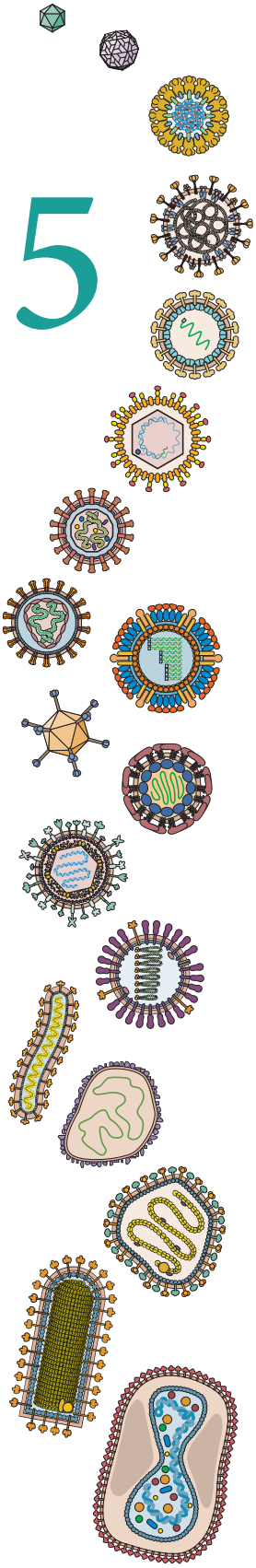


5 Attachment and Entry

LINKS FOR CHAPTER 5

- ⏭ *Video: Interview with Dr. Jeffrey M. Bergelson*
  http://bit.ly/Virology_Bergelson
- ⏭ *Video: Interview with Dr. Carolyn Coyne*
  http://bit.ly/Virology_Coyne
- ⏭ *Bond, covalent bond*
  http://bit.ly/Virology_Twiv210
- ⏭ *Breaking and entering*
  http://bit.ly/Virology_Twiv166
- ⏭ *A new cell receptor for rhinovirus*
  http://bit.ly/Virology_4-30-15
- ⏭ *Blocking HIV infection with two soluble cell receptors*
  http://bit.ly/Virology_2-26-15
- ⏭ *Changing influenza virus neuraminidase into a receptor binding protein*
  http://bit.ly/Virology_11-21-13

Who hath deceived thee so often as thyself?
BENJAMIN FRANKLIN

Introduction

Because viruses are obligate intracellular parasites, the genome must enter a cell for the viral reproduction cycle to occur. The physical properties of the virion are obstacles to this seemingly simple goal. Virus particles are too large to diffuse passively across the plasma membrane. Furthermore, the viral genome is encapsidated in a stable coat that shields the nucleic acid as it travels through the harsh extracellular environment. These impediments must all be overcome during the process of viral entry into cells. Encounter of a virus particle with the surface of a susceptible host cell induces a series of events that lead to entry of the viral genome into the cytoplasm or nucleus. The first step in entry is adherence of virus particles to the plasma membrane, an interaction mediated by binding to a specific **receptor** molecule on the cell surface.

The receptor plays an important role in **uncoating**, the process by which the viral genome is exposed, so that gene expression and genome replication can begin. Interaction of the virus particle with its receptor may initiate conformational changes that prime the capsid for uncoating. Alternatively, the receptor may direct the virus particle into endocytic pathways, where uncoating may be triggered by low pH or by the action of proteases. These steps bring the genome into the cytoplasm, which is the site of replication of most RNA-containing viruses. The genomes of viruses that replicate in the nucleus are moved to that location by cellular transport pathways. Viruses that replicate in the nucleus include most DNA-containing viruses (exceptions include poxviruses and giant viruses), RNA-containing retroviruses, influenza viruses, and Borna disease virus.

Virus entry into cells is **not** a passive process but relies on viral usurpation of normal cellular processes, including endocytosis, membrane fusion, vesicular trafficking, and transport into the nucleus. Because of the limited functions encoded by viral genomes, virus entry into cells depends absolutely on cellular processes.

Attachment of Virus Particles to Cells

General Principles

Infection of cells by many, but not all, viruses requires binding to a receptor on the cell surface. Exceptions include viruses of yeasts and fungi, which have no extracellular phases, and plant viruses, which are thought to enter cells in which the cell wall has been physically damaged, for example by insects or farm machinery. A receptor is a cell surface molecule that binds the virus particle and participates in entry. It may induce conformational changes in the virus particle that lead to membrane fusion or penetration, and it may also transmit signals that cause uptake. The receptor may also bring the bound particle into endocytic pathways.

Receptors for viruses comprise a variety of cell surface proteins, carbohydrates, and lipids, all with functions in the cell unrelated to virus entry. Many virus receptors have been identified in the past 30 years and include immunoglobulin-like proteins, ligand-binding receptors, glycoproteins, ion channels, gangliosides, carbohydrates, proteoglycans, and integrins. The receptor may be the only cell surface molecule required for entry into cells, or an additional cell surface molecule, or **coreceptor**, may be needed (Box 5.1). Different receptors may serve for virus entry in diverse cell types, and unrelated viruses may bind to the same receptor (e.g., the Coxsackievirus and adenovirus receptor).

The receptor may determine the **host range** of a virus, i.e., its ability to infect a particular animal or cell culture. For example, poliovirus infects primates and primate cell cultures but not mice or mouse cell cultures. Mouse cells synthesize a protein that is homologous to the poliovirus receptor,

PRINCIPLES *Attachment and entry*

- Virus particles are too large to diffuse across the plasma membrane, and thus entry must be an active process.
- Virus particles bind to receptors on their host cells to initiate entry.
- The cell receptor may determine the host range and tissue tropism of the virus.
- Viruses may bind multiple distinct receptors, and individual cellular proteins may be receptors for multiple viruses.
- Enveloped virus particles bind via their transmembrane glycoproteins; nonenveloped virus particles bind via the capsid surface or projections from the capsid.
- Attachment proteins may not lead to internalization and viral reproduction but may still be important for dissemination in the host.
- Some viruses uncoat at the plasma membrane, while others do so from intracellular vesicles.
- Many viruses enter host cells by the same cellular pathways used to take up macromolecules.
- The entry mechanism used by a particular virus may differ depending on the nature of the target cell.
- Viral particles and subviral particles depend on the cytoskeleton to move within an infected cell.
- Binding of virions to cell receptors may activate signaling pathways that facilitate virus entry and movement, or produce cellular responses that enhance virus propagation and/or affect pathogenesis.
- For viruses that undergo replication in the nucleus, import can occur either through use of the nuclear pore complex or during cell division, when the nuclear membrane breaks down.

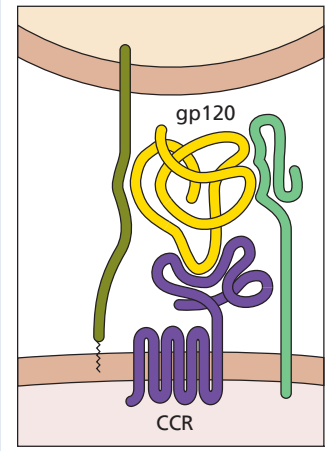
BOX 5.1

TERMINOLOGY

Receptors and coreceptors

By convention, the first cell surface molecule that is found to be essential for virus binding is called its **receptor**. Sometimes, such binding is not sufficient for entry into the cell. When binding to another cell surface molecule is needed, that protein is called a **coreceptor**. For example, human immunodeficiency virus binds to cells via a receptor, CD4, and then requires interaction with a second cell surface protein such as CXCR4, the coreceptor.

In practice, the use of receptor and coreceptor can be confusing and inaccurate. A particular cell surface molecule that is a coreceptor for one virus may be a receptor for another. Distinguishing receptors and coreceptors by the order in which they are bound is difficult to determine experimentally and is likely to be influenced by cell type and multiplicity of infection. Furthermore, as is the case for the human immunodeficiency viruses, binding only to the coreceptor may be sufficient for entry of some members. Usage of the terms "receptor" and "coreceptor" is convenient when describing virus entry, but the appellations may not be entirely precise.



but sufficiently different that poliovirus cannot attach to it. In this example, the poliovirus receptor is **the** determinant of poliovirus host range. However, production of the receptor in a particular cell type does **not** ensure that virus reproduction will occur. Some primate cell cultures produce the poliovirus receptor but cannot be infected. The restriction of viral reproduction in these cells is most probably due to a block in viral reproduction beyond the attachment step. Receptors can also be determinants of tissue **tropism**, the predilection of a virus to invade and reproduce in a particular cell type. However, there are many other determinants of tissue tropism. For example, the sialic acid residues on membrane glycoproteins or glycolipids, which are receptors for influenza virus, are found on many tissues, yet viral reproduction in the host is restricted. The basis of such restriction is discussed in Volume II, Chapter 2.

Our understanding of the earliest interactions of virus particles with cells comes almost exclusively from analysis of synchronously infected cells in culture. The initial association with cells is probably via electrostatic forces, as it is sensitive to low pH or high concentrations of salt, but higher affinity binding relies mainly on hydrophobic and other short-range forces between the viral and cellular surfaces. Although the **affinity** of a receptor for a single virus particle is low, the presence of multiple receptor-binding sites on the virion and the fluid nature of the plasma membrane allow engagement of multiple receptors. Consequently, the **avidity** (the strength conferred by multiple interactions) of virus particle binding to cells is usually very high. Binding can usually occur at 4°C (even though entry does not) as well as at body temperature (e.g., 37°C). Infection of cultured cells can therefore be synchronized by allowing binding to take place at a low temperature and then shifting the cells to a physiological temperature to allow the initiation of subsequent steps.

The first steps in virus attachment are governed largely by the probability that a virus particle and a cell will collide, and therefore by the concentrations of free particles and host cells. The rate of attachment can be described by the equation

$$dA/dt = k[V][H]$$

where A is attachment, t is time, and $[V]$ and $[H]$ are the concentrations of virus particles and host cells, respectively, and k is a constant that defines the rate of the reaction. It can be seen from this equation that if a mixture of viruses and cells is diluted after an adsorption period, subsequent binding of particles is greatly reduced. For example, a 100-fold dilution of the virus and cell mixture reduces the attachment rate 10,000-fold (i.e., $1/100 \times 1/100$). Dilution can be used to prevent subsequent virus adsorption and hence to synchronize an infection.

Many receptor molecules can move in the plasma membrane, leading to the formation of microdomains that differ in composition. Bound virus may therefore localize to specialized areas of the membrane such as lipid rafts, **caveolae**, or coated pits. Localization of virus particle-receptor complexes can also cause transmembrane signaling, changes in the cytoskeleton, and recruitment of clathrin.

Identification of Receptors for Virus Particles

The development of three crucial technologies in the past 30 years has enabled identification of many receptors for viruses. Production of monoclonal antibodies provided a powerful means of isolating and characterizing individual cell surface proteins. Hybridoma cell lines that secrete monoclonal antibodies that block virus attachment are

obtained after immunizing mice with intact cells. Such antibodies can be used to purify the receptor protein by affinity chromatography.

A second technology that facilitated the identification of receptors was the development of DNA-mediated transformation. This method was crucial for isolating genes that encode receptors, following introduction of DNA from susceptible cells into nonsusceptible cells (Fig. 5.1). Cells that acquire DNA encoding the receptor and carry the corresponding protein on their surface are able to bind virus specifically. Clones of such cells are recognized and selected, for example, by the binding of receptor-specific monoclonal antibodies. The receptor genes can then be isolated from these selected cells by using a third technology, molecular cloning. The power of these different technologies can lead to rapid progress: the receptor for a newly identified Middle Eastern respiratory syndrome coronavirus was identified just 4 months after the first description of the virus (Box 5.2). Although these technologies have led to the identification of many cell receptors for viruses, each method has associated uncertainties (Box 5.3).

The availability of receptor genes has made it possible to investigate the details of receptor interaction with viruses by site-directed mutagenesis. Receptor proteins can be synthesized in heterologous systems and purified, and their properties can be studied *in vitro*, while animal cells producing altered receptor proteins can be used to test the effects of alterations on virus attachment. Because of their hydrophobic membrane-spanning domains, many of these cell surface proteins are relatively insoluble and difficult to work with.

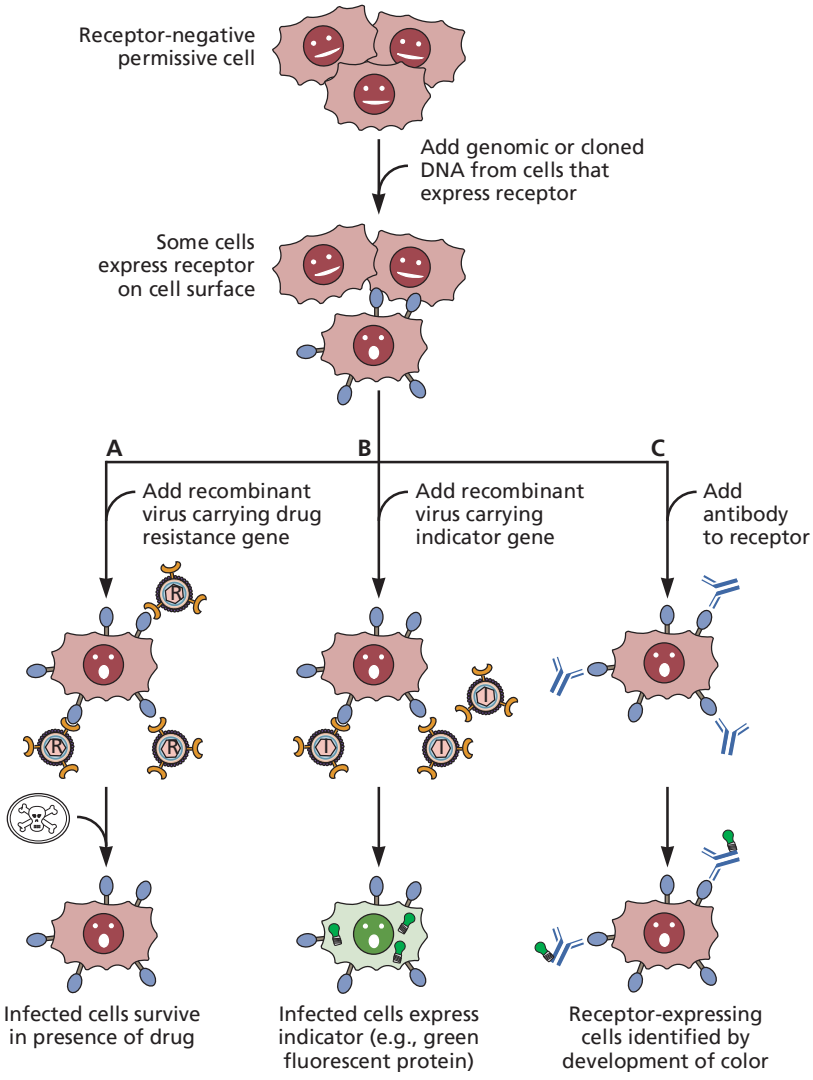


Figure 5.1 Experimental strategies for identification and isolation of genes encoding cell receptors for viruses. Genomic DNA or pools of DNA clones from cells known to synthesize the receptor are introduced into receptor-negative permissive cells. A small number of recipient cells produce the receptor. Three different strategies for identifying such rare receptor-expressing cells are outlined. **(A)** The cells are infected with a virus that has been engineered so that it carries a gene encoding drug resistance. Cells that express the receptor will become resistant to the drug. This strategy works only for viruses that persist in cells without killing them. **(B)** For lytic viruses, an alternative is to engineer the virus to express an indicator, such as green fluorescent protein or β-galactosidase. Cells that make the correct receptor and become infected with such viruses can be distinguished by a color change, such as green in the case of green fluorescent protein. **(C)** The third approach depends on the availability of an antibody directed against the receptor, which binds to cells that express the receptor gene. Bound antibodies can be detected by an indicator molecule. When complementary DNA (cDNA) cloned in a plasmid is used as the donor DNA, pools of individual clones (usually 10,000 clones per pool) are prepared and introduced individually into cells. The specific DNA pool that yields receptor-expressing cells is then subdivided, and the screening process is repeated until a single receptor-encoding DNA is identified.

BOX 5.2

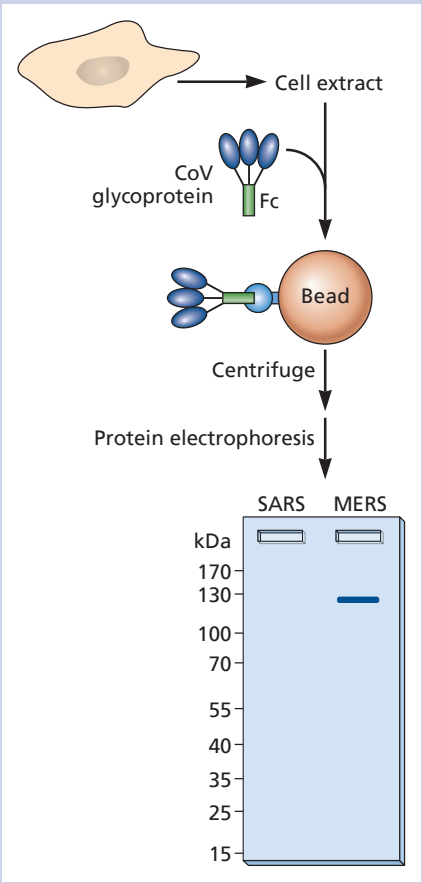
METHODS

Affinity isolation

To identify the receptor for the newly emerged Middle Eastern respiratory syndrome coronavirus, the gene encoding the viral spike glycoprotein gene was fused with sequences encoding the Fc domain of human IgG. The fusion protein was produced in cells and incubated with lysates of cells known to be susceptible to the virus, and the resulting complexes were fractionated by native polyacrylamide gel electrophoresis. A single polypeptide of ~110 kDa was obtained by this procedure. This polypeptide was excised from the polyacrylamide gel, and its amino acid sequence was determined by mass spectrometric analysis, identifying it as dipeptidyl peptidase 4. When this protein was subsequently synthesized in nonsusceptible Cos-7 cells by DNA-mediated transformation, the cells became susceptible to Middle Eastern respiratory syndrome coronavirus infection. That a single protein was identified by this procedure is remarkable: typically, this approach identifies many nonspecific binding proteins.

Raj VS, Mou H, Smits SL, Dekkers DH, Müller MA, Dijkman R, Muth D, Demmers JA, Zaki A, Fouchier RA, Thiel V, Drosten C, Rottier PJ, Osterhaus AD, Bosch BJ, Haagmans BL. 2013. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* **495:**251–256.

Identification of MERS-coronavirus cell receptor.



Soluble extracellular protein domains (with the virus binding sites) have been essential for structural studies of receptor-virus interactions. Receptor genes have also been used to produce transgenic mice that synthesize receptor proteins. Such transgenic animals can serve as useful models in the study of human viral diseases.

Virus-Receptor Interactions

Animal viruses have multiple receptor-binding sites on their surfaces. Of necessity, one or more of the capsid proteins of nonenveloped viruses specifically interact with the cell receptor. Typically, these form projections from or indentations in the surface. Receptor-binding sites for enveloped viruses are provided by oligomeric type 1 integral membrane glycoproteins encoded by the viral genome that have been incorporated into the cell-derived membranes of virus particles. Although the details vary among viruses, most virus-receptor interactions follow one of several mechanisms illustrated by the best-studied examples described below.

Nonenveloped Viruses Bind via the Capsid Surface or Projections

Attachment via surface features: canyons and loops. Members of the enterovirus genus of the *Picornaviridae* include human polioviruses, coxsackieviruses, echoviruses, enteroviruses, and rhinoviruses. The receptor for poliovirus, CD155, was identified by using a DNA transformation and cloning strategy (Fig. 5.1). It was known that mouse cells cannot be infected with poliovirus, because they do not produce the receptor. Transfection of poliovirus RNA into mouse cells in culture leads to poliovirus reproduction, indicating that there is no intracellular block to virus multiplication. Introduction of human DNA into mouse cells confers susceptibility to poliovirus infection. The human gene recovered from receptor-positive mouse cells proved to encode CD155, a glycoprotein that is a member of the immunoglobulin (Ig) superfamily (Fig. 5.2).

Mouse cells are permissive for poliovirus reproduction, and susceptibility is limited **only** by the absence of CD155. Consequently, it was possible to develop a small-animal model for poliomyelitis by producing transgenic mice that synthesize this receptor. Inoculation of CD155 transgenic mice with poliovirus by various routes produces paralysis, as is observed in human poliomyelitis. These CD155-synthesizing mice were the first new animal model created by transgenic technology for the study of viral disease. Similar approaches have subsequently led to animal models for viral diseases caused by measles virus and echoviruses.

