General Introduction

Value of Studying Virus Entry into Host Cells

- Increase in world population, explosion in international trade and travel, global warming and other factors led to an increased threat from infectious pathogens, including viruses -> information needed in battle against viruses
- Viruses as tools in molecular medicine, e.g. in gene therapy, delivery of drugs and targeted elimination of cancer cells
- Tools and model system in discovery of new concepts

How do Viruses Use Host Cell Machinery for Replication?

Viruses -> masters of camouflage and deception. Rodents, insects, migratory birds and global trade and travel -> viruses move around the world very fast. In the host -> penetration of mucus layers -> blood stream -> disperse with the help of motile cells and neuronal pathways.

Replication within living cells -> cellular machinery used for synthesis of genome and other components. Critical stage: Attachment, capsid and accessory proteins need to be transferred into the cell causing little damage and not leaving evidence. Viruses take advantage of endocytic pathways -> entry and uncoating done by the host cell. Successful internalization -> intracellular transport -> uncoating.

Viruses Use Cellular Membrane Trafficking Pathways for Infection

Transfer of viral genome and accessory proteins from an infected to a noninfected cell:

- Packaging viral genome (DNA or RNA) and accessory proteins
- Releasing package
- Protecting essential components during extracellular transmission
- Delivery to new host cell

DNA viruses need to enter the nucleus, whereas RNA viruses can replicate in the cytosol. Viruses use a **Trojan horse** strategy, the victim assists the intruder. Viruses use insider information that they have acquired during millions of years of coevolution with their hosts. In a typical animal virus particle, viral RNA or DNA is condensed in icosahedral or helical **capsids**. In **enveloped** viruses, the capsids are surrounded by a **lipid bilayer** that contains viral **spike glycoproteins**. Some viruses contain **reverse transcriptases**, **RNA polymerases**, **kinases** and other proteins that are important during uncoating, replication and other early intracellular steps.

To infect a target cell, a virus particle proceeds through a multistep entry process, where each step is tightly regulated in time and space. Progress through entry and uncoating depends on cues:

- Interaction with cell surface receptors
- Exposure to low pH
- Reimmersion into a reducing environment

-> Conformational changes and dissociation events. Some of the viral component proteins therefore occur in metastable and easily modified conformational states. When triggered by a cue, the metastable state can be relaxed to allow marked changes in viral properties without the input of external energy.

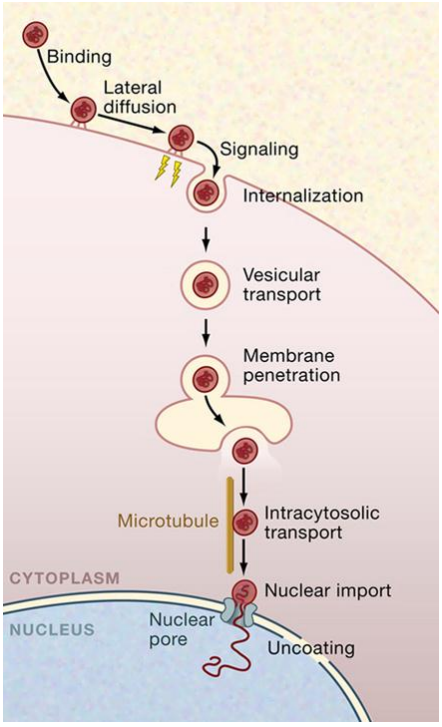


Figure 1: Steps in endocytic virus entry. Virus binding to attachment factors and receptors on the cell surface, followed by lateral movement of the virus-receptor complexes and the induction of signals that result in the endocytic internalization. After vesicular trafficking and delivery into the lumen of endosomes, caveosomes or the ER, a change in the virus conformation is induced by cellular cues. Penetration of the virus or its capsid through the vacuole membrane into the cytosolic compartment. Enveloped viruses use membrane fusion for penetration, nonenveloped induce lysis or pore formation. After targeting and transport along microtubules, the virus or the capsid binds to the nuclear pore complex, undergoes final conversion and releases the viral genome into the nucleus.

