viruses have been induced to fuse with liposomes or the plasma membrane of target cells (Gollins and Porterfield, 1986a). Penetration is pH-dependent, with the threshold for fusion being about pH 6.5. The mechanism of entry into macrophages is of particular interest, as it is mediated by antibodies and presumably occurs via the Fc receptor (Gollins and Porterfield, 1985; Peiris and Porterfield, 1979; Peiris *et al.*, 1981).

Gollins and Porterfield (1986c) have made an important observation which may be of value for future therapeutic strategies against flaviviruses. They found that among several neutralizing monoclonal antibodies to the West Nile virus, one was able to block penetration without affecting attachment or endocytic internalization. They concluded that the antibody specifically interacted with the acidic fusion-active form of the virus and prevented penetration in endosomes. A similar report has been made for rabies virus (Dietzschold *et al.*, 1987).

3. *Rhabdoviruses*

The entry of VSV and rabies virus into tissue culture cells resembles that of SFV and influenza virus, but less is known about it. Cell surface-bound virions are internalized through coated pits and delivered to endosomes (Matlin *et al.*, 1982b). Fusion is pH-dependent, occurring at pH 6.0 and below, and entry is inhibited by weak bases (Blumenthal *et al.*, 1987; Matlin *et al.*, 1982b; Miller and Lenard, 1981; Superti *et al.*, 1984; Tsing and Superti, 1984) and carboxylic ionophores (M. Marsh, unpublished observations). Vesicular stomatitis virus fuses rapidly and efficiently with a variety of natural and artificial target membranes (Blumenthal *et al.*, 1987; Matlin *et al.*, 1982b; Mifune *et al.*, 1982; White *et al.*, 1981; Yamada and Ohnishi, 1986). The G protein, which exists as a trimer in the viral membrane, has been successfully reconstituted into proteoliposomes (Metsikkö *et al.*, 1986). G Protein undergoes conformational changes at low pH which seem to be unique in being reversible when the pH is returned to neutrality (Doms *et al.*, 1987). One of the effects of the conformational change in the G protein is the stabilization of the homotrimeric structure (Doms *et al.*, 1987). However, the effects of these changes on the conformation of the G-protein trimers and the fusion properties of the G protein are still poorly understood. The sequence of G Protein is known (Rose and Gallione, 1981), but there is no obvious hydrophobic fusion sequence. It has been suggested that the N-terminal peptide may serve such a role (Schlegel and Wade, 1985), but this is unlikely in view of more recent *in vitro* mutation studies (Woodgett and Rose, 1986).

4. *Orthomyxoviruses*

The entry of influenza virus A has already been discussed extensively. Considerable information exists regarding the structure, receptor specificity, endocytosis, and low-pH-dependent fusion of these viruses. Penetration occurs from late endosomes (Yoshimura *et al.*, 1982) following uptake in coated and noncoated vesicles (Dourmashkin and Tyrell, 1974; Matlin *et al.*, 1982a). The low endosomal pH triggers the fusion reaction (Stegmann *et al.*, 1985; White *et al.*, 1982b) and infection is blocked by acidotropic weak bases (Matlin *et al.*, 1982a; Yoshimura *et al.*, 1982).

5. *Paramyxoviruses*

Paramyxoviruses, such as Sendai and Newcastle disease virus, are well-known fusogenic viruses (see Choppin and Compans, 1975; Hayward, 1987; White *et al.*, 1983). Unlike the fusion reactions already described for alphaviruses, rhabdoviruses, and orthomyxoviruses, fusion is pH-independent, occurring with almost equal efficiency throughout the relevant pH range. Consequently, paramyxovirus fusion can and does occur at the cell surface. During infection of cells with Sendai virus, intact virions can also be seen in coated vesicles and are presumably taken into endosomes. It remains to be demonstrated whether both or only one of the pathways are infectious.