Rhinoviruses multiply primarily in the upper respiratory tract and are responsible for causing up to 50% of all common colds. Over 150 rhinovirus genotypes have been identified and classified on the basis of genome sequence into three species, A, B, and C. Rhinoviruses bind to at least three

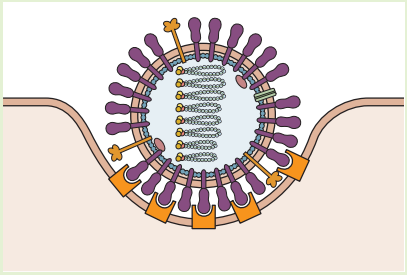
BOX 5.3

BACKGROUND

Criteria for identifying cell receptors for viruses

The use of monoclonal antibodies, molecular cloning, and DNA-mediated transformation provides a powerful approach for identifying cellular receptors for viruses, but each method has associated uncertainties. A monoclonal antibody that blocks virus attachment might recognize not the receptor but a closely associated membrane protein. To prove that the protein recognized by the monoclonal antibody **is** a receptor, DNA encoding the protein must be introduced into nonsusceptible cells to demonstrate that it can confer virus-binding activity. Any of the approaches outlined in Fig. 5.1 can result in identification of a cellular gene that encodes a putative receptor. However, the encoded protein might not be a receptor but may modify another cellular protein so that it can bind virus particles. One proof that the DNA codes for a receptor could come from the identification of a monoclonal antibody that blocks virus attachment and is directed against the encoded protein.

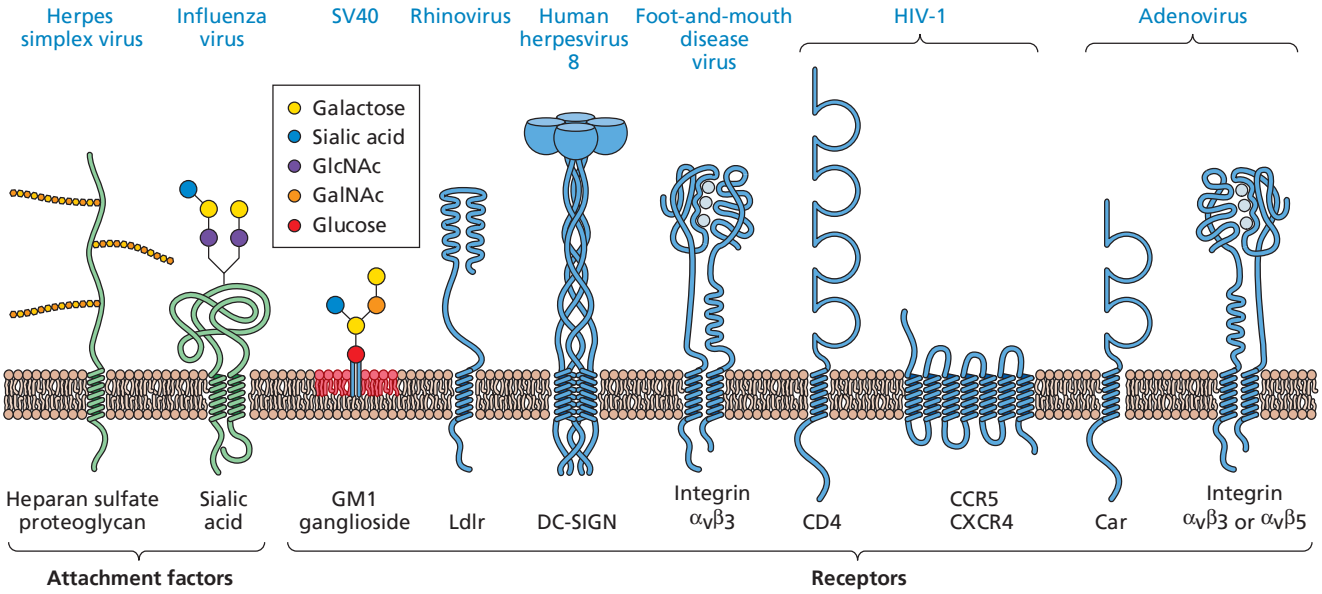
For some viruses, synthesis of the receptor on cells leads to binding but not infection. In such cases a coreceptor is required, either for internalization or for membrane fusion. The techniques of molecular cloning also can be used to identify coreceptors. For example, production of CD4 on mouse cells leads to binding of human immunodeficiency virus type 1 but not infection, because fusion of viral and cell membranes does not occur. To identify the coreceptor, a DNA clone was isolated from human cells that allowed membrane fusion catalyzed by the viral attachment protein in mouse cells synthesizing CD4.



different receptor molecules. The cell surface receptor bound by most A and B species rhinoviruses was identified by using a monoclonal antibody that blocks rhinovirus infection and that recognizes a cell surface protein. This monoclonal antibody was used to isolate a 95-kDa cell surface glycoprotein by affinity chromatography. Amino acid sequence analysis of the purified protein, which bound to rhinovirus *in vitro*, identified it as the integral membrane protein intercellular adhesion molecule 1 (Icam-1). Cell receptors for other rhinoviruses are the low-density lipoprotein receptor and cadherin-related family member 3.

The RNA genomes of picornaviruses are protected by capsids made up of four virus-encoded proteins, VP1, VP2, VP3, and VP4, arranged with icosahedral symmetry (see Fig. 4.12). The capsids of rhinoviruses and polioviruses have deep canyons surrounding the 12 5-fold axes of symmetry (Fig. 5.3),

Figure 5.2 Some cell attachment factors and receptors for viruses. Schematic diagrams of cell molecules that function during virus entry. GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Ldlr, low-density lipoprotein receptor; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; Car, coxsackievirus-adenovirus receptor.



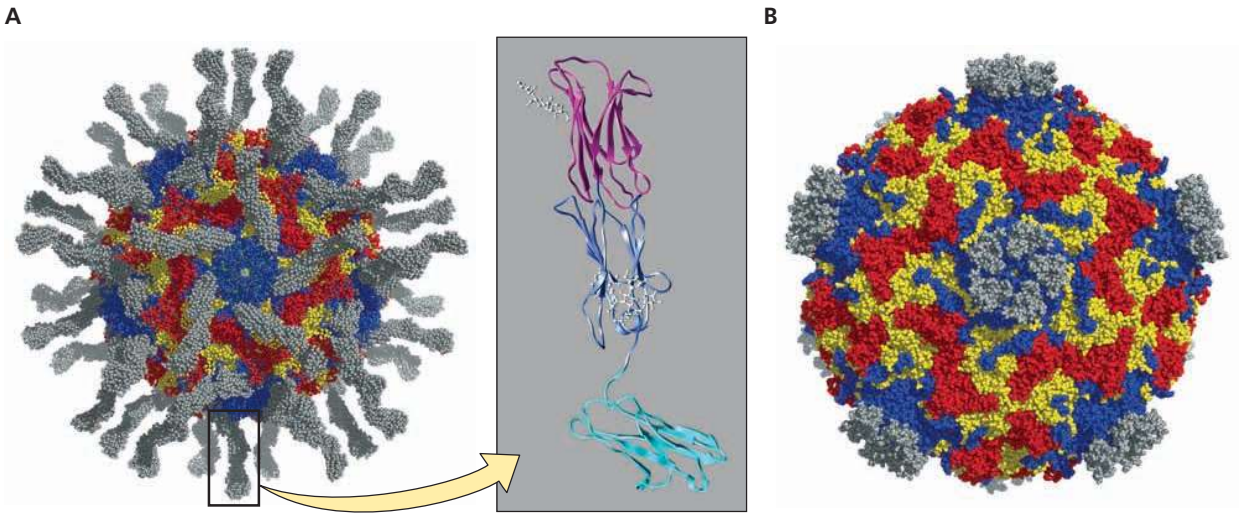


Figure 5.3 Picornavirus-receptor interactions. (A) Structure of poliovirus bound to a soluble form of CD155 (gray), derived by cryo-electron microscopy and image reconstruction. Capsid proteins are color coded (VP1, blue; VP2, yellow; VP3, red). One CD155 molecule is shown as a ribbon model in the panel to the right, with each Ig-like domain in a different color. The first Ig-like domain of CD155 (magenta) binds in the canyon of the viral capsid. **(B)** Structure of human rhinovirus type 2 bound to a soluble form of low-density lipoprotein receptor (gray). The receptor binds on the plateau at the 5-fold axis of symmetry of the capsid.

whereas cardioviruses and aphthoviruses lack this feature. The canyons in the capsids of some rhinoviruses and enteroviruses are the sites of interaction with cell surface receptors. Amino acids that line the canyons are more highly conserved than any others on the viral surface, and their substitution can alter the affinity of binding to cells. Poliovirus bound to a receptor fragment comprising CD155 domains 1 and 2 has been visualized in reconstructed images from cryo-electron microscopy. The results indicate that the first domain of CD155 binds to the central portion of the canyon in an orientation oblique to the surface of the virus particle (Fig. 5.3A).

Although canyons are present in the capsid of rhinovirus type 2, they are not the binding sites for the receptor, low-density lipoprotein receptor. Rather, this site on the capsid is located on the star-shaped plateau at the 5-fold axis of symmetry (Fig. 5.3B). Sequence and structural comparisons have revealed why different rhinovirus serotypes bind distinct receptors. A key VP1 amino acid, lysine, is conserved in all rhinoviruses that bind this receptor and interacts with a negatively charged region of low-density lipoprotein receptor. This lysine is not found in VP1 of rhinoviruses that bind Icam-1.

For picornaviruses with capsids that do not have prominent canyons, including coxsackievirus group A and foot-and-mouth disease virus, attachment is to VP1 surface loops that include amino acid sequence motifs recognized by their integrin receptors (Fig. 5.2).

Attachment via protruding fibers. The results of competition experiments indicated that members of two different virus families, group B coxsackieviruses and most human adenoviruses, share a cell receptor. This receptor is a 46-kDa member of the Ig superfamily called Car (coxsackievirus and adenovirus receptor). Binding to this receptor is not sufficient for infection by most adenoviruses. Interaction with a coreceptor, the α_v integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$, is required for uptake of the capsid into the cell by receptor-mediated endocytosis. An exception is adenovirus type 9, which can infect hematopoietic cells after binding directly to α_v integrins. Adenoviruses of subgroup B bind CD46, which is also a cell receptor for some strains of measles virus, an enveloped member of the *Paramyxoviridae*.

The nonenveloped DNA-containing adenoviruses are much larger than picornaviruses, and their icosahedral capsids are more complex, comprising at least 10 different proteins. Electron microscopy shows that fibers protrude from each adenovirus pentamer (Fig. 5.4; see the appendix in this volume, Fig. 1A). The fibers are composed of homotrimers of the adenovirus fiber protein and are anchored in the pentameric penton base; both proteins have roles to play in virus attachment and uptake.

For many adenovirus serotypes, attachment via the fibers is necessary but not sufficient for infection. A region comprising the N-terminal 40 amino acids of each subunit of the fiber protein is bound noncovalently to the penton base. The central shaft region is composed of repeating motifs of approximately 15 amino acids; the length of the shaft in different serotypes is determined by the number of these repeats. The three constituent shaft regions appear to form a rigid triple-helical structure in the trimeric fiber. The C-terminal 180 amino acids of each subunit interact to form a terminal knob. Genetic analyses and competition experiments indicate that determinants

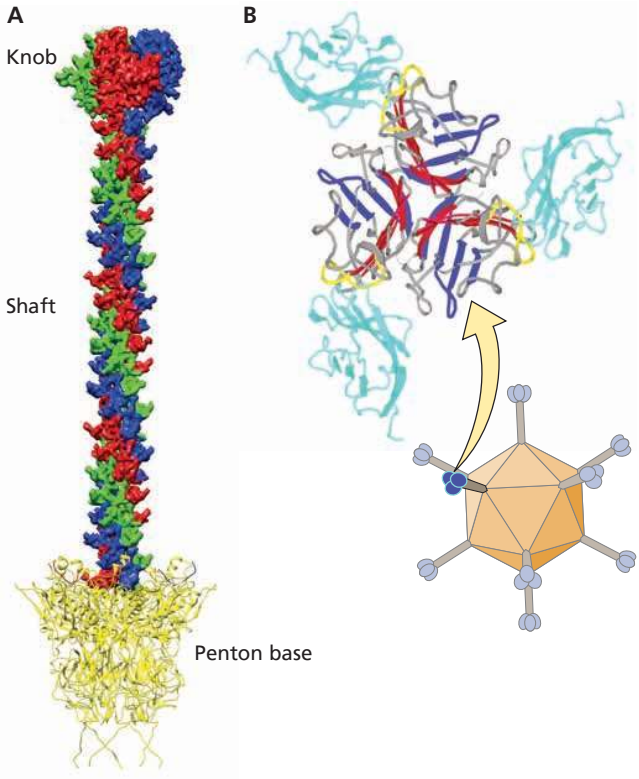


Figure 5.4 Structure of the adenovirus 12 knob bound to the Car receptor. (A) Structure of fiber protein, with knob, shaft, and tail domains labeled. Figure provided by Hong Zhou, University of California, Los Angeles, and Hongrong Liu, Hunan Normal University. **(B)** Ribbon diagram of the knob-Car complex as viewed down the axis of the viral fiber. The trimeric knob is in the center. The AB loop of the knob protein, which contacts Car, is in yellow. The first Ig-like domains of three Car molecules bound to the knob are colored blue. The binding sites of both molecules require trimer formation.

for the initial, specific attachment to host cell receptors reside in this knob. The structure of this receptor-binding domain bound to Car reveals that surface loops of the knob contact one face (Fig. 5.4).

Glycolipids, unusual cell receptors for polyomaviruses. The family *Polyomaviridae* includes simian virus 40 (SV40), mouse polyomavirus, and human BK virus. These viruses are unusual because they bind to ganglioside rather than protein receptors. Gangliosides are glycosphingolipids with one or more sialic acids linked to a sugar chain. There are over 40 known gangliosides, which differ in the position and number of sialic acid residues and are critical for virus binding. Simian virus 40, polyomavirus, and BK virus bind to three different types of ganglioside. Structural studies have revealed that sialic acid linked to galactose by an α(2,3) linkage binds to a pocket on the surface of the polyomavirus capsid (Fig. 5.5). Gangliosides are highly concentrated in lipid rafts (Chapter 2, Box 2.1) and participate in signal transduction, two properties

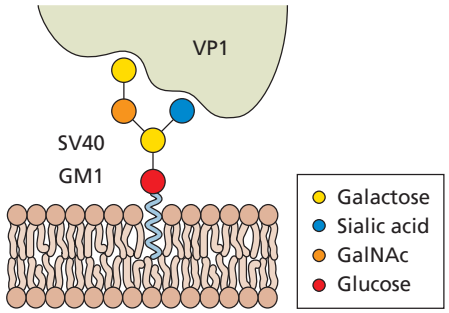


Figure 5.5 Interaction of polyomaviruses with ganglioside receptors. JC polyomavirus binds to a pentasaccharide with a terminal sialic acid linked by an α(2,6) bond to the penultimate galactose. The linear nature of this receptor differs from other branched polyomavirus ganglioside receptors. JC polyomavirus also appears to bind to a serotonin receptor for cell entry.

that are important during polyomavirus entry into cells. After binding a ganglioside, mouse polyomavirus interacts with α4β1 integrin to allow virus entry.

Enveloped Viruses Bind via Transmembrane Glycoproteins

The lipid membranes of enveloped viruses originate from those of the host cells. Membrane spanning viral proteins are inserted into these by the same mechanisms as cellular integral membrane proteins. Attachment sites on one or more of these envelope proteins bind to specific receptors. The two best-studied examples of enveloped virus attachment and its consequences are provided by the interactions of influenza A virus and the retrovirus human immunodeficiency virus type 1 with their receptors.

Influenza virus. The family *Orthomyxoviridae* comprises the three genera of influenza viruses, A, B, and C. These viruses bind to negatively charged, terminal sialic acid moieties present in oligosaccharide chains that are covalently attached to cell surface glycoproteins or glycolipids. The presence of sialic acid on most cell surfaces accounts for the ability of influenza virus particles to attach to many types of cell. The interaction of influenza virus with individual sialic acid moieties is of low affinity. However, the opportunity for multiple interactions among the numerous hemagglutinin (HA) molecules on the surface of the virus particle and multiple sialic acid residues on cellular glycoproteins and glycolipids results in a high overall avidity of the virus particle for the cell surface. The surfaces of influenza viruses were shown in the early 1940s to contain an enzyme that, paradoxically, removes the receptors for attachment from the surface of cells. Later, this enzyme was identified as the virus-encoded envelope glycoprotein neuraminidase, which cleaves the glycoside linkages of sialic acids (Fig. 5.6B). This enzyme is required for release of virus particles bound to the surfaces of

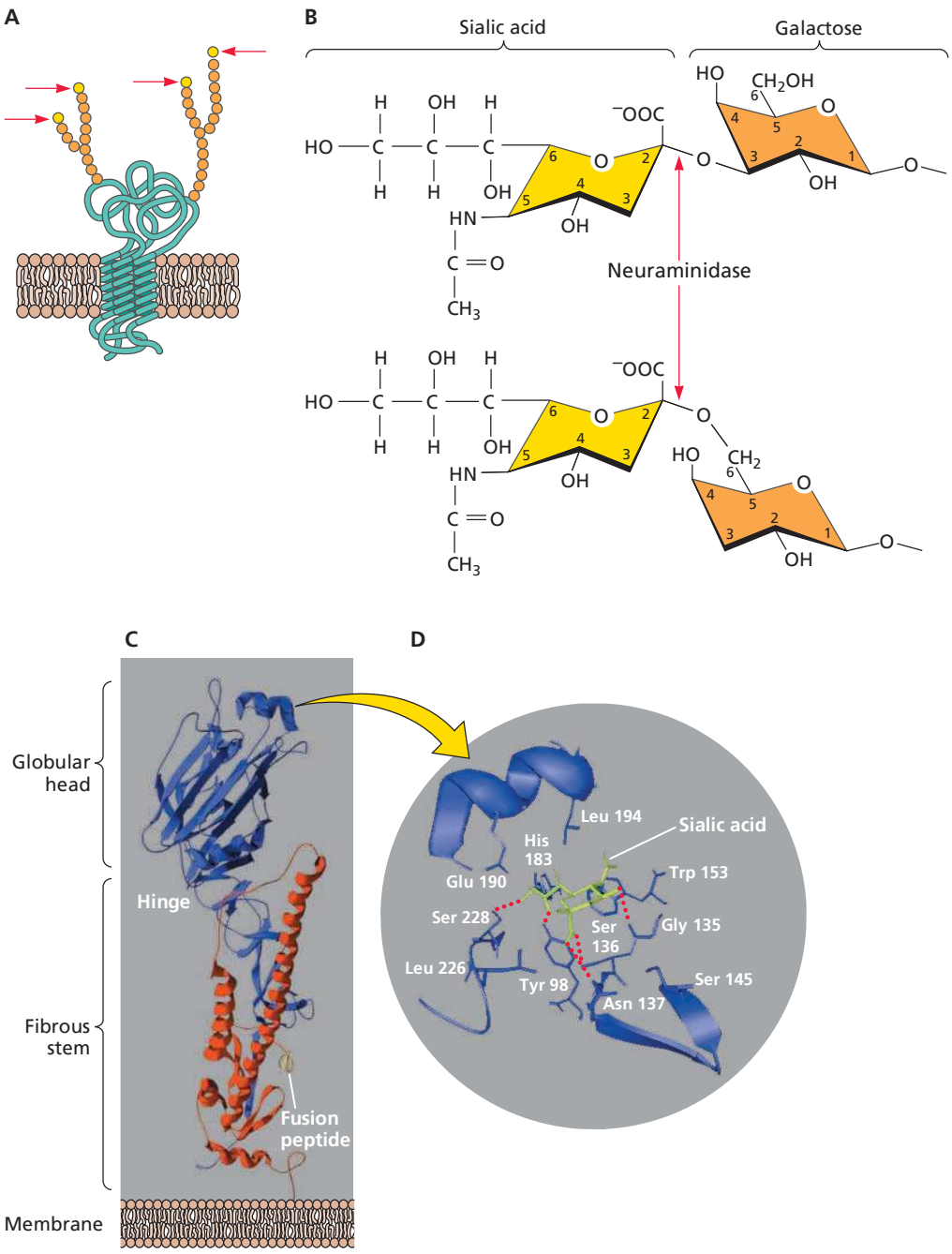


Figure 5.6 Interaction of sialic acid receptors with the hemagglutinin of influenza viruses. (A) An integral membrane glycoprotein; the arrows point to terminal sialic acid units that are attachment sites for influenza virus. **(B)** The structure of a terminal sialic acid moiety that is recognized by the viral envelope protein hemagglutinin. Sialic acid is attached to galactose by an α(2,3) (top) or an α(2,6) (bottom) linkage. The site of cleavage by the influenza virus envelope glycoprotein neuraminidase is indicated. The sialic acid shown is *N*-acetylneuraminic acid, which is the preferred receptor for influenza A and B viruses. These viruses do not bind to 9-*O*-acetyl-*N*-neuraminic acid, the receptor for influenza C viruses. **(C)** HA monomer modeled from the X-ray crystal structure of the natural trimer. HA1 (blue) and HA2 (red) subunits are held together by a disulfide bridge as well as by many noncovalent interactions. The fusion peptide at the N terminus of HA2 is indicated (yellow). **(D)** Close-up of the receptor-binding site with a bound sialic acid molecule. Side chains of the conserved amino acids that form the site and hydrogen-bond with the receptor are included.

infected cells, facilitating virus spread through the respiratory tract (Volume II, Chapter 9).