What is the Cellular Function of Membrane Trafficking Pathways?

Membrane trafficking functions:

- Uptake of molecules from the extracellular environment
- Secretion of molecules to the extracellular milieu
- Transfer of molecules from one compartment to another

-> Membrane enclosed carriers, highly dynamic, regulated, and interconnected.

Endocytosis: Endocytic cargo molecules internalized into vesicles from the plasma membrane through:

- Clathrin-mediated endocytosis (CME)
- Macropinocytosis
- Raft mediated endocytosis
- Non-clathrin dependent pathways (poorly understood)

Primary destination -> tubular shaped **early** (or sorting) **endosomes** (**EE**). Majority of material is recycled back to the plasma membrane either directly from the sorting endosomes (fast recycling) or through an intermediate compartment, the **recycling endosome** (**RE**, slow recycling). The mildly acidic pH of endosomes allows the disassociation of some receptor-ligand complexes, thereby allowing the nonligand bound receptors to undergo another round of binding after returning to the plasma membrane.

Cargo that is not recycled enters the **degradative** or **endolysosomal pathway**. Fusion and fission events and maturation processes of **multivesicular bodies** (**MVB**) are needed:

- Early to late endosome maturation
- Rab5 to Rab7 conversion
- Phosphoinositide conversion
- pH decrease
- Attachment to motor proteins
- Microtubule dependent transport of vesicles to perinuclear region (accumulation of **lysosomes**)

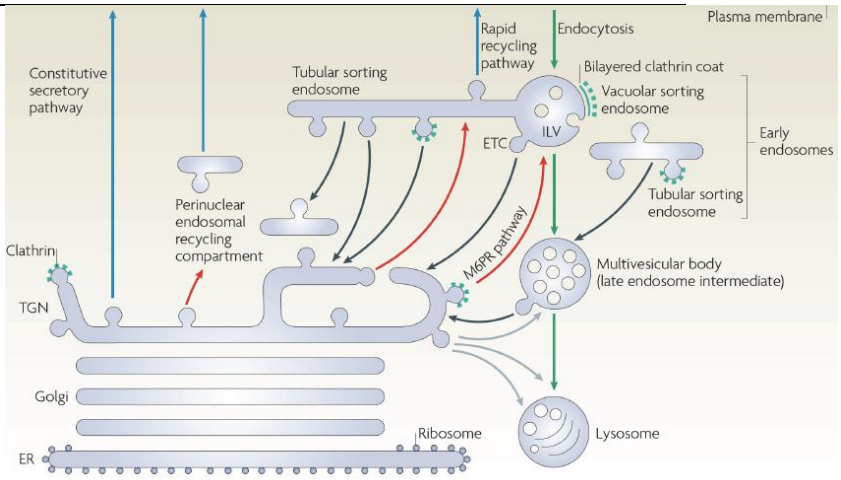


Figure 2: Membrane trafficking pathways, constitutive recycling and degradative pathway. Newly synthesized proteins from ER to Golgi, then plasma membrane. Endocytic and secretory pathway -> highly interconnected system!

Integral membrane proteins destined for degradation have to be actively sorted at the level of sorting endosomes whereas soluble luminal material can freely enter the degradative pathway. Membrane proteins are first incorporated into **intraluminal vesicles** (**ILVs**), which bud into the lumen of endosomes. The subpopulation of such endosomes is called **multivesicular bodies** (**MVBs**). After maturation, MVBs can fuse with lysosomes. ILVs form at the limiting membrane of EEs. The progressive accumulation of ILVs defines the maturation of EEs into MVBs. Mature MVBs are also known as **late endosomes** (**LE**). LE undergo multiple rounds of fusion and fission events with lysosomes, mixing their intraluminal content and allowing lysosomal hydrolases to degrade their cargo. Unlike ILVs and their contents, proteins and lipids at the limiting membrane of lysosomes are resistant to the hydrolases of the lysosomes.