Fusion is mediated by the F protein, which has a hydrophobic "fusion sequence" at the N terminus of the F1 subunit (Gething *et al.*, 1978). Only the proteolytically cleaved protein is active (Homma and Ohuchi, 1973; Scheid and Choppin, 1976). Several recent reports indicate that for maximal fusion, a second glycoprotein, HN, must also be present (Ohki, 1988). On the other hand, fusion can take place without receptor molecules in the target membrane (Citovsky and Loyter, 1985). Cholesterol in the target membrane provides for enhanced fusion activity (Asano and Asano, 1985; Citovsky *et al.*, 1988; Hus *et al.*, 1983; Yoshimura *et al.*, 1987). Even then, the fusion activity of Sendai and other paramyxoviruses is slow and inefficient compared to acid-activated viruses.

6. *Retroviruses*

The retroviruses are a diverse group of viruses that can induce tumors or immunodeficiency diseases in humans and animals. A common feature is that they contain a reverse transcriptase which, following penetration, converts the positive-sense RNA genome into a double-stranded DNA which can integrate into the host cell genome. Entry of the viruses occurs by membrane fusion, which is mediated by fusogenic

envelope glycoproteins (see White *et al.*, 1983). These proteins have a similar overall structure to the HA of influenza virus, although they differ from it in primary sequence. The proteins are synthesized as single-chain polyprotein precursors in the endoplasmic reticulum of infected cells. En route to the cell surface the proteins are trimerized (Hunter and Einfeld, 1988) and proteolytically cleaved. One of the resulting glycopolypeptides, the N-terminal gp120 of HIV for example, is not anchored in the bilayer and carries the receptor-binding site. It is analogous to the HA1 polypeptide. The other portion (gp41 for HIV) is a transmembrane protein and contains the newly created N terminus, which is highly hydrophobic. It is analogous to HA2. As with HA, proteolytic activation and the hydrophobic nature of the N terminus are essential for the fusion activity (Kowalski *et al.*, 1987).

The fusion activities of retroviruses can be acid-dependent or pH-independent (Table III). The acid-dependent viruses include the ecotropic murine leukemia viruses, which are internalized into host cells by endocytosis and inhibited by weak bases (Anderson and Nexø, 1983) and murine mammary tumor virus (MMTV), which fuses cells at <pH 5.3 (Redmond *et al.*, 1984). Many other retroviruses—in particular those that infect human cells—exhibit pH-independent fusion. This has been demonstrated most clearly for HIV-1 and HIV-2 (McClure *et al.*, 1987; Stein *et al.*, 1987). HIV fuses at the cell surface and entry is not affected by weak bases and carboxylic ionophores (McClure *et al.*, 1987; Stein *et al.*, 1987). Virus particles are also observed in endocytic coated pits and coated vesicles (Bauer *et al.*, 1987; D. Pauze, personal communication). It remains to be demonstrated whether either one or both of these routes leads to productive infection.

The CD4 receptor for HIV-1 and HIV-2, normally expressed on T-helper/inducer cells, functions as a virus receptor when expressed in human CD4-negative HeLa cells. Murine NIH 3T3 cells expressing CD4, however, bind HIV-1 but remain resistant to infection (Maddon *et al.*, 1986). An additional complication is suggested by experiments with VSV (HIV-1) pseudotype viruses: pseudotypes containing the VSV genome and the HIV envelope infect HeLa-CD4 cells but fail to replicate the VSV genome in NIH 3T3-CD4 cells. The block in these murine cells appears to be in entry, reflecting an important, and as yet unresolved, postbinding entry event.

7. Herpesviruses

Virions of the herpes group are considerably more complex than the viruses just discussed. They are believed to carry at least five antigenically distinct glycoproteins. Penetration occurs by membrane fusion. The gB protein is essential for entry and appears to be involved in

fusion but not in binding (Ali *et al.*, 1987). A role in fusion has also been suggested for the gD protein (Ali *et al.*, 1987; Campadelli-Fiume *et al.*, 1988; Fuller and Spear, 1987; Sarnieto *et al.*, 1979). Morphological studies indicate that virions are internalized, and that this may occur in coated vesicles. However, fusion can either occur at the plasma membrane or following endocytosis, and it remains unclear whether both routes give rise to productive infection (Campadelli-Fiume *et al.*, 1988).