Influenza virus HA is the viral glycoprotein that binds to the cell receptor sialic acid. The HA monomer is synthesized as a precursor that is glycosylated and subsequently cleaved to form HA1 and HA2 subunits. Each HA monomer consists of a long, helical stalk anchored in the membrane by HA2 and topped by a large HA1 globule, which includes the sialic acid-binding pocket (Fig. 5.6C, D). While attachment of all influenza A virus strains requires sialic acid, strains vary in their affinities for different sialyloligosaccharides. For example, human virus strains bind preferentially sialic acids attached to galactose via an α(2,6) linkage, the major sialic acid present on human respiratory epithelium (Fig. 5.6B). Avian virus strains bind preferentially to sialic acids attached to galactose via an α(2,3) linkage, the major sialic acid in the duck gut epithelium. Amino acids in the sialic acid-binding pocket of HA (Fig. 5.6D) determine which sialic acid is preferred and can therefore influence viral host range. It is thought that an amino acid change in the sialic acid-binding pocket of the 1918 influenza virus, which may have evolved from an avian virus, allowed it to recognize the α(2,6)-linked sialic acids that predominate in human cells.

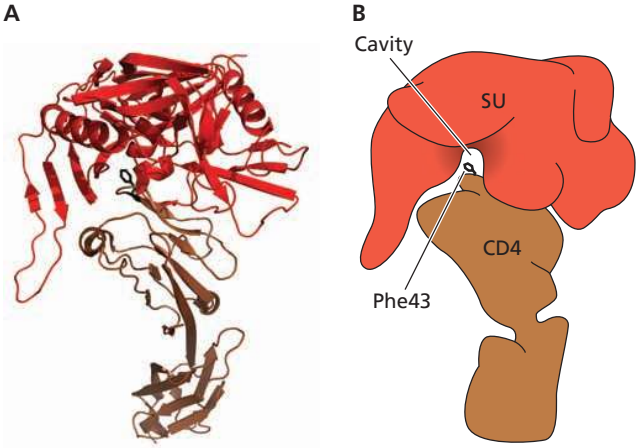


Figure 5.7 Interaction of human immunodeficiency virus type 1 SU with its cell receptor, CD4. (A) Ribbon diagram of SU (red) bound to CD4 (brown), derived from X-ray crystallographic data. The side chain of CD4 Phe43 is shown. **(B)** Cartoon of the CD4-SU complex. Mutagenesis has identified CD4 Phe43 as a residue critical for binding to SU. Phe43 is shown penetrating the hydrophobic cavity of SU. This amino acid, which makes 23% of the interatomic contacts between CD4 and SU, is at the center of the interface and appears to stabilize the entire complex.

Human immunodeficiency virus type 1. Animal retroviruses have long been of interest because of their ability to cause a variety of serious diseases, especially cancers (caused by oncogenic retroviruses) and neurological disorders (caused by lentiviruses). The acquired immunodeficiency syndrome (AIDS) pandemic has focused great attention on the lentivirus human immunodeficiency virus type 1 and its close relatives. The cell surface receptors of this virus have been among the most intensively studied and currently are the best understood.

When examined by electron microscopy, the envelopes of human immunodeficiency virus type 1 and other retroviruses appear to be studded with "spikes" (see Fig. 4.19). These structures are composed of trimers of the single viral envelope glycoprotein, which bind the cell receptor (Fig. 5.7). The monomers of the spike protein are synthesized as heavily glycosylated precursors that are cleaved by a cellular protease to form SU and TM. The latter is anchored in the envelope by a single membrane-spanning domain and remains bound to SU by numerous noncovalent bonds.

The cell receptor for human immunodeficiency virus type 1 is CD4 protein, a 55-kDa rodlike molecule that is a member of the Ig superfamily and has four Ig-like domains (Fig. 5.2). A variety of techniques have been used to identify the site of interaction with human immunodeficiency virus type 1, including site-directed mutagenesis and X-ray crystallographic studies of a complex of CD4 bound to the viral attachment protein SU (Fig. 5.7). The interaction site for SU in domain 1 of CD4 is in a region analogous to the site in CD155 that binds to poliovirus. Remarkably, two viruses with entirely different architectures bind to analogous surfaces of these Ig-like domains.

The atomic structure of a complex of human immunodeficiency virus type 1 SU, a two-domain fragment of CD4, and a neutralizing antibody against SU has been determined by X-ray crystallography (Fig. 5.7). The CD4-binding site in SU is a deep cavity, and the opening of this cavity is occupied by CD4 amino acid Phe43, which is critical for SU binding. Comparison with the structure of SU in the absence of CD4 indicates that receptor binding induces conformational changes in SU. These changes expose binding sites on SU for the chemokine receptors, which are required for fusion of viral and cell membranes (see "Uncoating at the Plasma Membrane" below).

Alphaherpesviruses. The alphaherpesvirus subfamily of the *Herpesviridae* includes herpes simplex virus types 1 and 2, pseudorabies virus, and bovine herpesvirus. Initial contact of these viruses with the cell surface is made by low-affinity binding of two viral glycoproteins, gC and gB, to glycosaminoglycans (preferentially heparan sulfate), abundant components of the extracellular matrix (Fig. 5.8). This interaction concentrates virus particles near the cell surface and facilitates subsequent attachment of the viral glycoprotein gD to an integral membrane protein, which is required for entry into the cell (Fig. 5.8). Members of at least two different protein families serve as entry receptors for alphaherpesviruses. One of these families, the nectins, comprises the poliovirus receptor CD155 and related proteins, yet another example of

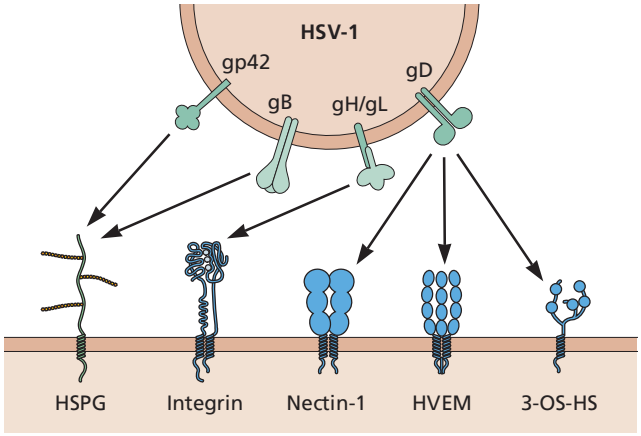


Figure 5.8 Cell receptors for herpes simplex virus type 1. Four viral glycoproteins, gp42, gB, gH/gL, and gD are shown binding with attachment molecule HSPG (heparin sulfate proteoglycan) or cell receptors (integrin, nectin-1, HVEM [herpesvirus entry mediator], 3-O-sulfate heparin sulfate). Virus entry does not require all interactions.

receptors shared by different viruses. When members of these two protein families are not present, 3-O-sulfated heparan sulfate can serve as an entry receptor for these viruses.

Multiple and Alternative Receptors

One type of receptor is not sufficient for infection by some viruses. Decay-accelerating protein (CD55), a regulator of the complement cascade, is the cell receptor for many enteroviruses, but infection also requires the presence of a coreceptor. Coxsackievirus A21 can bind to cell surface decay-accelerating protein, but this interaction does not lead to infection unless Icam-1 is also present. It is thought that Icam-1 inserts into the canyon where it triggers capsid uncoating. Some viruses bind to different cell receptors, depending on the nature of the virus isolate or the cell line. Often passage of viruses in cell culture selects variants that bind heparin sulfate. Infection of cells with foot-and-mouth disease virus type A12 requires the RGD-binding integrin $\alpha_v\beta_3$. However, the receptor for the O strain of this virus, which has been extensively passaged in cell culture, is not integrin $\alpha_v\beta_3$ but cell surface heparan sulfate. On the other hand, the type A12 strain cannot infect cells that lack integrin $\alpha_v\beta_3$, even if heparan sulfate is present. In a similar way, adaptation of Sindbis virus to cultured cells has led to the selection of variants that bind heparan sulfate. When receptors are rare, viruses that can bind to the more abundant glycosaminoglycan are readily selected.

Cell Surface Lectins and Spread of Infection

Cell surface **lectins** may bind to glycans present in viral glycoproteins, leading to dissemination within the host. An example is the lectin Dc-sign (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), a tetrameric C-type lectin present on the surface of dendritic cells. This lectin binds high-mannose, N-linked glycans, such as those produced in insect cells. Viruses that reproduce in insects are delivered to the human skin via a bite and may bind and sometimes infect dendritic cells. These cells then carry the viruses to other parts of the body, particularly lymph nodes. However, not all viruses that bind Dc-sign replicate in insect cells. In humans, Dc-sign on the surface of dendritic cells binds human immunodeficiency virus type 1 virus particles, but cell entry does not take place. When the dendritic cells migrate to the lymph node, infectious virus is released where it can enter and reproduce in T cells. While the interaction of human immunodeficiency virus type 1 with Dc-sign is nonproductive, it leads to viral dissemination in the host.

Entry into Cells

Uncoating at the Plasma Membrane

The particles of many enveloped viruses, including members of the family *Paramyxoviridae* such as Sendai virus and measles virus, fuse directly with the plasma membrane at neutral pH. These virions bind to cell surface receptors via a viral integral membrane protein (Fig. 5.9). Once the viral and cell membranes have been closely juxtaposed by this receptor-ligand interaction, fusion is induced by a second viral glycoprotein known as fusion (F) protein, and the viral nucleocapsid is released into the cell cytoplasm (Fig. 5.10).

F protein is a type I integral membrane glycoprotein (the N terminus lies outside the viral membrane) with similarities to influenza virus HA in its synthesis and structure. It is a homotrimer that is synthesized as a precursor called F0 and cleaved during transit to the cell surface by a host cell protease to produce two subunits, F1 and F2, held together by disulfide bonds. The newly formed N-terminal 20 amino acids of the F1 subunit, which are highly hydrophobic, form a region called the **fusion peptide** because it inserts into target membranes to initiate fusion. Viruses with the uncleaved F0 precursor can be produced in cells that lack the protease responsible for its cleavage. Such virus particles are noninfectious; they bind to target cells but the viral genome does not enter. Cleavage of the F0 precursor is necessary for fusion, not only because the fusion peptide is made available for insertion into the plasma membrane, but also to generate the metastable state of the protein that can undergo the conformational rearrangements needed for fusion.

Because cleaved F-protein-mediated fusion can occur at neutral pH, it must be controlled, both to ensure that virus particles fuse with only the appropriate cell and to prevent aggregation of newly assembled virions. The fusion peptide of F1 is buried between two subunits of the trimer in the pre-fusion protein. Conformational changes in F protein lead to refolding of the protein, assembly of an α-helical coiled

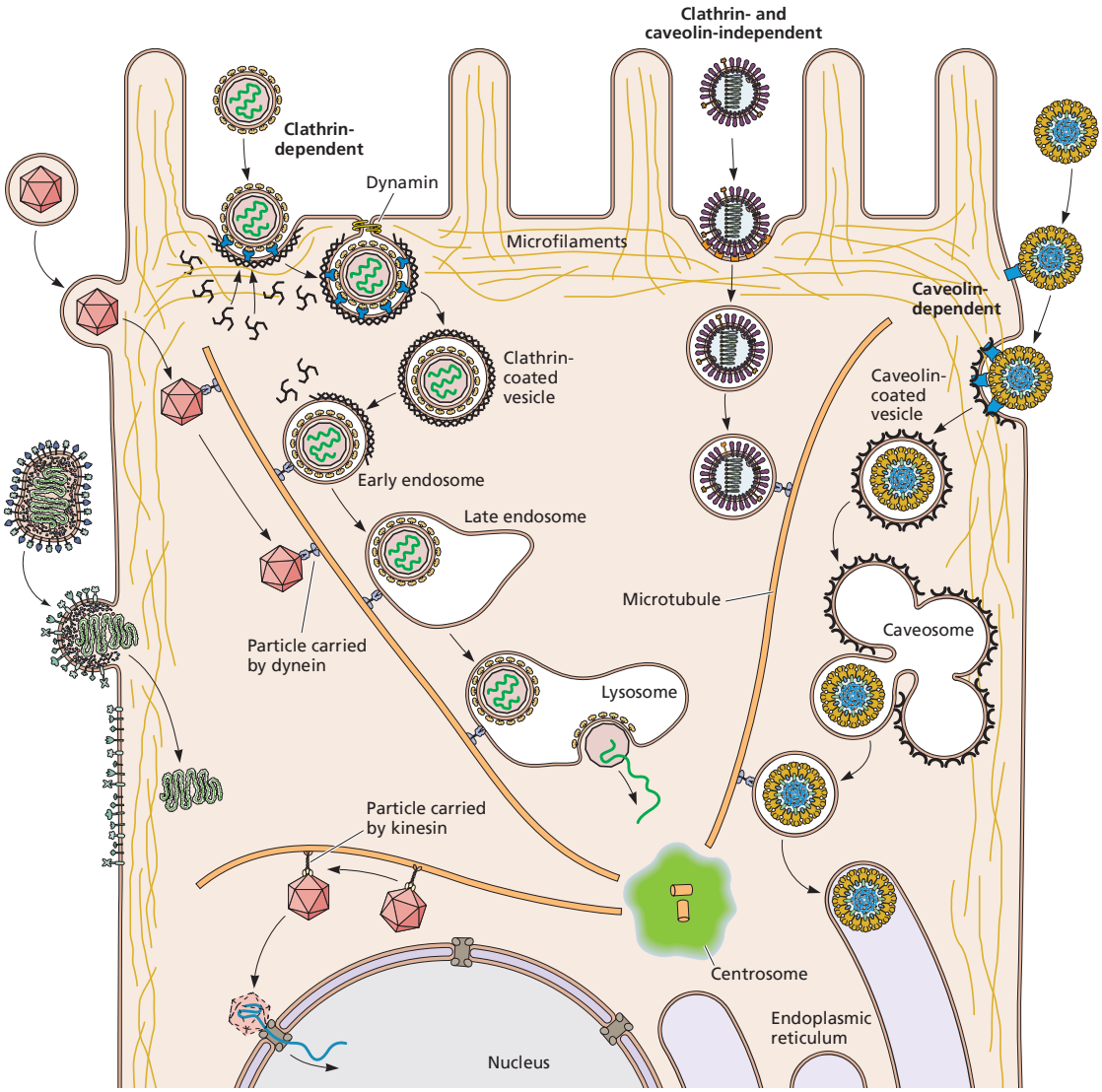


Figure 5.9 Virus entry and movement in cells. Examples of genome uncoating at the plasma membrane are shown on the left side of the cell. Fusion at the plasma membrane releases the nucleocapsid into the cytoplasm. In some cases, the subviral particle is transported on microtubules toward the nucleus, where the nucleic acid is released. Uptake of virions by clathrin-dependent endocytosis commences with binding to a specific cell surface receptor. The ligand-receptor complex diffuses into an invagination of the plasma membrane coated with the protein clathrin on the cytosolic side (clathrin-coated pits). The coated pit further invaginates and pinches off, a process that is facilitated by the GTPase dynamin. The resulting coated vesicle then fuses with an early endosome. Endosomes are acidic, as a result of the activity of vacuolar proton ATPases. Particle uncoating usually occurs from early or late endosomes. Late endosomes then fuse with lysosomes. Virus particles may enter cells by a dynamin- and caveolin-dependent endocytic pathway (right side of the cell). Three types of caveolar endocytosis have been identified. Dynamin 2-dependent endocytosis by caveolin 1-containing **caveolae** is observed in cells infected with simian virus 40 and polyomavirus. Dynamin 2-dependent, noncaveolar, lipid raft-mediated endocytosis occurs during echovirus and rotavirus infection, while dynamin-independent, noncaveolar, raft-mediated endocytosis is also observed during simian virus 40 and polyomavirus infection. This pathway brings virions to the endoplasmic reticulum via the caveosome, a pH-neutral compartment. Clathrin- and caveolin-independent endocytic pathways of viral entry have also been described (center of cell). Movement of endocytic vesicles within cells occurs on microfilaments or microtubules, components of the cytoskeleton. Microfilaments are two-stranded helical polymers of the ATPase actin. They are dispersed throughout the cell but are most highly concentrated beneath the plasma membrane, where they are connected via integrins and other proteins to the extracellular matrix. Transport along microfilaments is accomplished by myosin motors. Microtubules are 25-nm hollow cylinders made of the GTPase tubulin. They radiate from the **centrosome** to the cell periphery. Movement on microtubules is carried out by kinesin and dynein motors.

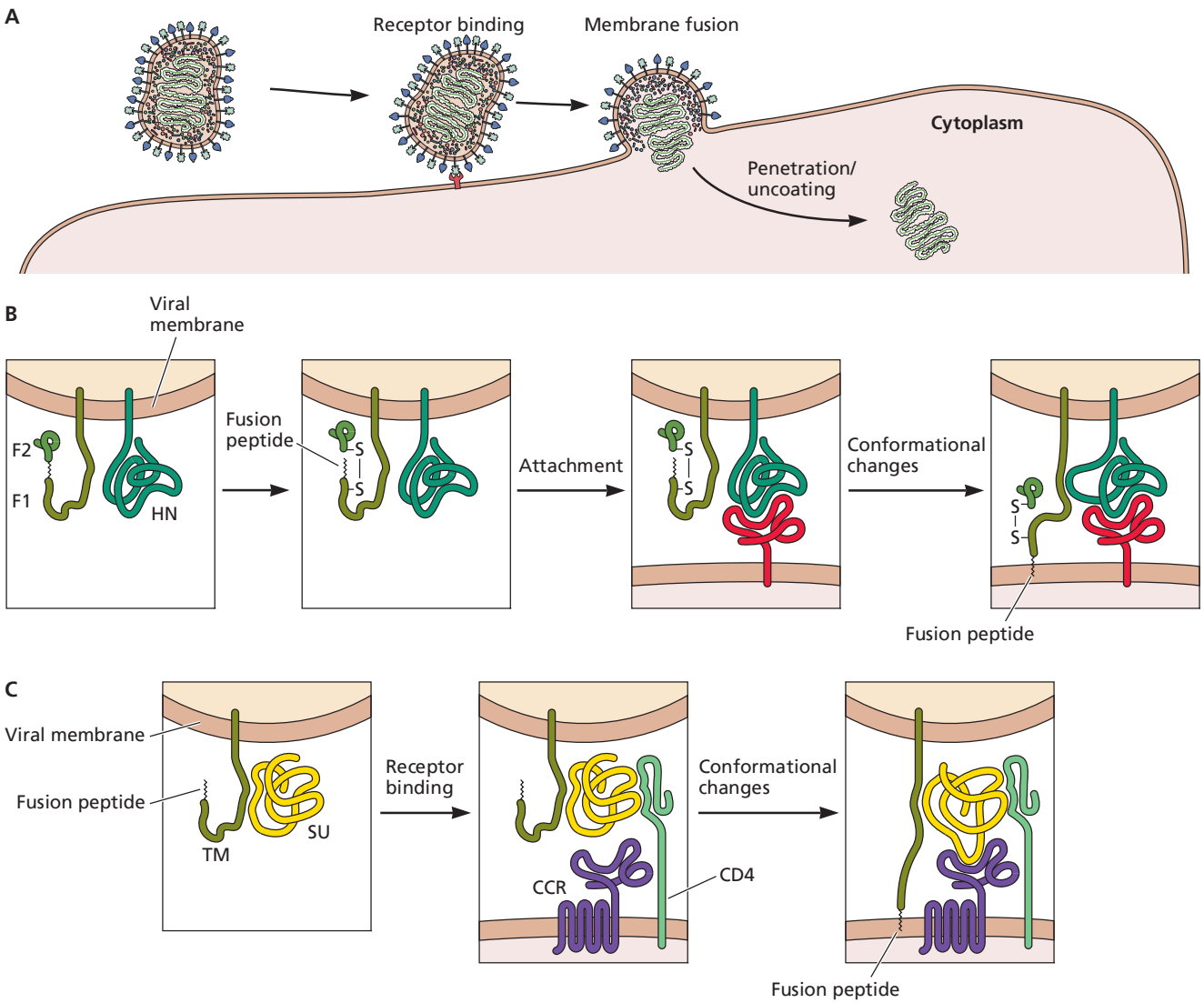


Figure 5.10 Penetration and uncoating at the plasma membrane. (A) Overview. Entry of a member of the *Paramyxoviridae*, which bind to cell surface receptors via the HN, H, or G glycoprotein. The fusion protein (F) then catalyzes membrane fusion at the cell surface at neutral pH. The viral nucleocapsid, as RNP, is released into the cytoplasm, where RNA synthesis begins. **(B)** Model for F-protein-mediated membrane fusion. Binding of HN to the cell receptor (red) induces conformational changes in HN that in turn induce conformational changes in the F protein, moving the fusion peptide from a buried position nearer to the cell membrane. **(C)** Model of the role of chemokine receptors in human immunodeficiency virus type 1 fusion at the plasma membrane. For simplicity, the envelope glycoprotein is shown as a monomer. Binding of SU to CD4 exposes a high-affinity chemokine receptor-binding site on SU. The SU-chemokine receptor interaction leads to conformational changes in TM that expose the fusion peptide and permit it to insert into the cell membrane, catalyzing fusion in a manner similar to that proposed for influenza virus (cf. Fig. 5.12 and 5.13).

coil, and movement of the fusion peptide toward the cell membrane (Fig. 5.10). Such movement of the fusion peptide has been described in atomic detail by comparing structures of the F protein before and after fusion.