Connection to other pathways is important, e.g. to cargo trafficking to and from the **trans-Golgi network** (**TGN**). Newly synthesized lysosomal hydrolases are recognized by mannose-6-phosphate receptors (M6PRs) in the TGN, which mediate their delivery into lysosomes. A small minority is delivered to the plasma membrane and following endocytosis subsequently transported through the EE and LE on their way to lysosomes. The other and more common route of delivery is direct intracellular transport to the endolysosomal system. It is generally believed that cargo can enter the endolysosomal system both at the EE and LE phases of the pathway. Recycling of M6PRs back to the TGN occurs at the level of maturing endosomes and is mediated by a group of proteins that form the so-called retromer complex.

Another pathway is **autophagy**. Autophagocytosis is a self-degradative process by which cytosolic molecules and organelles are enwrapped by membrane extensions, which ultimately enclose portions of the cytoplasm, thereby forming vacuolar organelles termed **autophagosomes**. Autophagosomes fuse with endosomes in the degradative pathway and thus deliver their contents to the lysosomes for degradation.

Chapter 1: Exploiting Endocytosis for Virus Entry and Infection

Introduction

Bacteriophages: Hypodermic syringes, contractile tail as DNA delivery apparatus, pierce through the two membranes of gram-negative bacteria and inject DNA into the cytosol. Cue that triggers this process comes from attachment to host cell receptors.

Animal viruses are not that elaborate, as they don't have to overcome a cell wall and an outer membrane. Moreover, animal cells provide endocytic mechanisms that provide advantages for viruses.

Transport in vesicles is advantageous:

- Vesicles ferry incoming viruses from the periphery to the perinuclear area of the host cell -> conditions for infection favorable, distance to nucleus minimal
- Bypass obstacles (cytoplasmic crowding, meshwork of microfilaments)
- Neurons: Long distances from axon to cell body
- Sensing location (decreasing pH, change in redox environment)
- Set timing of penetration and uncoating
- Specific proteases (furin, cathepsins) provide proteolytic activation of viruses
- Avoidance of leaving any evidence (delay in detection by immunosurveillance)

Even if viruses can infect cells by other pathways, endocytosis is often preferred because of advantages!