The receptor for EBV, the CR2 complement receptor, is expressed primarily on B lymphocytes. The virions are up to 250 nm in diameter and thus considerably larger than the average coated vesicle. Epstein-Barr virus is, indeed, observed to enter into noncoated vesicles, possibly by phagocytosis (Nemerow *et al.*, 1985; Tanner *et al.*, 1987). Cytomegalovirus is believed to require exposure to low pH, as infection can be blocked by weak bases (J. McKeating, personal communication).

B. Nonenveloped Viruses

Compared to enveloped viruses, less is known about the penetration and uncoating of nonenveloped viruses. Endocytic uptake of adenoviruses, reoviruses, polyomaviruses, picornaviruses, and other viruses has been observed both biochemically and morphologically (Dales, 1973, 1978; Griffiths and Consigli, 1986; Silverstein and Dales, 1968), and a requirement for exposure to acid pH is inferred for several nonenveloped viruses from experiments in which infection is inhibited by acidotropic weak bases or carboxylic ionophores (Madshus *et al.*, 1984a-c; Pastan *et al.*, 1986; Stuzenbecker *et al.*, 1987). The general mechanism of penetration through a cellular membrane probably involves a conformational change in the viral particle which confers on it hydrophobic, membrane-binding properties. This change may allow the viruses to become embedded in the cellular membrane and provide a mechanism for releasing the genome through the membrane.

1. Picornaviruses

A deep "canyon" or pit on the surface picornaviruses has been proposed as the receptor binding site. The amino acids lining the cavity are more conserved between the various viruses than other surface residues (Rossman and Palmerberg, 1988). Endocytic uptake of various picornaviruses has been observed morphologically and analyzed biochemically (Dales, 1973; Mandel, 1967; Zeichardt *et al.*, 1985). It seems to be required for infectious entry of poliovirus type 1 and rhinoviruses. Infectivity is blocked by acidotropic amines, carboxylic ionophores, and reagents that inhibit endocytosis or endosome acid-

ification (2-deoxy-D-glucose, NaN_3) (Madshus *et al.*, 1984a–c). The block can be bypassed if cells with bound viruses are briefly incubated at low pH (Madshus *et al.*, 1984a). In isolated virions low pH induces changes in the capsid. In many cases this leads to loss of the VP4 protein and RNA, as well as to an increase in hydrophobicity and a change in isoelectric point. When polioviruses are incubated with cells or isolated membranes a similar change occurs, resulting in subviral particles that have lost the smallest structural protein VP4 and the viral RNA. It is possible that the extrusion of VP4, which is myristylated and therefore rather hydrophobic, may be significant in the translocation and mechanism (Paul *et al.*, 1987; Chow *et al.*, 1987). The conformational change in the virus particles also leads to exposure of the amino terminus of VP1, which helps attach the virus to lipid vesicles (Hogle, 1988). The changes are observed during normal entry into cells, and they are inhibited by weak bases, suggesting that low pH is responsible for conformational changes in the virions during penetration (Madshus *et al.*, 1984a,b). In addition to exposure to low pH, a pH gradient across the membrane appears to be required for penetration (Madshus *et al.*, 1984a). Thus acetic acid-induced acidification of the cytoplasm can inhibit poliovirus infection.

It has been suggested that occupation of receptor binding sites on the surface of the picornavirus capsids might facilitate disruption of the pentamer–pentamer contacts in the capsid and provide a port by which VP4 and RNA can exit the virion, accompanied by a change in the isoelectric point of the virus (Rossmann *et al.*, 1985). Experiments on the pH dependence of the conformational changes observed in poliovirus type 1 support this idea. The changes are influenced both by temperature and association of the virus with a cell. In free virus the conformational changes are half-maximal at pH 5.0, following binding to cells they are half-maximal at pH 6.1–6.5 (Madshus *et al.*, 1984a,b). Thus receptor binding may assist in unlocking the capsid.