The trigger that initiates conformational changes in the F protein is not known. The results of experiments in which hemagglutinin-neuraminidase (HN) and F glycoproteins are synthesized in cultured mammalian cells indicate that the fusion activity of F protein is absent or inefficient if HN is not present. It has therefore been hypothesized that an interaction between HN and F proteins is essential for fusion. It is thought that binding of HN protein to its cellular receptor induces

conformational changes, which in turn trigger conformational change in the F protein, exposing the fusion peptide and making the protein fusion competent (Fig. 5.10). The requirement for HN protein in F fusion activity has been observed only with certain paramyxoviruses, including human parainfluenza virus type 3 and mumps virus.

As a result of fusion of the viral and plasma membranes, the viral nucleocapsid, which is a ribonucleoprotein (RNP) consisting of the (−) strand viral RNA genome and the viral proteins L, NP, and P, is released into the cytoplasm (Fig. 5.10). Once in the cytoplasm, the L, NP, and P proteins begin the synthesis of viral messenger RNAs (mRNAs), a process discussed in Chapter 6. Because members of the *Paramyxoviridae* replicate in the cytoplasm, fusion of the viral and plasma membranes achieves uncoating and delivery of the viral genome to this cellular compartment in a single step.

Fusion of human immunodeficiency virus type 1 with the plasma membrane requires participation not only of the cell receptor CD4 but also of an additional cellular protein. These proteins are cell surface receptors for small molecules produced by many cells to attract and stimulate cells of the immune defense system at sites of infection; hence, these small molecules are called **chemotactic cytokines** or **chemokines**. The chemokine receptors on such cells comprise a large family of proteins with seven membrane-spanning domains and are coupled to intracellular signal transduction pathways. There are two major coreceptors for human immunodeficiency virus type 1 infection. CXCr4 (a member of a family of chemokines characterized by having their first two cysteines separated by a single amino acid) appears to be a specific coreceptor for virus strains that infect T cells preferentially. The second is CCr5, a coreceptor for the macrophage-tropic strains of the virus. The chemokines that bind to this receptor activate both T cells and macrophages, and the receptor is found on both types of cell. Individuals who are homozygous for deletions in the CCr5 gene and produce nonfunctional coreceptors have no discernible immune function abnormality, but they appear to be resistant to infection with human immunodeficiency virus type 1. Even heterozygous individuals seem to be somewhat resistant to the virus. Other members of the CC chemokine receptor family (CCr2b and CCr3) were subsequently found to serve as coreceptors for the virus.

Attachment to CD4 appears to create a high-affinity binding site on SU for CCr5. The atomic structure of SU bound to CD4 revealed that binding of CD4 induces conformational changes that expose binding sites for chemokine receptors (Fig. 5.10). Studies of CCr5 have shown that the first N-terminal extracellular domain is crucial for coreceptor function, suggesting that this sequence might interact with SU. An antibody molecule fused to both the CD4 and CCr5 binding sites is being explored as a therapeutic compound to block infection (Box 5.4).

Human immunodeficiency virus type 1 TM mediates envelope fusion with the cell membrane. The high-affinity SU-CCr5 interaction may induce conformational changes in TM to expose the fusion peptide, placing it near the cell membrane, where it can catalyze fusion (Fig. 5.10). Such changes are similar to those that influenza virus HA undergoes upon exposure to low pH. X-ray crystallographic analysis of fusion-active human immunodeficiency virus type 1 TM revealed that its structure is strikingly similar to that of the low-pH fusogenic form of HA (see "Acid-Catalyzed Membrane Fusion" below).

Uncoating during Endocytosis

Many viruses enter cells by the same pathways by which cells take up macromolecules. The plasma membrane, the limiting membrane of the cell, permits nutrient molecules to enter and waste molecules to leave, thereby ensuring an appropriate internal environment. Water, gases, and small hydrophobic molecules such as ethanol can freely traverse the lipid bilayer, but most metabolites and ions cannot. These essential components enter the cell by specific transport processes. Integral membrane proteins are responsible for the transport of ions, sugars, and amino acids, while proteins and large particles are taken into the cell by phagocytosis or endocytosis. The former process (Fig. 5.11) is nonspecific, which means that any particle or molecule can be taken into the cell, and only occurs in specialized cell types such as dendritic cells and macrophages.

Clathrin-Mediated Endocytosis

A wide range of ligands, fluid, membrane proteins, and lipids are selectively taken into cells from the extracellular milieu by **clathrin-mediated endocytosis** (Fig. 5.9 and 5.11), also the mechanism of entry of many viruses. Ligands in the extracellular medium bind to cells via specific plasma membrane receptor proteins. The receptor-ligand assembly diffuses along the membrane until it reaches an invagination that is coated on its cytoplasmic surface by a cage-like lattice composed of the fibrous protein clathrin (Fig. 5.9). Such clathrin-coated pits can comprise as much as 2% of the surface area of a cell, and some receptors are clustered over these areas even in the absence of their ligands. Following the accumulation of receptor-ligand complexes, the clathrin-coated pit invaginates and then pinches off to form a clathrin-coated vesicle. Within a few seconds, the clathrin coat is lost and the vesicles fuse with small, smooth-walled vesicles located near the cell surface, called early **endosomes**. The lumen of early endosomes is mildly acidic (pH 6.5 to 6.0), a result of energy-dependent transport of protons into the interior of the vesicles by a membrane proton pump. The contents of the early endosome are then transported via endosomal carrier vesicles to late endosomes located close to the nucleus. The lumen of late endosomes is more acidic (pH 6.0 to 5.0). Late endosomes in turn

BOX 5.4

EXPERIMENTS

Blocking human immunodeficiency virus infection with two soluble cell receptors

Because viruses must bind to cell surface molecules to initiate replication, the use of soluble receptors to block virus infection has long been an attractive therapeutic option. Soluble CD4 receptors that block infection with human immunodeficiency virus type 1 (HIV-1) have been developed, but these have not been licensed because of their suboptimal potency. A newly designed soluble receptor for HIV-1 overcomes this problem and provides broad and effective protection against infection of cells and of nonhuman primates.

A soluble form of CD4 fused to an antibody molecule can block infection of most HIV-1 isolates and has been shown to be safe in humans, but its affinity for gp120 is low. Furthermore, human immunodeficiency virus can also be spread from cell to cell by fusion, a process that is not blocked by circulating, soluble CD4. Similarly, peptide mimics of the CCR5 coreceptor have been shown to block infection, but their affinity for gp120 is also low.

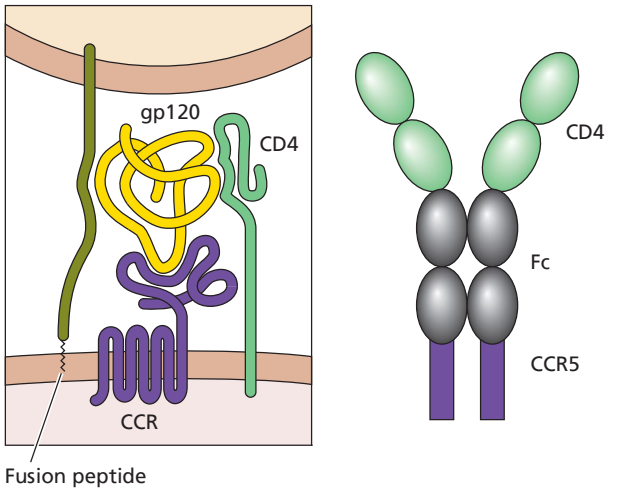
Combining the two gp120-binding molecules solved the problem of low affinity and in addition provided protection against a wide range of virus isolates. The entry inhibitor, called eCD4-Ig, is a fusion of the first two domains of CD4 to the Fc domain of an antibody molecule, with the CCR5-mimicking peptide at the carboxy terminus (illustrated). It binds strongly to gp120 and blocks infection with many different isolates of HIV-1, HIV-2, simian immunodeficiency virus (SIV), and HIV-1 resistant to broadly neutralizing monoclonal antibodies. The molecule blocks viral infection at concentrations that might be achieved in humans (1.5 to 5.2 micrograms per milliliter).

When administered to mice, eCD4-Ig protected the animals from HIV-1. Rhesus macaques inoculated with an adenovirus-associated virus (AAV) recombinant containing the gene for eCD4-Ig were protected from infection with large quantities of virus for up to 34 weeks after immunization. Concentrations of eCD4-Ig in the sera of these animals ranged from 17 to 77 micrograms per milliliter.

These results show that eCD4-Ig blocks HIV infection with a wide range of isolates more effectively than previously studied broadly neutralizing antibodies. Emergence of HIV variants resistant to neutralization with eCD4-Ig would likely produce viruses that infect cells less efficiently, reducing their transmission. eCD4-Ig is therefore an attractive candidate for therapy of HIV-1 infections. Whether sustained production of the protein in humans will cause disease remains to be determined. Because expression of the AAV genome persists for long periods, it might be advantageous to include a kill-switch in the vector: a way of turning it off if something should go wrong.

Gardner MR, Kattenhorn LM, Kondur HR, von Schaewen M, Dorfman T, Chiang JJ, Haworth KG, Decker JM, Alpert MD, Bailey CC, Neale ES, Jr, Fellinger CH, Joshi VR, Fuchs SP, Martinez-Navio JM, Quinlan BD, Yao AY, Mouquet H, Gorman J, Zhang B, Poignard P, Nussenzweig MC, Burton DR, Kwong PD, Piatak M, Jr, Lifson JD, Gao G, Desrosiers RC, Evans DT, Hahn BH, Ploss A, Cannon PM, Seaman MS, Farzan M. 2015. AAV-expressed eCD4-Ig provides durable protection from multiple SHIV challenges. *Nature* **519:**87–91.

Left, binding of HIV-1 SU (gp120) to CD4 and a chemokine receptor, CCR. Right, illustration of soluble eCD4-Ig. The Fab domains of the antibody molecule are replaced with the first two Ig-like domains of CD4, and the gp120-binding part of CCR5 is added to the C terminus of the Fc domain.



fuse with **lysosomes**, which are vesicles containing a variety of enzymes that degrade sugars, proteins, nucleic acids, and lipids. Viruses with a high pH threshold for fusion, such as vesicular stomatitis virus, enter from early endosomes; most enter the cytoplasm from late endosomes, and a few enter from lysosomes.

Clathrin-mediated endocytosis is a continuous but regulated process. For example, the uptake of vesicular stomatitis virus into cells may be influenced by over 90 different cellular protein kinases. Influenza virus, vesicular stomatitis virus, and reovirus particles are taken into cells, not into preexisting pits but mainly by clathrin-coated pits that form after virus binds to the cell surface. It is not known how virus binding to the plasma membrane induces the formation of the clathrin-coated pit.

Caveolar and Lipid Raft-Mediated Endocytosis

Although uptake of most viruses occurs by the clathrin-mediated endocytic pathway, some viruses enter by caveolin- or raft-mediated endocytosis (Fig. 5.9). The caveolar pathway

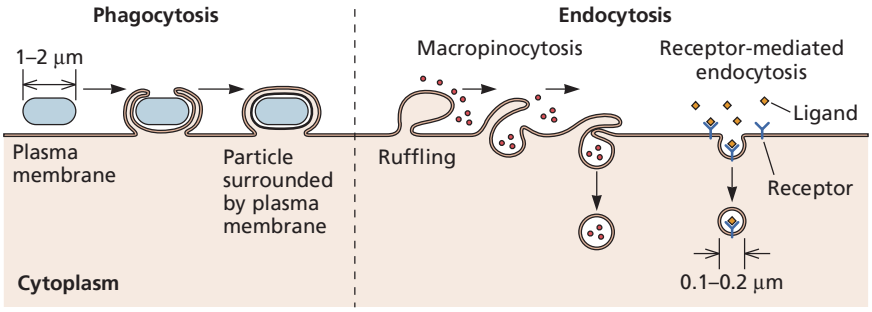


Figure 5.11 Mechanisms for the uptake of macromolecules from extracellular fluid. During phagocytosis, large particles such as bacteria or cell fragments that come in contact with the cell surface are engulfed by extensions of the plasma membrane. Phagosomes ultimately fuse with lysosomes, resulting in degradation of the material within the vesicle. Macrophages use phagocytosis to ingest bacteria and destroy them. Endocytosis comprises the invagination and pinching off of small regions of the plasma membrane, resulting in the nonspecific internalization of molecules (macropinocytosis) or the specific uptake of molecules bound to cell surface receptors (receptor-mediated endocytosis). Macropinocytosis is a mechanism for the uptake of extracellular fluid. It is triggered by ligand binding which initiates formation of plasma membrane ruffling, which traps material in large vacuoles. Adapted from J. Darnell et al., *Molecular Cell Biology* (Scientific American Books, New York, NY, 1986), with permission.

requires cholesterol (a major component of lipid rafts). Caveolae are distinguished from clathrin-coated vesicles by their flask-like shape, their smaller size, the absence of a clathrin coat, and the presence of a marker protein called caveolin. In the uninfected cell, caveolae participate in transcytosis, signal transduction, and uptake of membrane components and extracellular ligands. Binding of a virus particle to the cell surface activates signal transduction pathways required for pinching off of the vesicle, which then moves within the cytoplasm. Disassembly of filamentous actin also occurs, presumably to facilitate movement of the vesicle deeper into the cytoplasm. There it fuses with the **caveosome**, a larger membranous organelle that contains caveolin (Fig. 5.9). In contrast to endosomes, the pH of the caveosome lumen is neutral. Some viruses (e.g., echovirus type 1) penetrate the cytoplasm from the caveosome. Others (simian virus 40, polyomavirus, coxsackievirus B3) are sorted to the endoplasmic reticulum (ER) by a transport vesicle that lacks caveolin. These viruses enter the cytoplasm by a process mediated by thiol oxidases present in the lumen of the endoplasmic reticulum and by a component of the protein degradation pathway present in the membrane.

The study of virus entry by endocytosis can be confusing because some viruses may enter cells by multiple routes, depending on cell type and multiplicity of infection. For example, herpes simplex virus can enter cells by three different routes and influenza A virus may enter cells by both clathrin-dependent and clathrin-independent pathways.

Macropinocytosis

Macropinocytosis is a process by which extracellular fluid is taken into cells via large vacuoles. It is triggered by ligands and dependent on actin and a signaling pathway. It differs from phagocytosis by the signaling pathways needed and can take place in many cell types. This process serves as a pathway of entry for many viruses, including vaccinia virus, herpesviruses, and ebolaviruses. Upon receptor binding, viruses that enter cells via macropinocytosis trigger a signaling cascade that leads to changes in cortical actin and ruffling of the plasma membrane (Fig. 5.11). When these plasma membrane extensions retract, the viruses are brought into macropinosomes and eventually leave these vesicles via membrane fusion.

Membrane Fusion

The membranes of enveloped viruses fuse with those of the cell as a first step in delivery of the viral nucleic acid. Membrane fusion takes place during many other cellular processes, such as cell division, myoblast fusion, and exocytosis.

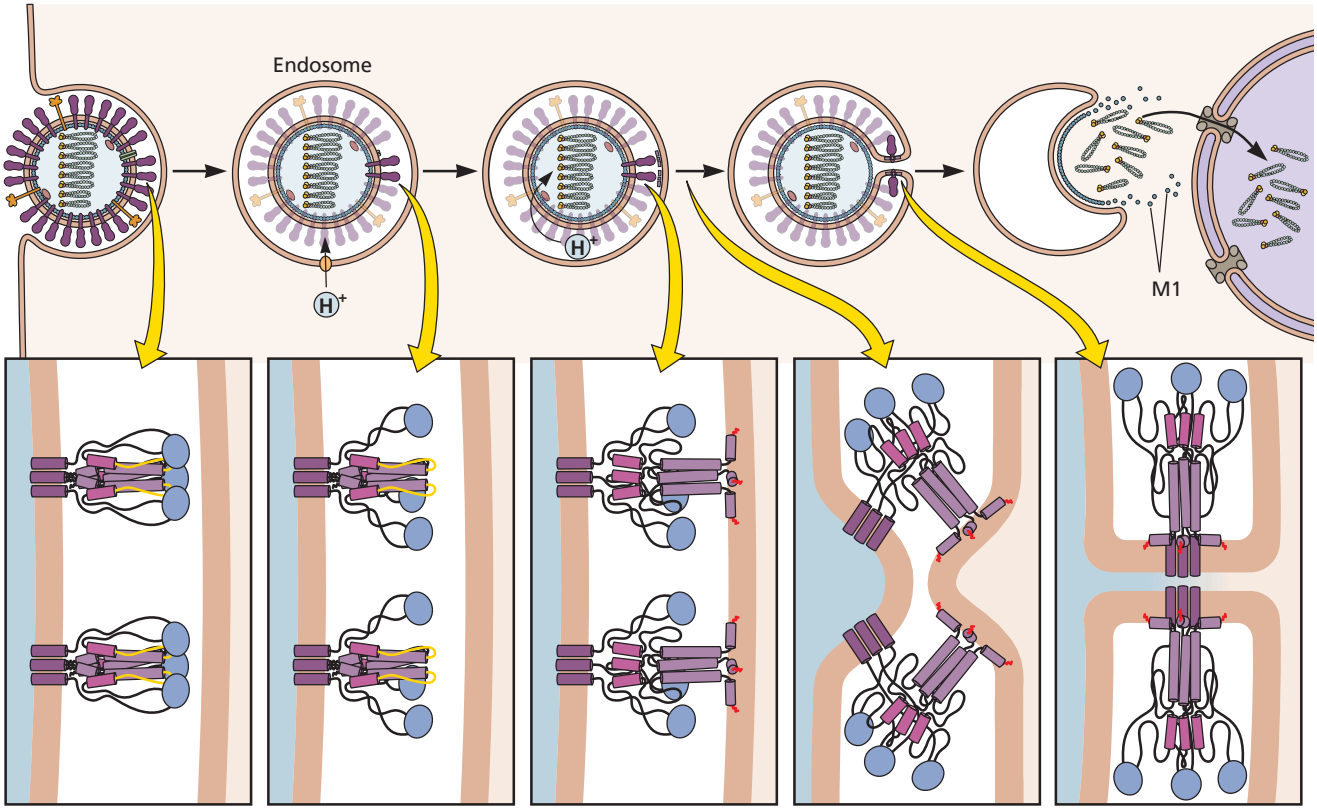
Membrane fusion must be regulated in order to maintain the integrity of the cell and its intracellular compartments. Consequently, membrane fusion does not occur spontaneously but proceeds by specialized mechanisms mediated by proteins. The two membranes must first come into close proximity. In cells, this reaction is mediated by interactions of integral membrane proteins that protrude from the lipid bilayers, a targeting protein on one membrane and a docking protein on the other. During entry of enveloped viruses, the virus and cell membranes are first brought into close contact by interaction of a viral glycoprotein with a cell receptor. The next step, fusion, requires an even closer approach of the membranes, to within 1.5 nm of each other. This step depends on the removal of water molecules from the membrane surfaces,

an energetically unfavorable process. This step is hypothesized to occur when the viral glycoprotein undergoes a structural rearrangement called "hairpinning" (Fig. 5.12).