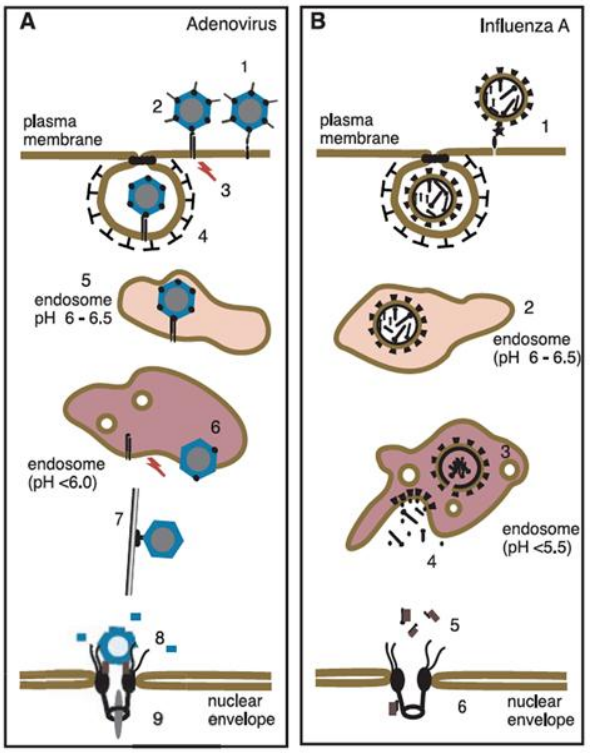


Figure 3: Entry of adenovirus 2: Fibers bind to the CAR (1); fibers dissociate, pentons expose RGD sequences, bind to integrins (2); clustering integrins activate phosphatidylinositol-3-OH kinase, Rac, and cdc42 -> cytoskeleton rearrangements (3). Virus-integrin complex internalizes in clathrin-coated vesicles (4); transport to EE (5), pH of 6 triggers conformational change of the penton base, virus escapes into cytosol (endosomal lysis) (6). Virus particles bind dynein, transport along MT to NPC (7). Capsids dock to CAN/Nup214, disassemble (8); release viral DNA for transport through the pore (9). **Entry of influenza A**: Viral HA binds to sialic acid-containing glycoproteins or glycolipids (1); viruses internalized by clathrin-coated pits are transported by way of the EE to LE (2); low pH activates the M2 protein ion channel in the viral membrane, allowing the internal capsid to be acidified (3); HA-mediated fusion occurs between the viral envelope and the endosomal membrane triggered by low pH (5.5) (4). Viral ribonucleoproteins (RNPs) separate from each other, bind importin β and move through the NPC (5) and into the nucleus (6).

Anvantages of Endocytosis for Virus Entry

Adenovirus 2 and influenza A both use the endocytic entry pathway. **Adenoviruses** are non-enveloped DNA viruses with an icosahedral capsid composed of hexon proteins, penton base complexes, and homotrimeric fibers. **Influenza A** is an enveloped negative-stranded RNA virus of the myxo-virus family. It attaches to cell surface glycoproteins bearing **sialic acid residues**. These seem to act as attachment factors, and

internalization requires an active signal (receptors not yet identified).

Advantages using endocytic pathways:

- Free ride into the cytoplasm (vesicle are designed to traverse the barriers imposed by the cytoskeleton and highly structured cytoplasm)
- Entering endosomal structures, lysosomes, ER and occasionally the Golgi
- Exposure to compartmental environments, differ from extracellular milieu, mildly acidic pH (cue triggering penetration and uncoating)
- Penetration from intracellular vacuoles -> no viral glycoproteins exposed on the cell surface -> no immune detection (influenza A)
- Lytic penetration (adenoviruses) -> Less damaging to the cell than lysis of plasma membrane

Risk:

- Possible delivery to the lysosome -> therefore activation pH matching EE (pH 6-6.5) or LE (5-6)

Experiment: pH-dependency influenza A vs. **Semliki-forest virus** (**SFV**), an enveloped positive-stranded RNA toga alphavirus -> constitutively inactive mutant of rab5 (EE) blocked entry of both SFV (metastable conversion triggered at pH 6.2) and influenza (metastable conversion triggered at pH 5.4), whereas the corresponding rab7 mutant (LE) only blocked influenza virus entry.

Using real-time video microscopy, fluorescent virus particle can be observed binding to the cell surface -> diffuse along the membrane -> get trapped in coated pits or caveolae -> enter by endocytosis -> move along MT etc.

Viruses as Endocytic Cargo

- Size of animal viruses: 30 nm (parvoviruses) to 400 nm (poxviruses), most viruses 60-150 nm
- Roughly spherical shape, some fibrous and highly elongated (influenza)
- Viruses not deformed by host cell binding, rather plasma membrane (invagination)
- Surface covered by receptor-binding proteins, as **capsid proteins** in an icosahedral grid or **spike glycoproteins** covering the viral envelope
- Contact with many receptors -> high avidity and binding virtually irreversible
- Multivalent binding -> receptor clustering -> association with lipid domains -> signaling
- Once delivered, virus particles similar size to ILVs, localized to bulbous, vacuolar domains of endosomes, sorted to degradative pathway

Viruses are useful tools used as model cargo in endocytosis and membrane trafficking studies:

- Easily recognizable by EM

- Tagging with fluorescent groups of proteins (single particle detection, live cell tracking)
- Amplification by infection -> quantification of successful entry
- Virus mutants, fluorescent viruses, antibodies, expression systems, modified host cells…

Penetration: Fusion vs. Lysis/Pore Formation

Enveloped viruses use **membrane fusion** for penetration:

- Catalyzed by fusion proteins in the viral envelope (metastable state conversion)
- Simple machinery, viral fusion factors used only once
- Receptor binding or low pH -> irreversible conformational change -> triggers fusion activity
- Elegant and effective way for delivering capsids into cytosol
- No need for macromolecules to cross hydrophobic membrane barriers
- Viral envelope = transport vesicle; capsid = cargo