Although acidotropic reagents and carboxylic ionophores inhibit the entry of poliovirus, HRV type 2, and foot-and-mouth disease virus (Carrillo *et al.*, 1984; Madshus *et al.*, 1984b), they do not block the entry of all picornaviruses. Infection by murine encephalomyocarditis (EMC) virus is, in fact, enhanced by monensin (Madshus *et al.*, 1984b).

2. Adenovirus

The endocytosis of adenovirus has been observed morphologically, and a low-pH-dependent step has been implicated in its penetration (Dales, 1973, 1978; Pastan *et al.*, 1986). Morphological experiments show that virions are internalized in coated vesicles and delivered to endosomes (Dales, 1978; Pastan *et al.*, 1986). Further studies at the

morphological and biochemical level suggest that the viruses are released into the cytosol in intact form by lysis of endosomes (Dales, 1978; Pastan *et al.*, 1986). It has, moreover, been suggested that the lytic reaction is catalyzed by a viral factor which is activated by low pH (Pastan *et al.*, 1986). Cells treated with weak bases (chloroquine and methylamine) are protected from infection, and the virions do not undergo acid-induced changes which render the viral proteins more hydrophobic (Pastan *et al.*, 1986). As already mentioned, morphological evidence suggests that, following penetration from endosomes, the virion releases its genome into the nucleus through nuclear pores (Dales, 1978).

3. *Reovirus*

Reoviruses are internalized by receptor-mediated endocytosis, through coated pits and coated vesicles, and delivered to endosomes and lysosomes (Silverstein and Dales, 1968; Sturzenbecker *et al.*, 1987). A variation in the theme of pH-induced changes seems to occur. The viral outer capsids are proteolytically digested to produce partially uncoated subviral particles which are able to penetrate.

Entry of reovirus types 1 and 3 into mouse L cells is inhibited by NH_4Cl (Sturzenbecker *et al.*, 1987). The inhibitor acts early in entry, within 1 hour of virus addition, and does not influence the binding, endocytosis, or intracellular routing of the virus. It blocks the digestion of the outer capsid proteins. If virions are digested with protease prior to infection, subviral particles are produced that are infectious and insensitive to NH_4Cl . It is unknown whether the acid-dependent protease is viral or cellular, but it is clear that digestion is an essential step in entry.

The fact that low pH and proteolytic digestion are important for entry in tissue culture cells does not mean that the same process occurs in the intact animal. Infection appears to occur through specialized M cells of the small intestine, and in order to reach these cells the viruses must pass through the acid hydrolytic environment of the stomach. How this "pretreatment" affects the virus is unclear (Wolf *et al.*, 1983).

VIII. CONCLUSION

Interest in early virus-cell interactions is rapidly growing. For enveloped viruses some well-characterized paradigms have now been established, and relatively clear-cut diagnostic tests are available to determine general properties of entry in other systems. The main chal-

lenges for the future are the identification of cell surface receptors, the characterization of the virus-receptor interactions at the molecular level, the elucidation of translocation, membrane fusion, and uncoating mechanisms, and the analysis of early cytoplasmic and nuclear events. The membrane translocation mechanisms of nonenveloped viruses remain particularly enigmatic.

Most of the successes so far have been limited to tissue culture cells. The question of entry in the whole organism, and its role in determining cell tropism and pathogenesis, must now be addressed. More work should also be invested in therapeutic and prophylactic strategies based on our knowledge about early virus-cell interactions.

It will be important to keep abreast with progress in a wide field of adjoining disciplines such as cell biology, molecular and structural biology, membrane biochemistry, and physiology. These areas provide the basis for understanding early virus-cell interactions. The exchange of information with other disciplines goes both ways: the work on virus entry has already greatly enhanced our understanding of endocytosis, membrane fusion, and organelle acidification. While each virus will turn out to have special features in respect to entry, the overall principles will probably be common to many. We are not yet at a point where these principles are known, and continued parallel work with a variety of different viruses is needed.

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