The precise mechanism by which lipid bilayers fuse is not completely understood, but the action of fusion proteins is thought to result in the formation of an opening called a **fusion pore**, allowing exchange of material across the membranes (Box 5.5). The viral glycoprotein bound to a cell receptor, or a different viral integral membrane protein, then catalyzes the fusion of the juxtaposed membranes. Viral fusion proteins are integral membrane proteins, often glycoproteins, that form homo- or hetero-oligomers.

Virus-mediated fusion must be regulated to prevent viruses from aggregating or to ensure that fusion does not occur in the incorrect cellular compartment. In some cases, fusogenic potential is masked until the fusion protein interacts with other integral membrane proteins. In others, low pH is required to expose fusion domains. The activity of fusion proteins may also be regulated by cleavage of a precursor. This requirement probably prevents premature activation of fusion potential during virus assembly. Viral fusion proteins are often primed for fusion by proteolytic cleavage as they move through the trans-Golgi network as described in Chapter 12. Proteases that catalyze such cleavage are typically furin family convertases that either cleave the fusion proteins directly (orthomyxoviruses, retroviruses, paramyxoviruses) or cleave a protein that masks the fusion protein (alphaviruses, flaviviruses).

Figure 5.12 Influenza virus entry. The globular heads of native HA mediate binding of the virus to sialic acid-containing cell receptors. The virus-receptor complex is endocytosed, and import of H^+ ions into the endosome acidifies the interior. Upon acidification, the viral HA undergoes a conformational rearrangement that produces a fusogenic protein. The loop region of native HA (yellow) becomes a coiled coil, moving the fusion peptides (red) to the top of the molecule near the cell membrane. At the viral membrane, the long α-helix (purple) packs against the trimer core, pulling the globular heads to the side. The long coiled coil bends, or hairpins, bringing the fusion peptides and the transmembrane domains together. This movement moves the cell and viral membranes close together so that fusion can occur. To allow release of vRNP into the cytoplasm, the H^+ ions in the acidic endosome are pumped into the particle interior by the M2 ion channel. As a result, vRNP is primed to dissociate from M1 after fusion of the viral and endosomal membranes. The released vRNPs are imported into the nucleus through the nuclear pore complex via a nuclear localization signal-dependent mechanism (see "Import of Influenza Virus Ribonucleoprotein" below). Adapted from C. M. Carr and P. S. Kim, *Science* **266:**234–236, 1994, with permission.

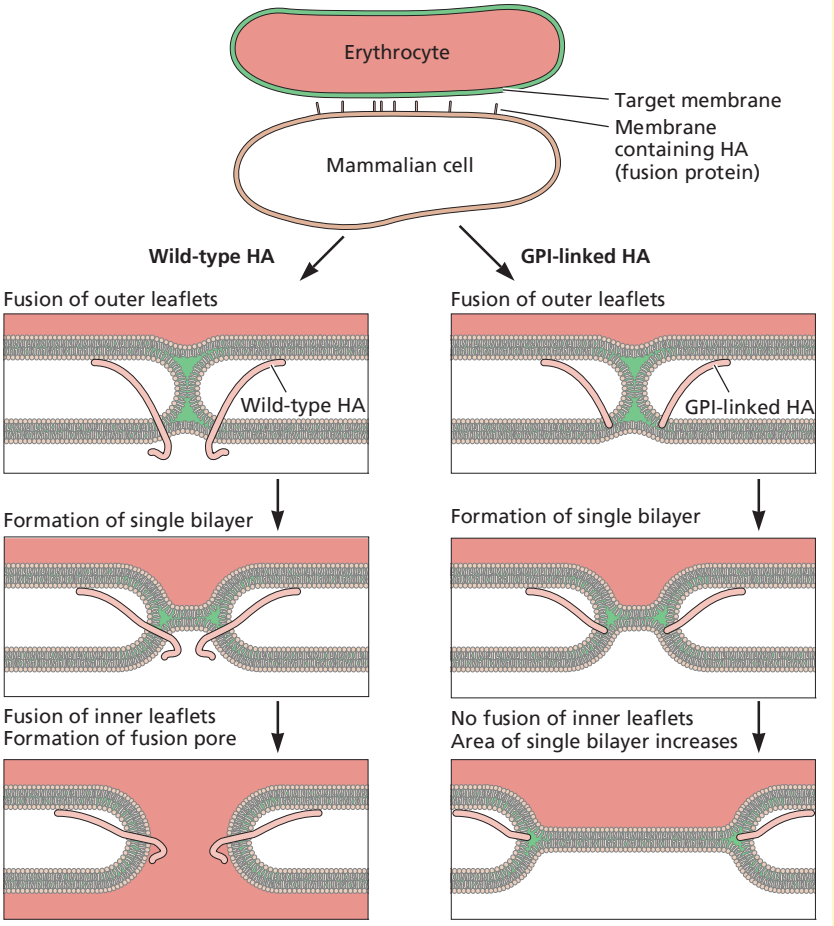


BOX 5.5

EXPERIMENTS

Membrane fusion proceeds through a hemifusion intermediate

Fusion is thought to proceed through a hemifusion intermediate in which the outer leaflets of two opposing bilayers fuse (see figure), followed by fusion of the inner leaflets and the formation of a fusion pore. Direct evidence that fusion proceeds via a hemifusion intermediate has been obtained with influenza virus HA (see figure). **(Left)** Cultured mammalian cells expressing wild-type HA are fused with erythrocytes containing two different types of fluorescent dye, one in the cytoplasm and one in the lipid membrane. Upon exposure to low pH, HA undergoes conformational change and the fusion peptide is inserted into the erythrocyte membrane. The green dye is transferred from the lipid bilayer of the erythrocyte to the bilayer of the cultured cell. The HA trimers tilt, causing reorientation of the transmembrane domain and generating stress within the hemifusion diaphragm. Fusion pore formation relieves the stress. The red dye within the cytoplasm of the erythrocyte is then transferred to the cytoplasm of the cultured cell. **(Right)** An altered form of HA was produced, lacking the transmembrane and cytoplasmic domains and with membrane anchoring provided by linkage to a glycosylphosphatidylinositol (GPI) moiety. Upon exposure to low pH, the HA fusion peptide is inserted into the erythrocyte membrane, and green dye is transferred to the membranes of the mammalian cell. When the HA trimers tilt, no stress is transmitted to the hemifusion diaphragm because no transmembrane domain is present, and the diaphragm becomes larger. Fusion pores do not form, and there is no mixing of the contents of the cytoplasm, indicating that complete membrane fusion has not occurred. These results prove that hemifusion, or fusion of only the inner leaflet of the bilayer, can occur among whole cells. The findings also demonstrate that the transmembrane domain of the HA polypeptide plays a role in the fusion process.



Glycosylphosphatidylinositol-anchored influenza virus HA induces hemifusion. (Left) Model of the steps of fusion mediated by wild-type HA. **(Right)** Effect on fusion by an altered form of HA lacking the transmembrane and cytoplasmic domains. Adapted from G. B. Melikyan et al., *J. Cell Biol* **131:**679–691, 1995, with permission.

Proteolytic cleavage not only is a mechanism to regulate where fusion occurs, but also generates the metastable states of viral glycoproteins that can subsequently undergo the conformational rearrangements required for fusion activity without additional energy. These structural changes expose the hidden fusion peptide so that it can insert into the target membrane and likely provide the energy needed to overcome the hydration force that prevents spontaneous membrane fusion. As a consequence the fusion protein is anchored in both viral and cellular membranes.

Acid-Catalyzed Membrane Fusion

The entry of influenza virus from the endosomal pathway is one of the best-understood viral entry mechanisms. At the cell surface, the virus attaches to sialic acid-containing receptors via the viral HA glycoprotein (Fig. 5.12). The virus-receptor complex is then internalized into the clathrin-dependent receptor-mediated endocytic pathway. When the endosomal pH reaches approximately 5.0, HA undergoes an acid-catalyzed conformational rearrangement, exposing a fusion peptide. The viral and endosomal membranes

then fuse, allowing penetration of the viral RNP (vRNP) into the cytoplasm. Because influenza virus particles have a low pH threshold for fusion, uncoating occurs in late endosomes.

The fusion reaction mediated by the influenza virus HA protein is a remarkable event when viewed at atomic resolution (Fig. 5.13). In native HA, the fusion peptide is joined to the three-stranded coiled-coil core by which the HA monomers interact via a 28-amino-acid sequence that forms an extended loop structure buried deep inside the molecule, about 100 Å from the globular head. In contrast, in the low-pH HA structure, this loop region is transformed into a three-stranded coiled coil. In addition, the long α-helices of the coiled coil bend upward and away from the viral membrane. The result is that the fusion peptide is moved a great distance toward the endosomal membrane (Fig. 5.13). Despite these dramatic changes, HA remains trimeric and the globular heads can still bind sialic acid. In this conformation, HA holds the viral and endosome membranes 100 Å apart, too distant for fusion to occur. To bring the viral and cellular membranes closer, it is thought that the HA molecule bends, bringing together the fusion peptide and the transmembrane segment (Fig. 5.12). This movement brings the two membranes close enough to fuse.

In contrast to cleaved HA, the precursor HA0 is stable at low pH and cannot undergo structural changes. Cleavage of the covalent bond between HA1 and HA2 might simply allow movement of the fusion peptide, which is restricted in the uncleaved molecule. Another possibility is suggested by the observation that cleavage of HA is accompanied by movement of the fusion peptide into a cavity in the protein (Fig. 5.13). This movement buries ionizable residues of the fusion peptide, perhaps setting the low-pH "trigger." It should be emphasized that after cleavage, the N terminus of HA2 is tucked into the hydrophobic interior of the trimer (Fig. 5.13). This rearrangement presumably buries the fusion peptide so that newly synthesized virions do not aggregate and lose infectivity.

Viral fusion proteins have been placed into three structural classes called I, II, and III. Common characteristics of all three classes include insertion of a fusion peptide into the target membrane and refolding of the fusion protein so that cell and viral membranes are brought together. In class I fusion proteins (Fig. 5.14), the fusion peptides are presented to membranes on top of a three-stranded α-coiled coil.

The envelope proteins of alphaviruses and flaviviruses exemplify class II viral fusion proteins. Defining characteristics include a three-domain subunit, a fusion loop, and tight association with a second viral protein. Proteolytic cleavage of the second protein converts the fusion protein to a metastable state that can undergo structural rearrangements at low pH to promote fusion. In contrast, the fusion peptide of the influenza virus HA is adjacent to the cleavage point and becomes the N terminus of the mature fusion protein. The envelope proteins of alphaviruses (E1) and flaviviruses (E) do not form coiled coils, as do type I fusion proteins. Rather, they contain predominantly β-barrels that tile the surface of the virus particles as dimers. At low pH they extend toward the endosome membrane, allowing insertion of the fusion peptide (Fig. 5.15).

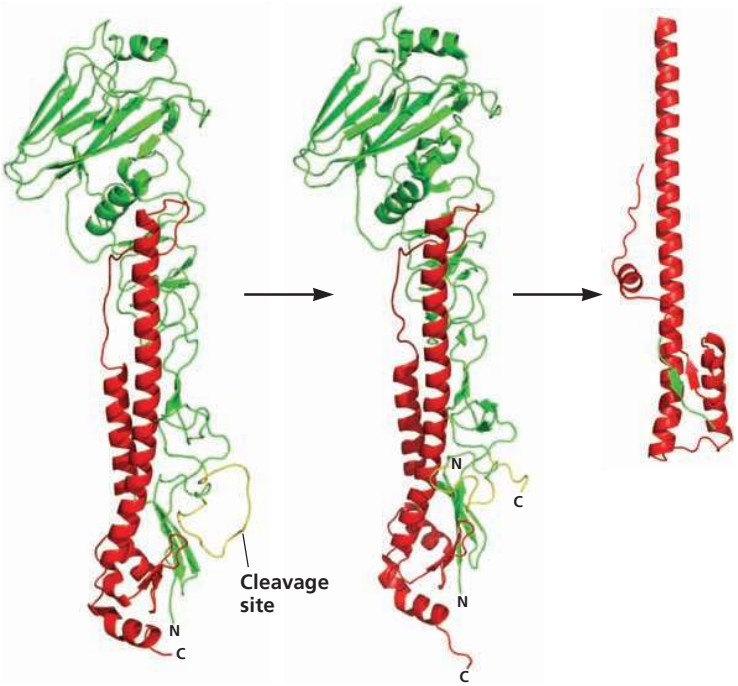


Figure 5.13 Cleavage- and low-pH-induced structural changes in the extracellular domains of influenza virus HA. **(Left)** Structure of the uncleaved HA0 precursor extracellular domain at neutral pH. HA1 subunits are green, HA2 subunits are red, residues 323 of HA1 to 12 of HA2 are yellow, and the locations of some of the N and C termini are indicated. The viral membrane is at the bottom, and the globular heads are at the top. The cleavage site between HA1 and HA2 is in a loop adjacent to a deep cavity. **(Middle)** Structure of the cleaved HA trimer at neutral pH. Cleavage of HA0 generates new N and C termini, which are separated by 20 Å. The N and C termini visible in this model are labeled. The cavity is now filled with residues 1 to 10 of HA2, part of the fusion peptide. **(Right)** Structure of the low-pH trimer. The protein used for crystallization was treated with proteases, and therefore the HA1 subunit and the fusion peptide are not present. This treatment is necessary to prevent aggregation of HA at low pH. At neutral pH the fusion peptide is close to the viral membrane, linked to a short α-helix, and at acidic pH this α-helix is reoriented toward the cell membrane, carrying with it the fusion peptide. The structures are aligned on a central α-helix that is unaffected by the conformational change. Only monomers of all three structures are shown. Adapted from J. Chen et al., *Cell* **95:**409–417, 1998, with permission.

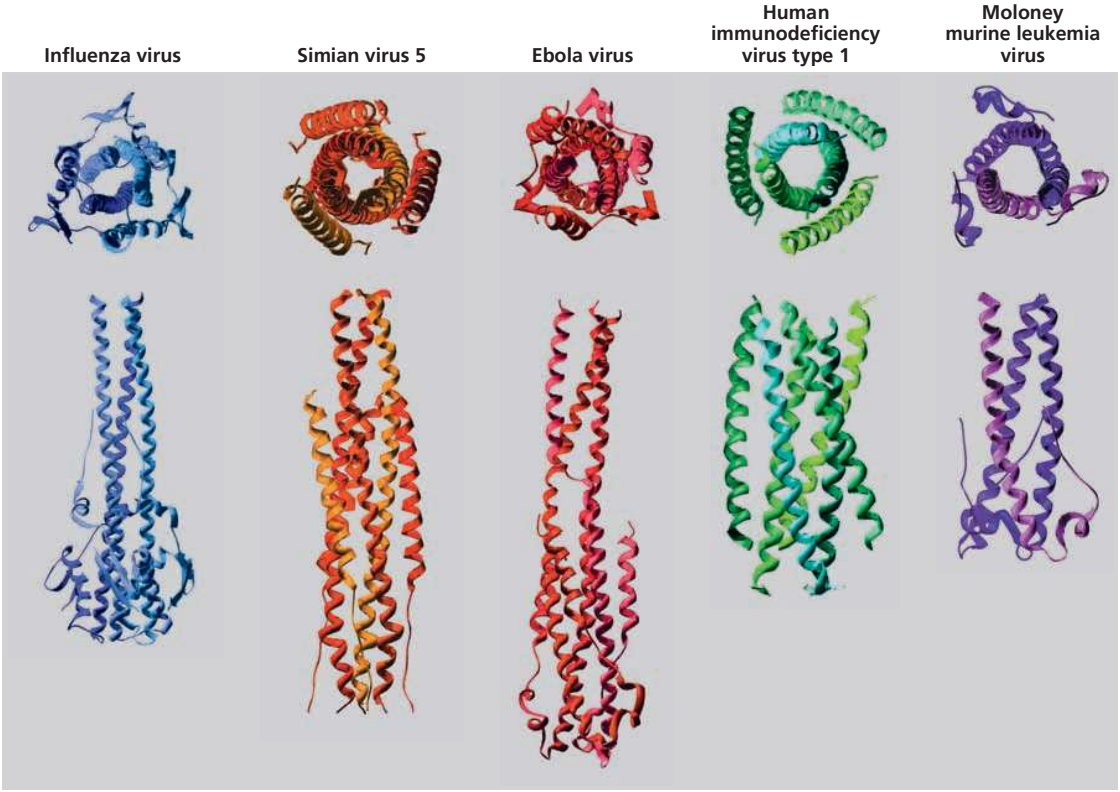
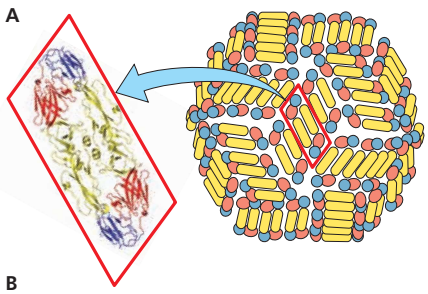


Figure 5.14 Similarities among five viral fusion proteins. **(Top)** View from the top of the structures. **(Bottom)** Side view. The structure shown for HA is the low-pH, or fusogenic, form. The structure of simian virus 5 F protein is of peptides from the N- and C-terminal heptad repeats. Structures of retroviral TM proteins are derived from interacting human immunodeficiency virus type 1 peptides and a peptide from Moloney murine leukemia virus and are presumed to represent the fusogenic forms because of structural similarity to HA. In all three molecules, fusion peptides would be located at the membrane-distal portion (the tops of the molecules in the bottom view). All present fusion peptides to cells on top of a central three-stranded coiled coil supported by C-terminal structures. Adapted from K. A. Baker et al., *Mol. Cell* **3:**309–319, 1999, with permission.



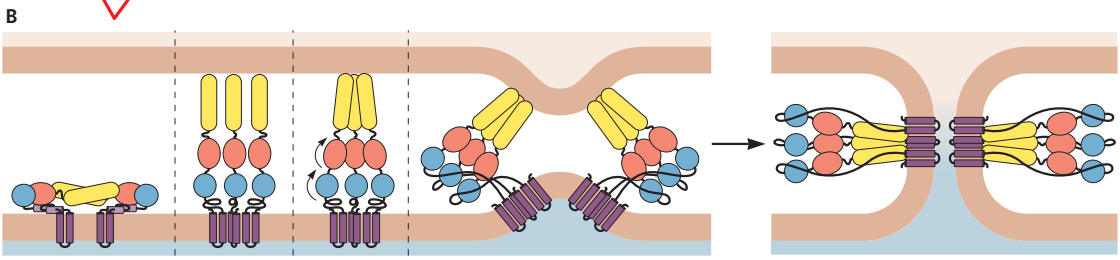


Figure 5.15 Membrane fusion by flavivirus envelope glycoprotein E. Low pH causes conformational changes in the viral glycoproteins to produce the fusion-active forms. **(A)** Ninety dimers of E tile the surface of the virus particle. **(B)** The fusion loop is located at the tip of domain II (yellow) where it is buried in dimers of the glycoprotein E. At low pH, the dimers are disrupted, the proteins extend to form trimers, and the fusion peptide is directed toward the cell membrane. The glycoprotein then undergoes a rearrangement that brings the fusion peptides and transmembrane segments (purple) together. This movement brings the viral and cell membranes together, allowing fusion.

In the alphavirus particle, the second viral protein, E2, acts as a clamp to hold the fusion protein in place; at low pH the clamp is released. In contrast to the situation with other viruses, proteolytic cleavage of E1 is not required to produce a fusogenic protein. However, protein processing controls fusion potential in another way: in the endoplasmic reticulum, E1 protein is associated with the precursor of E2, called p62. In this heterodimeric form, p62-E1, E1 protein cannot be activated for fusion by mildly acidic conditions. Only after p62 has been cleaved to E2 can low pH induce disruption of E1-E2 heterodimers and formation of fusion-active E1 homotrimers.

Receptor Priming for Low-pH Fusion: Two Entry Mechanisms Combined

During the entry of avian leukosis virus into cells, binding of the virus particle to the cell receptor primes the viral fusion protein for low-pH-activated fusion. Avian leukosis virus, like many other retroviruses with simple genomes, was believed to enter cells at the plasma membrane in a pH-independent mechanism resembling that of members of the *Paramyxoviridae* (Fig. 5.10). It is now known that binding of the viral membrane glycoprotein (SU) to the cellular receptors of avian leukosis viruses induces conformational rearrangements that convert the viral protein from a native metastable state that is insensitive to low pH to a second metastable state. In this state, exposure to low pH within the endosomal compartment leads to membrane fusion and release of the viral capsid.

An Endosomal Fusion Receptor

The study of Ebolavirus entry into cells has revealed a new mechanism in which the viral fusion protein binds to a specific fusion receptor in the endosome membrane. Previously, all known cases of fusion catalyzed by viral glycoproteins have taken place when the fusion peptide inserts into the endosomal membrane by virtue of its hydrophobic properties.

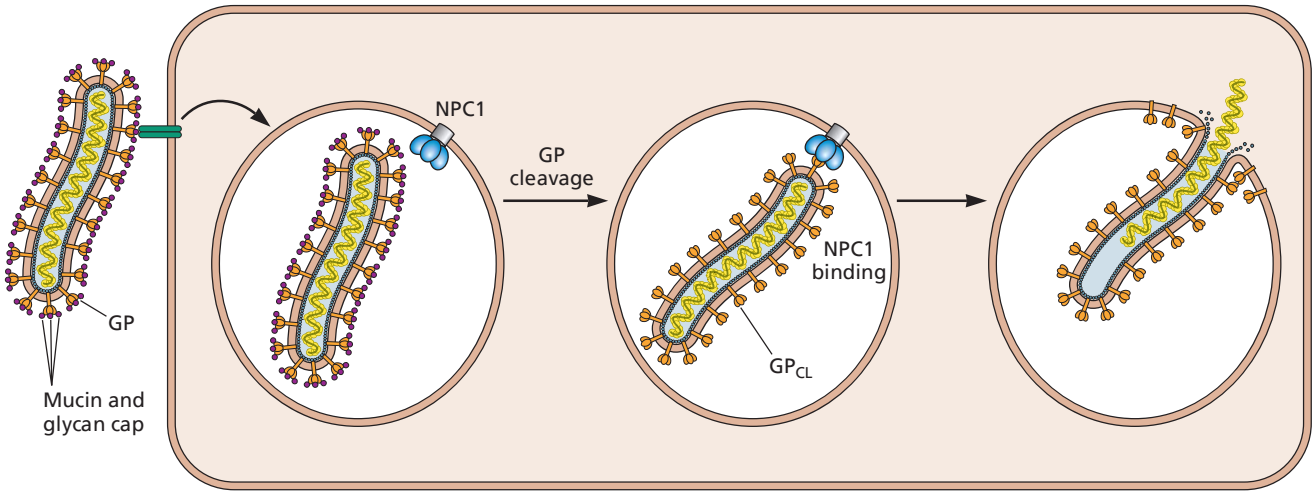
Following attachment to cells via the viral glycoprotein GP, viral particles are internalized and move to late endosomes. There, cysteine proteases cleave GP to remove heavily glycosylated sequences at the C terminus of the protein, producing GP1 and GP2 subunits. The cleaved glycoprotein then binds Niemann-Pick C1 protein, catalyzing fusion of the viral and endosomal membranes. Niemann-Pick C1 is a multiple membrane-spanning protein that resides in the late endosomes and lysosomes and participates in the transport of lysosomal cholesterol to the ER and other cellular sites. Individuals with Niemann-Pick type C1 disease lack the protein and consequently have defects in cholesterol transport; fibroblasts from these patients are resistant to Ebola virus infection (Fig. 5.16).

The binding site on the viral glycoprotein for Niemann-Pick C1 protein is located beneath the heavily glycosylated mucin and glycan cap of the protein, explaining why proteolytic removal of these sequences is needed for binding of viral GP. These observations demonstrate that Niemann-Pick C1 is an intracellular receptor for Ebola virus that promotes a late step in viral entry. It is believed that this receptor assists in dissociating GP1 and GP2, allowing conformational rearrangements needed for membrane fusion.

Release of Viral Ribonucleoprotein

The genomes of many enveloped RNA viruses are present as vRNP in the virus particle. One mechanism for release of

Figure 5.16 Entry of Ebolavirus into cells. Virus particles bind cells via an unidentified attachment receptor and enter by endocytosis. The mucin and glycan cap on the viral glycoprotein is removed by cellular cysteine proteases, exposing binding sites for NPC1. The latter is required for fusion of the viral and cell membranes, releasing the nucleocapsid into the cytoplasm. Courtesy of Kartik Chandran, Albert Einstein College of Medicine.



vRNP during virus entry has been identified by studies of influenza virus. Each influenza virus vRNP is composed of a segment of the RNA genome bound by nucleoprotein (NP) molecules and the viral RNA polymerase. This structure interacts with viral M1 protein, an abundant protein in virus particles that underlies the envelope and provides rigidity (Fig. 5.12). The M1 protein also contacts the internal tails of HA and neuraminidase proteins in the viral envelope. This arrangement presents two problems. Unless M1-vRNP interactions are disrupted, vRNPs might not be released into the cytoplasm. Furthermore, the vRNPs must enter the nucleus, where mRNA synthesis takes place. However, vRNP cannot enter the nucleus if M1 protein remains bound, because this protein masks a nuclear localization signal (see "Import of Influenza Virus Ribonucleoprotein" below).

The influenza virus M2 protein, the first viral protein discovered to be an ion channel, provides the solution to both problems. The envelope of the virus particle contains a small number of molecules of M2 protein, which form a homotetramer. When purified M2 was reconstituted into synthetic lipid bilayers, ion channel activity was observed, indicating that this property requires only M2 protein. The M2 protein channel is structurally much simpler than other ion channels and is the smallest channel discovered to date.

The M2 ion channel is activated by the low pH of the endosome before HA-catalyzed membrane fusion occurs. As a result, protons enter the interior of the virus particle. It has been suggested that the reduced pH of the particle interior leads to conformational changes in the M1 protein, thereby disrupting M1-vRNP interactions. When fusion between the viral envelope and the endosomal membrane takes place, vRNPs are released into the cytoplasm free of M1 and can then be imported into the nucleus (Fig. 5.12). Support for this model comes from studies with the anti-influenza virus drug amantadine, which specifically inhibits M2 ion channel activity (Volume II, Fig. 9.11). In the presence of this drug, influenza virus particles can bind to cells, enter endosomes, and undergo HA-mediated membrane fusion, but vRNPs are not released from endosomes.

Uncoating in the Cytoplasm by Ribosomes

Some enveloped RNA-containing viruses, such as Semliki Forest virus, contain nucleocapsids that are disassembled in the cytoplasm by pH-independent mechanisms. The icosahedral nucleocapsid of this virus is composed of a single viral protein, C protein, which encloses the (+) strand viral RNA. This structure is surrounded by an envelope containing viral glycoproteins E1 and E2, which are arranged as heterodimers clustered into groups of three, each cluster forming a spike on the virus surface.

Fusion of the viral and endosomal membranes exposes the nucleocapsid to the cytoplasm (Fig. 5.17). To begin translation

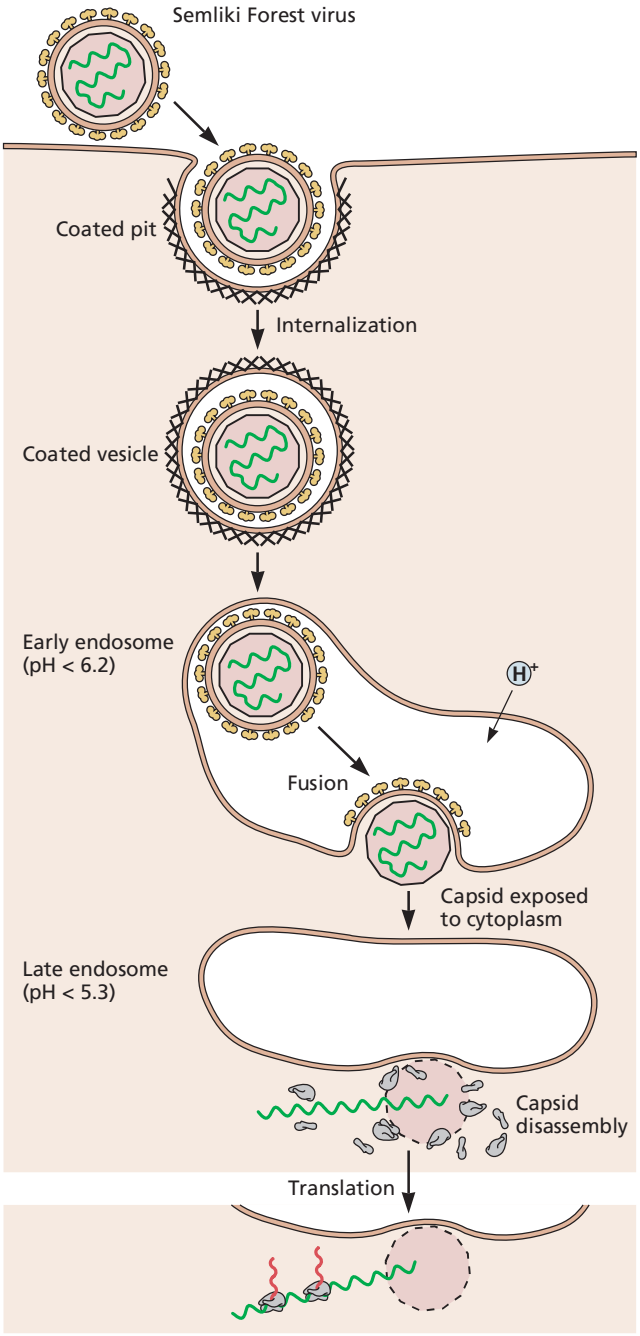


Figure 5.17 Entry of Semliki Forest virus into cells. Semliki Forest virus enters cells by clathrin-dependent receptor-mediated endocytosis, and membrane fusion is catalyzed by acidification of endosomes. Fusion results in exposure of the viral nucleocapsid to the cytoplasm, although the nucleocapsid remains attached to the cytosolic side of the endosome membrane. Cellular ribosomes then bind the capsid, disassembling it and distributing the capsid protein throughout the cytoplasm. The viral RNA is then accessible to ribosomes, which initiate translation. Adapted from M. Marsh and A. Helenius, *Adv. Virus Res.* **36:**107–151, 1989, with permission.

of (+) strand viral RNA, the nucleocapsid must be disassembled, a process mediated by an abundant cellular component—the ribosome. Each ribosome binds three to six molecules of C protein, disrupting the nucleocapsid. This process occurs while the nucleocapsid is attached to the cytoplasmic side of the endosomal membrane (Fig. 5.17) and ultimately results in disassembly. The uncoated viral RNA remains associated with cellular membranes, where translation and replication begin.

Disrupting the Endosomal Membrane

Adenoviruses are composed of a double-stranded DNA genome packaged in an icosahedral capsid (Chapter 4). Internalization of most adenovirus serotypes by receptor-mediated endocytosis requires attachment of fiber to an integrin or Ig-like cell surface receptor and binding of the penton base to a second cell receptor, the cellular vitronectin-binding integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Attachment is mediated by amino acid sequences in each of the five subunits of the adenovirus penton base that mimic the normal ligands of cell surface integrins. As the virus particle is transported via the endosomes from the cell surface toward the nuclear membrane, it undergoes multiple uncoating steps as structural proteins are removed sequentially (Fig. 5.18). As the endosome becomes acidified, the viral capsid is destabilized, leading to release of proteins from the capsid. Among these is protein VI, which causes disruption of the endosomal membrane, thereby delivering the remainder of the particle into the cytoplasm. An N-terminal amphipathic α-helix of protein VI is probably responsible for its pH-dependent membrane disruption activity. This region of the protein appears to be masked in the native capsid by the hexon protein. The liberated subviral particle then docks onto the nuclear pore complex (see "Import of DNA Genomes" below).

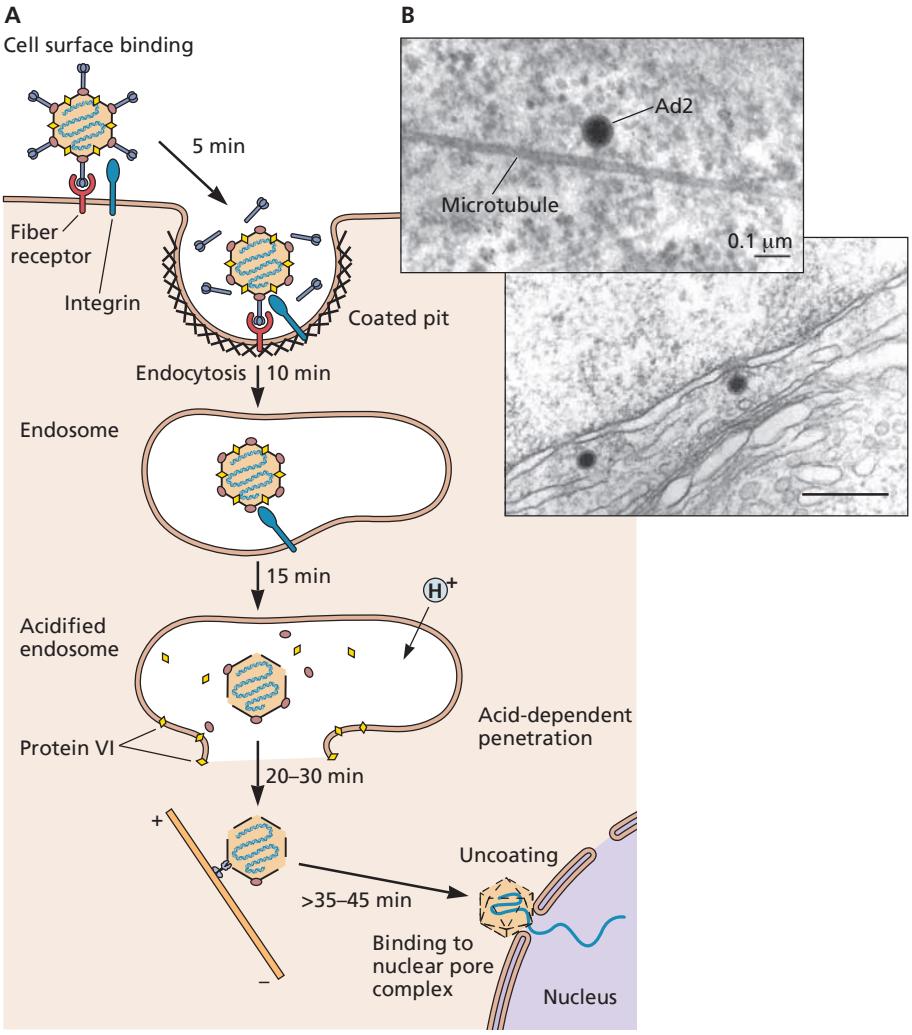


Figure 5.18 Stepwise uncoating of adenovirus. **(A)** Adenoviruses bind the cell receptor via the fiber protein. Interaction of the penton base with an integrin receptor leads to internalization by endocytosis. Fibers are released from the capsid during uptake. Low pH in the endosome causes destabilization of the capsid and release of protein VI (yellow diamonds). The hydrophobic N terminus of protein VI disrupts the endosome membrane, leading to release of a subviral particle into the cytoplasm. The capsid is transported in the cytoplasm along microtubules and docks onto the nuclear pore complex. **(B)** Electron micrograph of adenovirus type 2 particles bound to a microtubule (top) and bound to the cytoplasmic face of the nuclear pore complex (bottom). Bar in bottom panel, 200 nm. (A) Data from U. F. Greber et al., *Cell* **75:**477–486, 1993, and L. C. Trotman et al., *Nat. Cell Biol.* **3:**1092–1100, 2001. (B) Reprinted from U. F. Greber et al., *Trends Microbiol.* **2:**52–56, 1994, with permission. Courtesy of Ari Helenius, Urs Greber, and Paul Webster, University of Zurich.

Forming a Pore in the Endosomal Membrane

The genomes of nonenveloped picornaviruses are transferred across the cell membrane by a different mechanism, as determined by structural information at the atomic level and complementary genetic and biochemical data obtained from studies of cell entry. The interaction of poliovirus with its Ig-like cell receptor, CD155, leads to major conformational rearrangements in the virus particle and the production of an expanded form called an altered (A) particle (Fig. 5.19A). Portions of two capsid proteins, VP1 and VP4, move from the inner surface of the capsid to the exterior. These polypeptides are thought to form a pore in the cell membrane that allows transport of viral RNA into the cytoplasm (Fig. 5.19B). In support of this model, ion channel activity can be detected when A particles are added to lipid bilayers.

The properties of a virus with an amino acid change in VP4 indicate that this protein is required for an early stage of cell entry. Virus particles with such amino acid alterations can bind to target cells and convert to A particles but are blocked at a subsequent, unidentified step. During poliovirus assembly, VP4 and VP2 are part of the precursor VP0, which remains uncleaved until the viral RNA has been encapsidated. The cleavage of VP0 during poliovirus assembly therefore primes the capsid for uncoating by separating VP4 from VP2.

In cells in culture, release of the poliovirus genome occurs from within early endosomes located close (within 100 to 200 nm) to the plasma membrane (Fig. 5.19A). Uncoating is dependent upon actin and tyrosine kinases, possibly for movement of the capsid through the network of actin filaments, but not on dynamin, clathrin, caveolin, or flotillin (a marker protein for clathrin- and caveolin-independent endocytosis), endosome acidification, or microtubules. The trigger for RNA release from early endosomes is not known but is clearly dependent on prior interaction with CD155. This conclusion derives from the finding that antibody-poliovirus complexes can bind to cells that produce Fc receptors but cannot infect them. As the Fc receptor is known to be endocytosed, these results suggest that interaction of poliovirus with CD155 is required to induce conformational changes in the particle that are required for uncoating.

A critical regulator of the receptor-induced structural transitions of poliovirus particles appears to be a hydrophobic tunnel located below the surface of each structural unit (Fig. 5.19C). The tunnel opens at the base of the canyon and extends toward the 5-fold axis of symmetry. In poliovirus type 1, each tunnel is occupied by a molecule of sphingosine. Similar lipids have been observed in the capsids of other picornaviruses. Because of the symmetry of the capsid, each virus particle may contain up to 60 lipid molecules.

The lipids are thought to contribute to the stability of the native virus particle by locking the capsid in a stable conformation. Consequently, removal of the lipid is probably necessary to endow the particle with sufficient flexibility to permit the RNA to leave the shell. These conclusions come from the study of antiviral drugs known as WIN compounds (named after Sterling-Winthrop, the pharmaceutical company at which they were discovered). These compounds displace the lipid and fit tightly in the hydrophobic tunnel (Fig. 5.19C). Polioviruses containing bound WIN compounds can bind to the cell receptor, but A particles are not produced. WIN compounds may therefore inhibit poliovirus infectivity by preventing the receptor-mediated conformational alterations required for uncoating. The properties of poliovirus mutants that cannot replicate in the absence of WIN compounds underscore the role of the lipids in uncoating. These drug-dependent mutants spontaneously convert to altered particles at 37°C, in the absence of the cell receptor, probably because they do not contain lipid in the hydrophobic pocket. The lipids are therefore viewed as switches, because their presence or absence determines whether the virus is stable or will be uncoated. The interaction of the virus particle with its receptor probably initiates structural changes in the virion that lead to the release of lipid. Consistent with this hypothesis is the observation that CD155 docks onto the poliovirus capsid just above the hydrophobic pocket.

It is usually assumed that the 5′-end of (+) strand RNAs is the first to leave the capsid, to allow immediate translation by ribosomes. This assumption is incorrect for rhinovirus type 2: exit of viral RNA starts from the 3′-end. This directionality is a consequence of how the viral RNA is packaged in the virion, with the 3′-end near the location of pore formation in the altered particle. Whether such directionality is a general feature of nonenveloped (+) strand RNA viruses is unknown.

Uncoating in the Lysosome

Most virus particles that enter cells by receptor-mediated endocytosis leave the pathway before the vesicles reach the lysosomal compartment. This departure is not surprising, for lysosomes contain proteases and nucleases that would degrade virus particles. However, these enzymes play an important role during the uncoating of members of the *Reoviridae*.

Orthoreoviruses are naked icosahedral viruses containing a double-stranded RNA genome of 10 segments. The viral capsid is a double-shelled structure composed of eight different proteins. These virus particles bind to cell receptors via protein σ1 and are internalized into cells by endocytosis (Fig. 5.20A). Infection of cells by reoviruses is sensitive to bafilomycin A1, an inhibitor of the endosomal proton pump, indicating that acidification is required for entry. Low pH activates lysosomal proteases, which then modify several capsid proteins, enabling the virus to cross the vesicle membrane. One viral outer capsid protein is cleaved and another is removed from the particle, producing an infectious subviral particle. These particles have the viral μ1 protein, a myristoylated protein

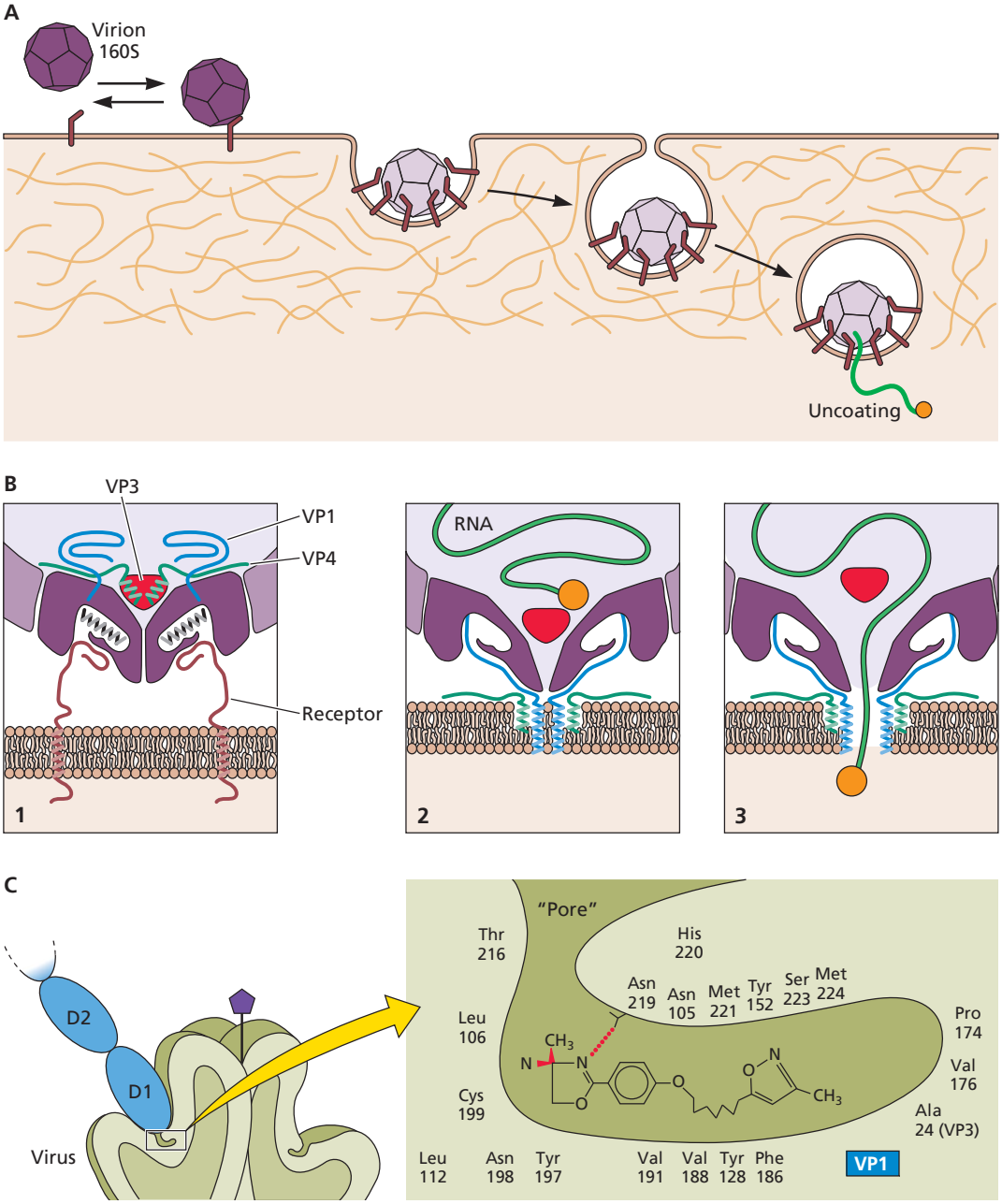


Figure 5.19 Model for poliovirus entry into cells. (A) Overview. The native virion (160S) binds to its cell receptor, CD155, and undergoes a receptor-mediated conformational transition resulting in the formation of altered (A) particles. The viral RNA, shown as a curved green line, leaves the capsid from within early endosomes close to the plasma membrane. **(B)** Model of the formation of a pore in the cell membrane after poliovirus binding. 1, poliovirus (shown in cross section, with capsid proteins purple) binds to CD155 (brown). 2, a conformational change leads to displacement of the pocket lipid (black). The pocket may be occupied by sphingosine in the capsid of poliovirus type 1. The hydrophobic N termini of VP1 (blue) are extruded and insert into the plasma membrane. 3, a pore is formed in the membrane by VP4 and the VP1 N termini, through which the RNA is released from the capsid into the cytosol. **(C)** Schematic diagram of the hydrophobic pocket below the canyon floor. Inset shows a WIN compound in the hydrophobic pocket. Adapted from J. M. Hogle and V. R. Racaniello, p. 71–83, *in* B. L. Semler and E. Wimmer (ed.), *Molecular Biology of Picornaviruses* (ASM Press, Washington, DC, 2002).

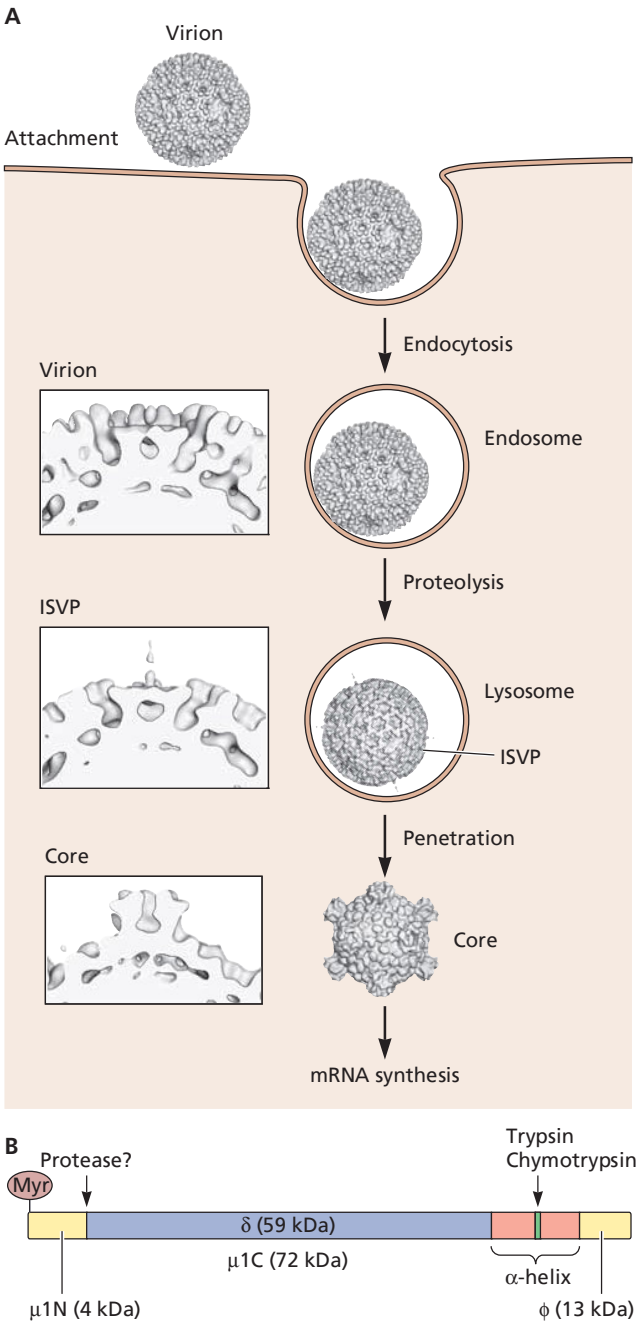


Figure 5.20 Entry of reovirus into cells. (A) The different stages in cell entry of reovirus. After the attachment of σ1 protein to the cell receptor, the virus particle enters the cell by receptor-mediated endocytosis. Proteolysis in the late endosome produces the infectious subviral particle (ISVP), which may then cross the lysosomal membrane and enter the cytoplasm as a core particle. The intact virion is composed of two concentric, icosahedrally organized protein capsids. The outer capsid is made up largely of σ3 and μ1. The dense core shell is formed mainly by λ1 and σ2. In the ISVP, 600 σ3 subunits have been released by proteolysis, and the σ1 protein changes from a compact form to an extended flexible fiber. The μ1 protein, which is thought to mediate interaction of the ISVP with membranes, is present as two cleaved fragments, μ1N and μ1C (see schematic of μ1 in panel B). The N terminus of μ1N is modified with myristate, suggesting that the protein functions in the penetration of membranes. A pair of amphipathic α-helices flank a C-terminal trypsin/chymotrypsin cleavage site at which μ1C is cleaved by lysosomal proteases. Such cleavage may release the helices to facilitate membrane penetration. The membrane-penetrating potential of μ1C in the virion may be masked by σ3; release of the σ3 in ISVPs might then allow μ1C to interact with membranes. The core is produced by the release of 12 σ1 fibers and 600 μ1 subunits. In the transition from ISVP to core, domains of λ2 rotate upward and outward to form a turretlike structure. (Insets) Close-up views of the emerging turretlike structure as the virus progresses through the ISVP and core stages. This structure may facilitate the entry of nucleotides into the core and the exit of newly synthesized viral mRNAs. **(B)** Schematic of the μ1 protein, showing locations of myristate, protease cleavage sites, and amphipathic α-helices. Virus images reprinted from K. A. Dryden et al., *J. Cell Biol.* **122:**1023–1041, 1993, with permission. Courtesy of Norm Olson and Tim Baker, Purdue University.

that interacts with membranes, on the surface. Consequently, subviral particles penetrate the lysosomal membrane and escape into the cytosol. Isolated infectious subviral particles cause cell membranes to become permeable to toxins and produce pores in artificial membranes. These particles can initiate an infection by penetrating the plasma membrane, entering the cytoplasm directly. Their infectivity is not sensitive to bafilomycin A1, further supporting the idea that these particles are primed for membrane entry and do not require further acidification for this process. The core particles generated from infectious subviral particles after penetration into the cytoplasm carry out viral mRNA synthesis.

Movement of Viral and Subviral Particles within Cells

Viral and subviral particles move within the host cell during entry and egress (Chapters 12 and 13). However, movement of molecules larger than 500 kDa does not occur by passive diffusion, because the cytoplasm is crowded with organelles, high concentrations of proteins, and the cytoskeleton. Rather, viral particles and their components are transported via the actin and microtubule cytoskeletons. Such movement can be visualized in live cells by using fluorescently labeled viral proteins (Chapter 2).

The cytoskeleton is a dynamic network of protein filaments that extends throughout the cytoplasm. It is composed of three types of filament—microtubules, intermediate filaments, and microfilaments. Microtubules are organized in a polarized manner, with minus ends situated at the microtubule-organizing center near the nucleus, and plus ends located at the cell periphery. This arrangement permits directed movement of cellular and viral components over long distances. Actin filaments (microfilaments) typically assist in virus movement close to the plasma membrane (Fig. 5.9).

Transport along actin filaments is accomplished by myosin motors, and movement on microtubules is carried out by kinesin and dynein motors. Hydrolysis of adenosine triphosphate (ATP) provides the energy for the motors to move their cargo along cytoskeletal tracks. Dyneins and kinesins participate in movement of viral components during both entry and egress (Chapters 12 and 13). In some cases, the actin cytoskeleton is remodeled during these processes, for example, when viruses bud from the plasma membrane.

There are two basic ways for viral or subviral particles to travel within the cell—within a membrane vesicle such as an endosome, which interacts with the cytoskeletal transport machinery, or directly in the cytoplasm (Fig. 5.9). In the latter case, some form of the virus particle must bind directly to the transport machinery. After leaving endosomes, the subviral particles derived from adenoviruses and parvoviruses are transported along microtubules to the nucleus. Although adenovirus particles have an overall net movement toward the nucleus, they exhibit bidirectional plus- and minus-end-directed microtubule movement. Adenovirus binding to cells activates two different signal transduction pathways that increase the net velocity of minus-end-directed motility. The signaling pathways are therefore required for efficient delivery of the viral genome to the nucleus. Adenovirus subviral particles are loaded onto microtubules through interaction of the capsid protein, hexon, with dynein. The particles move towards the **centrosome** and are then released and dock onto the nuclear pore complex, prior to viral genome entry into the nucleus.

Some virus particles move along the surfaces of cells prior to entry until a clathrin-coated pit is encountered. If the cell receptor is rare or inaccessible, particles may first bind to more abundant or accessible receptors, such as carbohydrates, and then migrate to receptors that allow entry into the cell. For example, after binding, polyomavirus particles move laterally ("surf") on the plasma membrane for 5 to 10 s before they are internalized. They can be visualized moving along the plasma membrane toward the cell body on **filopodia**, thin extensions of the plasma membrane (Fig. 5.11). Movement along filopodia occurs by an actin-dependent mechanism. Filopodial bridges mediate cell-to-cell spread of a retrovirus in cells in culture. The filopodia originate from uninfected cells and contact infected cells with their tips. The interaction of the viral envelope glycoprotein on the surface of infected cells with the receptor on uninfected cells stabilizes the interaction. Particles move along the outside of the filipodial bridge to the uninfected cell. Such transport is a consequence of actin-based movement of the viral receptor toward the uninfected cell.

A number of different viruses enter the peripheral nervous system and spread to the central nervous system via axons. As no viral genome encodes the molecular motors or cytoskeletal structures needed for long-distance axonal transport, viral adapter proteins are required to allow movement within nerves. An example is axonal transport of alphaherpesviruses. After fusion at the plasma membrane, the viral nucleocapsid is carried by retrograde transport to the neuronal cell body. Such transport is accomplished by the interaction of a major component of the tegument, viral protein VP1/2, with minus-end-directed dynein motors. In contrast, other virus particles are carried to the nerve cell body within endocytic vesicles. After endocytosis of poliovirus, virus particles remain attached to the cellular receptor CD155. The cytoplasmic domain of the receptor engages the dynein light chain Tctex-1 to allow retrograde transport of virus-containing vesicles.

Virus-Induced Signaling via Cell Receptors

Binding of virus particles to cell receptors not only concentrates the particles on the cell surface, but also activates signaling pathways that facilitate virus entry and movement within the cell or produce cellular responses that enhance virus propagation and/or affect pathogenesis. Virus binding may lead to activation of protein kinases that trigger cascades of responses at the plasma membrane, cytoplasm, and nucleus (Chapter 14). Second messengers that participate in signaling include phosphatidylinositides, diacylglycerides, and calcium; regulators of membrane trafficking and actin dynamics also contribute to signaling. Virus-receptor interactions also stimulate antiviral responses (Volume II, Chapter 3).

Signaling triggered by binding of coxsackievirus B3 to cells makes receptors accessible for virus entry. The coxsackievirus and adenovirus receptor, Car, is not present on the apical surface of epithelial cells that line the intestinal and respiratory tracts. This membrane protein is a component of tight junctions and is inaccessible to virus particles. Binding of group B coxsackieviruses to its receptor, CD55, which is present on the apical surface, activates Abl kinase, which in turn triggers Rac-dependent actin rearrangements. These changes allow movement of virus particles to the tight junction, where they can bind Car and enter cells.

Signaling is essential for the entry of simian virus 40 into cells. Binding of this virus particle to its glycolipid cell receptor, GM1 ganglioside, causes activation of tyrosine kinases. The signaling that ensues induces reorganization of actin filaments, internalization of the virus in caveolae, and transport of the caveolar vesicles to the endoplasmic reticulum. The activities of more than 50 cellular protein kinases regulate the entry of this virus into cells.

Import of Viral Genomes into the Nucleus

The reproduction of most DNA viruses, and some RNA viruses including retroviruses and influenza viruses, begins in the cell nucleus. The genomes of these viruses must therefore

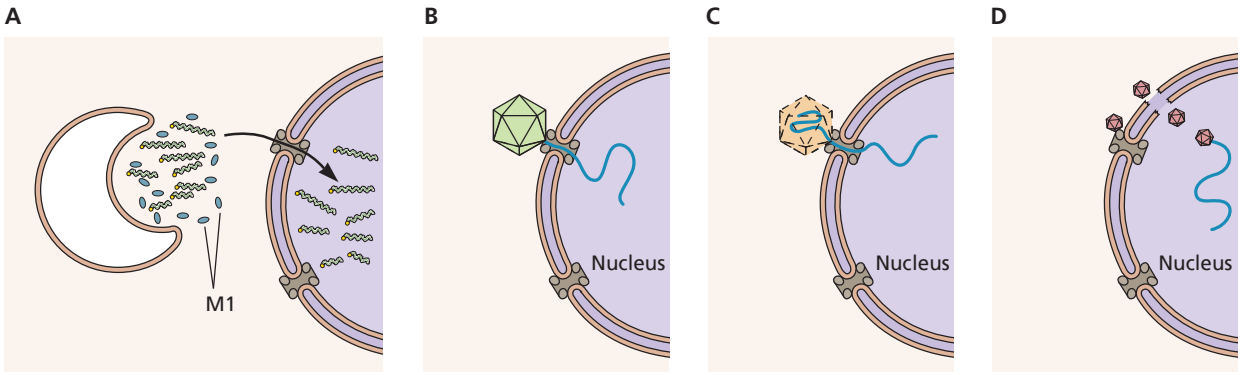


Figure 5.21 Different strategies for entering the nucleus. **(A)** Each segment of the influenza virus genome is small enough to be transported through the pore complex. **(B)** The herpes simplex virus type 1 capsid docks onto the nuclear pore complex and is minimally disassembled to allow transit of the viral DNA into the nucleus. **(C)** The adenovirus subviral particle is dismantled at the nuclear pore, allowing transport of the viral DNA into the nucleus. **(D)** The capsids of some viruses (parvovirus and hepadnavirus) are small enough to enter the nuclear pore complex without disassembly but do not enter by this route. These virus particles bind the nuclear pore complex, which causes disruption of the nuclear envelope followed by nuclear entry.

be imported from the cytoplasm. One way to accomplish this movement is via the cellular pathway for protein import into the nucleus. An alternative, observed in cells infected by some retroviruses, is to enter the nucleus after the nuclear envelope breaks down during cell division. When the nuclear envelope is reformed, the viral DNA is incorporated into the nucleus together with cellular chromatin. This strategy restricts infection to cells that undergo mitosis.

Many subviral particles are too large to pass through the nuclear pore complex. There are several strategies to overcome this limitation (Fig. 5.21). The influenza virus genome, which consists of eight segments that are each small enough to pass through the nuclear pore complex, is uncoated in the cytoplasm. Adenovirus subviral particles dock onto the nuclear pore complex and are disassembled by the import machinery, allowing the viral DNA to pass into the nucleus. Herpes simplex virus capsids also dock onto the nuclear pore but remain largely intact, and the nucleic acid is injected into the nucleus through a portal in the nucleocapsid. The DNA of some bacteriophages is packaged in virus particles at high pressure, which provides sufficient force to insert the viral DNA genome into the bacterial cell. A similar mechanism may allow injection of herpesviral DNA (Box 5.6). This mechanism would overcome the problem that transport through the nuclear pore complex depends upon hydrophobic interactions with nucleoporins: the charged and hydrophilic viral nucleic acids would have difficulty passing through the pore.

Nuclear Localization Signals

Proteins that reside within the nucleus are characterized by the presence of specific nuclear targeting sequences. Such **nuclear localization signals** are both necessary for nuclear entry of the proteins in which they are present and sufficient to direct heterologous, nonnuclear proteins to enter this organelle. Nuclear localization signals identified by these criteria share a number of common properties: they are generally fewer than 20 amino acids in length, and they are usually rich in basic amino acids. Despite these similarities, no consensus nuclear localization sequence can be defined.

Most nuclear localization signals belong to one of two classes, simple or bipartite sequences (Fig. 5.22). A particularly well characterized example of a simple nuclear localization signal is that of simian virus 40 large T antigen, which comprises five contiguous basic residues flanked by hydrophobic amino acids. This sequence is sufficient to relocate normally cytoplasmic proteins to the nucleus. Many other viral and cellular nuclear proteins contain short, basic nuclear localization signals, but these signals are not identical in primary sequence to that of T-antigen. The presence of a nuclear localization signal is all that is needed to target a macromolecular substrate for import into the nucleus.

The Nuclear Pore Complex

The nuclear envelope is composed of two typical lipid bilayers separated by a luminal space (Fig. 5.23). Like all other cellular membranes, it is impermeable to macromolecules such as proteins. However, the nuclear pore complexes that stud the nuclear envelopes of all eukaryotic cells provide aqueous channels that span both the inner and outer nuclear membranes for exchange of small molecules, macromolecules, and macromolecular assemblies between nuclear and cytoplasmic compartments. Numerous experimental techniques, including direct visualization of gold particles attached to proteins or RNA molecules as they are transported, have established that

BOX 5.6

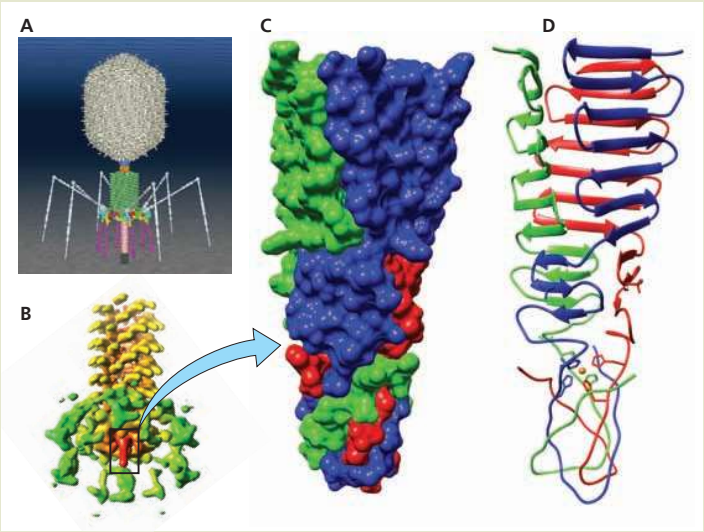
DISCUSSION

The bacteriophage DNA injection machine

The mechanisms by which the bacteriophage genome enters the bacterial host are unlike those for viruses of eukaryotic cells. One major difference is that the bacteriophage particle remains on the surface of the bacterium as the nucleic acid passes into the cell. The DNA genome of some bacteriophages is packaged under high pressure (up to 870 lb/in^2) in the capsid and is injected into the cell in a process that has no counterpart in the entry process of eukaryotic viruses. The complete structure of bacteriophage T4 illustrates this remarkable process (see figure). To initiate infection, the tail fibers attach to receptors on the surface of *Escherichia coli*. Binding causes a conformational change in the baseplate, which leads to contraction of the sheath. This movement drives the rigid tail tube through the outer membrane, using a needle at the tip. When the needle touches the peptidoglycan layer in the periplasm, the needle dissolves and three lysozyme domains in the baseplate are activated. These disrupt the peptidoglycan layer of the bacterium, allowing DNA to enter.

Browning C, Shneider MM, Bowman VD, Schwarzer D, Leiman PG. 2011. Phage pierces the host cell membrane with the iron-loaded spike. *Structure* **20:**236–339.

Structure of bacteriophage T4. (A) A model of the 2,000-Å bacteriophage as produced from electron microscopy and X-ray crystallography. Components of the virion are color coded: virion head (beige), tail tube (pink), contractile sheath around the tail tube (green), baseplate (multicolored), and tail fibers (white and magenta). In the illustration, the virion contacts the cell surface, and the tail sheath is contracted prior to DNA release into the cell. Courtesy of Michael Rossmann, Purdue University. **Structure of bacteriophage membrane-piercing spike. (B)** CryoEM reconstruction of phi92 basplate. The spike is shown in red. **(C, D)** Trimers of bacteriophage phi92 gp138, shown as surface **(C)** and ribbon diagrams **(D)**. From P.G. Leiman et al., *Cell* **118:**419-430, 2004, with permission.



nuclear proteins enter and RNA molecules exit the nucleus by transport through the nuclear pore complex. The functions of the nuclear pore complex in both protein import and RNA export are not completely understood, not least because this important cellular machine is large (molecular mass, approximately 125 × 10^6 kDa in vertebrates), built from many different proteins, and architecturally complex (Fig. 5.23).

The nuclear pore complex allows passage of cargo in and out of the nucleus by either passive diffusion or facilitated translocation. Passive diffusion does not require interaction between the cargo and components of the nuclear pore complex and becomes inefficient as molecules approach 9 nm in diameter. Objects as large as 39 nm in diameter can pass through nuclear pore complexes by facilitated translocation. This process requires specific interactions between the cargo and components of the nuclear pore complex.

The Nuclear Import Pathway

Import of a protein into the nucleus via nuclear localization signals occurs in two distinct, and experimentally separable, steps (Fig. 5.23C). A protein containing such a signal first binds to a soluble cytoplasmic receptor protein. This complex then engages with the cytoplasmic surface of the nuclear pore complex, a reaction often called docking, and is translocated through the nuclear pore complex. In the nucleus, the complex is disassembled, releasing the protein cargo.

Different groups of proteins are imported by specific receptor systems. In what is known as the "classical system" of import, cargo proteins containing basic nuclear localization signals bind to the cytoplasmic nuclear localization signal receptor protein importin-α (Fig. 5.23C). This complex then binds importin-β, which mediates docking with the nuclear pore complex by binding to members of a family of nucleoporins. Some of these nucleoporins are found in the cytoplasmic filaments of the nuclear pore complex (Fig. 5.23), which associate with import substrates. The complex is translocated to the opposite side of the nuclear envelope, where the cargo is released. Other importins can bind cargo proteins directly without the need for an adapter protein.

A single translocation through the nuclear pore complex does not require energy consumption. However, maintenance of a gradient of the guanosine nucleotide-bound forms of Ran,

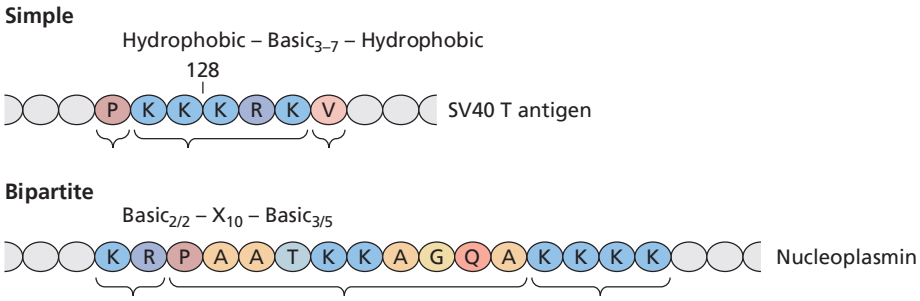


Figure 5.22 Nuclear localization signals. The general form and a specific example of simple and bipartite nuclear localization signals are shown in the one-letter amino acid code, where X is any residue. Bipartite nuclear targeting signals are defined by the presence of two clusters of positively charged amino acids separated by a spacer region of variable sequence. Both clusters of basic residues, which often resemble the simple targeting sequences of proteins like simian virus 40 T antigen, are required for efficient import of the proteins in which they are found. The subscript indicates either length (3–7) or composition (e.g., 3/5 means at least 3 residues out of 5 are basic). Gold particles with diameters as large as 26 nm are readily imported following their microinjection into the cytoplasm, as long as they are coated with proteins or peptides containing a nuclear localization signal.

with Ran-GDP and Ran-GTP concentrated in the cytoplasm and nucleus, respectively, is absolutely essential for continued transport. For example, conversion of Ran-GDP to Ran-GTP in the nucleus, catalyzed by the guanine nucleotide exchange protein Rcc-1, promotes dissociation of imported proteins from importins (Fig. 5.23).

Import of Influenza Virus Ribonucleoprotein

Influenza virus is among the few RNA-containing viruses with genomes that replicate in the cell nucleus. After vRNPs separate from M1 and are released into the cytosol, they are rapidly imported into the nucleus (Fig. 5.12). Such import depends on the presence of a nuclear localization signal in the NP protein, a component of vRNP: naked viral RNA does not dock onto the nuclear pore complex, nor is it taken up into the nucleus.

Import of DNA Genomes

The capsids of many DNA-containing viruses are larger than 39 nm and cannot be imported into the nucleus from the cytoplasm. One mechanism for crossing the nuclear membrane comprises docking of a capsid onto the nuclear pore complex, followed by delivery of the viral DNA into the nucleus. Adenoviral and herpesviral DNAs are transported into the nucleus via this mechanism. However, the strategies for DNA import are distinct: adenovirus DNA is covered with proteins and is recognized by the import system, while HSV DNA is naked and is injected.

Partially disassembled adenovirus capsids dock onto the nuclear pore complex by interaction with Nup214 (Fig. 5.24). Release of the viral genome requires capsid protein binding to kinesin-1, the motor protein that mediates transport on microtubules from the nucleus to the cell periphery. As the capsid is held on the nuclear pore, movement of kinesin-1 towards the plasma membrane is thought to pull apart the capsid. The released protein VII-associated viral DNA is then imported into the nucleus, where viral transcription begins. Herpesvirus capsids also dock onto the nuclear pore complex, and interaction with nucleoporins destabilizes a viral protein, pUL25, which locks the genome inside the capsid. This event causes the naked viral DNA, which is packaged in the nucleocapsid under very high pressure, to exit through the portal.

The 26-nm capsid of parvoviruses is small enough to fit through the nuclear pore (39 nm), and it has been assumed that these virus particles enter by this route. However, there is no experimental evidence that parvovirus capsids pass intact through the nuclear pore. Instead, virus particles bind to the nuclear pore complex, followed by disruption of the nuclear envelope and the nuclear lamina, leading to entry of virus particles (Fig. 5.21). After release from the endoplasmic reticulum, the 45-nm capsid of SV40 also docks onto the nuclear pore, initiating disruption of the nuclear envelope and lamina. Such nuclear disruption appears to involve cell proteins that also participate in the increased nuclear permeability that takes place during mitosis, raising the possibility that nuclear entry of these viral genomes is a consequence of remodeling a cell process.

Import of Retroviral Genomes

Fusion of retroviral and plasma membranes releases the viral core into the cytoplasm. The retroviral core consists of the viral RNA genome, coated with NC protein, and the enzymes reverse transcriptase (RT) and integrase (IN), enclosed by capsid (CA) protein. Retroviral DNA synthesis commences in the cytoplasm, within the nucleocapsid core, and after 4 to 8 h of DNA synthesis the preintegration complex, comprising

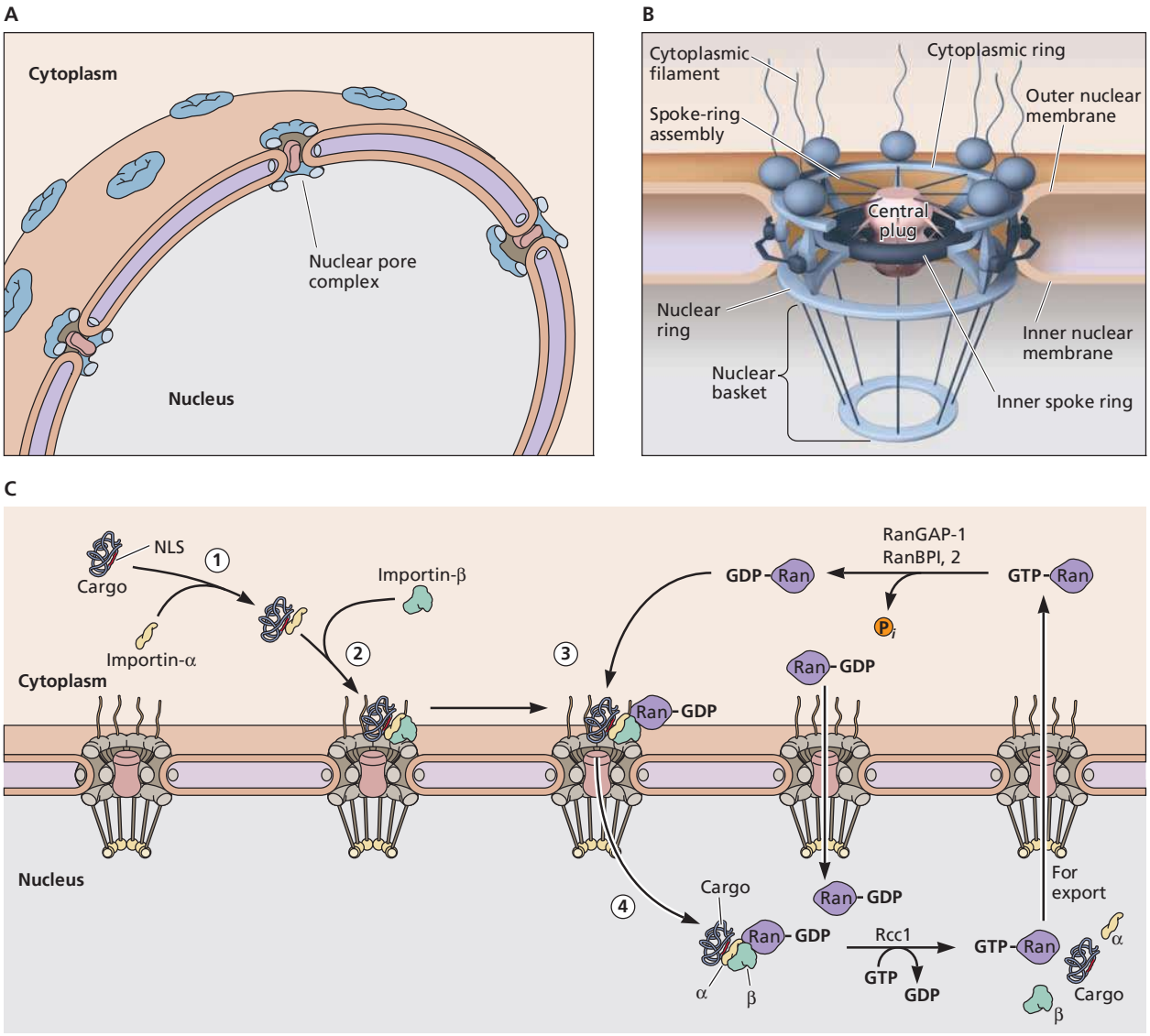


Figure 5.23 Structure and function of the nuclear pore complex. (A) Overview of the nuclear membrane, showing the topology of the nuclear pore complexes. **(B)** Schematic drawing of the nuclear pore complex, showing the spoke-ring assembly at its waist and its attachment to cytoplasmic filaments and the nuclear basket. The latter comprises eight filaments, extending 50 to 100 nm from the central structure and terminating in a distal annulus. The nuclear pore channel is shown containing the transporter. **(C)** An example of the classical protein import pathway for proteins with a simple nuclear localization signal (NLS). This pathway is illustrated schematically from left to right. Cytoplasmic and nuclear compartments are shown separated by the nuclear envelope studded with nuclear pore complexes. In step 1, a nuclear localization signal on the cargo (red) is recognized by importin-α. In step 2, importin-β binds the cargo–importin-α complex and docks onto the nucleus, probably by associating initially with nucleoporins present in the cytoplasmic filaments of the nuclear pore complex. Translocation of the substrate into the nucleus (step 4) requires additional soluble proteins, including the small guanine nucleotide-binding protein Ran (step 3). A Ran-specific guanine nucleotide exchange protein (Rcc1) and a Ran-GTPase-activating protein (RanGap-1) are localized in the nucleus and cytoplasm, respectively. The action of RanGAP-1, with the accessory proteins RanBp1 and RanBp2, maintains cytoplasmic Ran in the GDP-bound form. When Ran is in the GTP-bound form, nuclear import cannot occur. Following import, the complexes are dissociated when Ran-GDP is converted to Ran-GTP by Rcc1. Ran-GTP participates in export from the nucleus. The nuclear pool of Ran-GDP is replenished by the action of the transporter Ntf2/p10, which efficiently transports Ran-GDP from the cytoplasm to the nucleus. Hydrolysis of Ran-GTP in the cytoplasm and GTP-GDP exchange in the nucleus therefore maintain a gradient of Ran-GTP/Ran-GDP. The asymmetric distribution of RanGap-1 and Rcc1 allows for the formation of such a gradient. This gradient provides the driving force and directionality for nuclear transport. A monomeric receptor called transportin mediates the import of heterogeneous nuclear RNA-binding proteins that contain glycine- and arginine-rich nuclear localization signals. Transportin is related to importin-β, as are other monomeric receptors that mediate nuclear import of ribosomal proteins. (B) Adapted from Q. Yang, M. P. Rout, and C. W. Akey, *Mol. Cell* **1:**223–234, 1998, with permission.

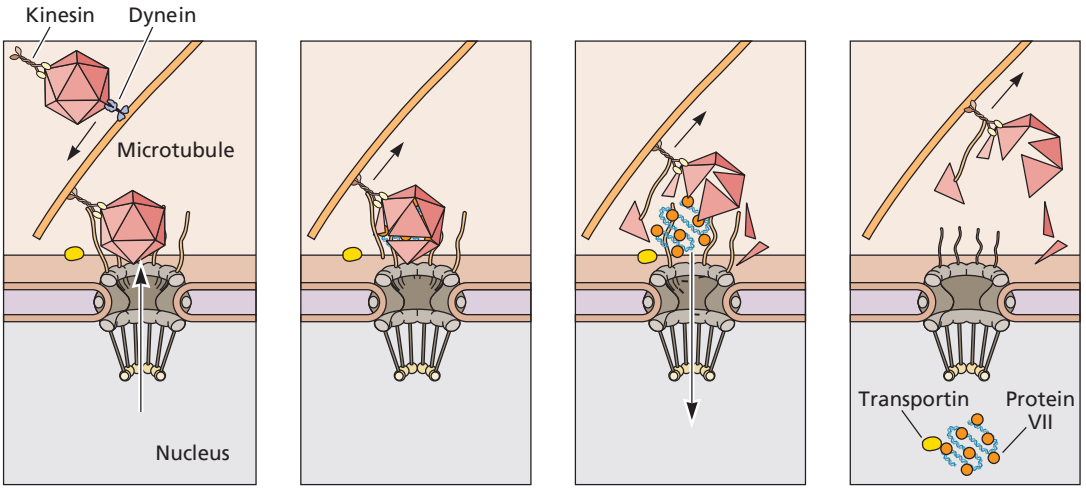


Figure 5.24 Uncoating of adenovirus at the nuclear pore complex. After release from the endosome, the partially disassembled capsid moves towards the nucleus by dynein-driven transport on microtubules. The particle docks onto the nuclear pore complex protein Nup214. The capsid also binds kinesin-1 light chains, which move away from the nucleus, pulling the capsid apart. The viral DNA, bound to protein VII, is delivered into the nucleus by the import protein transportin and other nuclear import proteins.

viral DNA, IN, and other proteins, localizes to the nucleus. There the viral DNA is integrated into the cellular genome, and viral transcription begins. The mechanism of nuclear import of the preintegration complex is poorly understood, but it is quite clear that this structure is too large to pass through the nuclear pore complex. The betaretrovirus Moloney murine leukemia virus can efficiently infect only dividing cells. The viral preintegration complex is tethered to chromatin when the nuclear membrane is broken down during mitosis and remains associated with chromatin as the nuclear membrane reforms in daughter cells, avoiding the need for active transport.

In contrast to Moloney murine leukemia virus, other retroviruses can reproduce in nondividing cells. The preintegration complex of these viruses must therefore be transported into an intact nucleus. The exact mechanism for nuclear entry is still unclear, but for the lentivirus human immunodeficiency virus type 1, there is evidence that CA-mediated attachment of the preintegration complex to the nuclear pore is required. In addition, various viral proteins that contain nuclear localization signals may facilitate the process (e.g., Vpr, MA, and IN).

Perspectives

The study of how viruses enter cells provides critical insight into the very first steps of virus reproduction. Virus entry comprises binding to receptors, transport within the cell, dismantling of the virus particle, and release of the genome. It has become clear that there are many pathways for virus entry into cells. Clathrin- and dynamin-dependent endocytosis is no longer the sole entry pathway known; other routes are caveolin-dependent endocytosis and clathrin- and caveolin-independent endocytosis. The road used seems to depend on the virus, the cell type, and the conditions of infection. As most of our current knowledge has been derived from studies with cells in culture, a crucial unanswered question is whether these same pathways of viral entry are utilized during infection of the heterogeneous tissues of living animals.

Virus binding to the cell surface leads to major alterations in cell activities, effects mediated by signal transduction. These responses include providing access to coreceptors, formation of pits, pinching off of vesicles, and rearrangement of actin filaments to facilitate vesicle movement. The precise signaling pathways required need to be elucidated. Such efforts may identify specific targets for inhibiting virus movement in cells.

The development of single-particle tracking methods has advanced considerably in the past 5 years. As a consequence, our understanding of the routes that viruses travel once they are inside the cell has improved markedly. The role of cellular transport pathways in bringing viral genomes to the site appropriate for their replication within the cell is beginning to be clarified. Yet many questions remain. How are viruses or subviral particles transported on the cytoskeletal network? What are the signals for their attachment and release from microtubules and filaments?

The genomes of many viruses are replicated in the nucleus. Some viral genomes enter this cellular compartment by transport through the nuclear pore complex. Studies of adenoviral DNA import into the nucleus have revealed an active role for components of both the nuclear pore complex and the

microtubule network in subviral particle disassembly. Other viral DNA genomes, such as that of the herpesviruses, pass naked through the nuclear pore, raising the question of how these hydrophilic molecules move through the hydrophobic pore, against a steep gradient of nucleic acid in the nucleus. Nuclear import of retroviral DNA is barely understood. What signal allows transport of the large preintegration complex of retroviruses through the nuclear pore?

Nearly all the principles and specific features discussed in this chapter were derived from studies of viral infection in cultured cells. How viruses attach to and enter cells of a living animal remains an uncharted territory. Methods are being developed to study virus entry in whole animals, and the results will be important for understanding how viruses spread and breach host defenses to reach target cells.

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