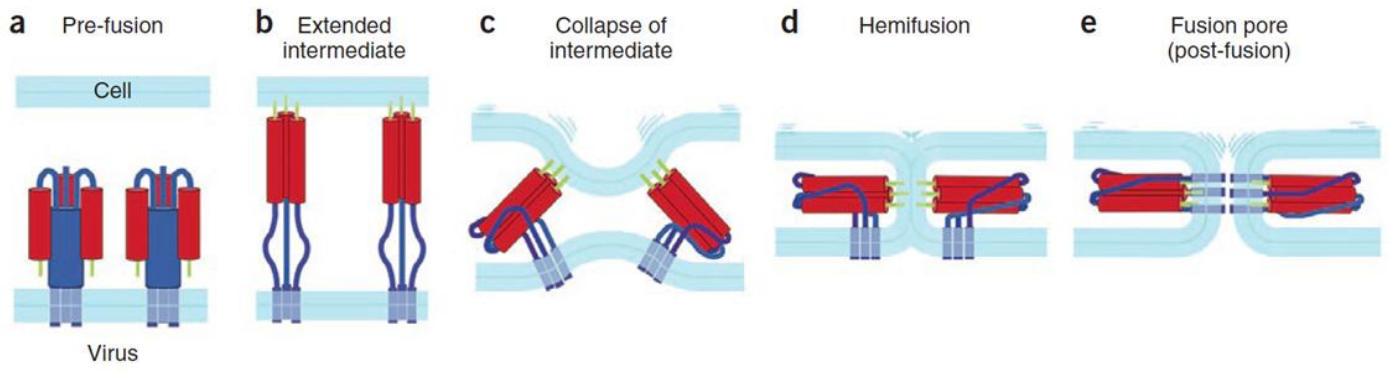


Figure 4: Membrane fusion promoted by a viral fusion protein: Protein in the pre-fusion conformation, with its fusion peptide or loop sequestered (a). Extended intermediate, the protein opens up, extending the fusion peptide or loop to interact with the target bilayer, the part of the protein that bears the fusion peptide forms a trimer cluster (b). Collapse of the extended intermediate. A C-terminal segment of the protein folds back along the outside of the trimer core, the segments from the three subunits fold back independently, so that at any point in the process they can extend to different distances along the trimer axis, and the entire trimer can bow outward, away from the deforming membrane (c). Hemifusion. When collapse of the intermediate has proceeded far enough to bring the two bilayers into contact, the apposed, proximal leaflets merge into a hemifusion stalk (d). Fusion pore formation. As the hemifused bilayers open into a fusion pore, the final zipping up of the C-terminal ectodomain segments snaps the refolded trimer into its fully symmetric, post-fusion conformation, preventing the pore from resealing.

Fusion factors are divided into two main classes:

- Type I fusion proteins: Homotrimeric spike glycoproteins, subunits joined by long coiled coils. They are membrane proteins, synthesized as precursor proteins, folded, and assembled into oligomers in the ER. When going through the secretory pathway, they undergo postsynthetic proteolytic cleavages -> they get conformationally metastable and fusion competent, cooperative conversion into lower energy conformation possible. When triggered, resulting conformation exposes hydrophobic fusion sequences, that insert into the target membrane, and

the released free energy is used to force the membranes closer together in a focal site, resulting in fusion (ex. influenza A).

- Type II fusion proteins: Carry internal fusion sequences, are synthesized and assembled as heterodimers with another membrane protein. Low pH triggers a change in the quarternary structure, and the fusion subunits dissociate from their partners and join together as active homotrimers (ex. alpha viruses, SFV).

Because nonenveloped viruses do not have a membrane, they penetrate either by lysis or by creating a pore-like structure into the membrane. Penetration of nonenveloped viruses also involves cooperative changes in virus particles triggered by receptor binding, low pH or changes in the reducing environment. The viruses become more hydrophobic and interact with membranes directly. Details remain obscure.

In adenoviruses, the penton base becomes lytic at low pH, and the virus is released from ruptured endosomes intact with the rest of endosomal content. The case of pre formation is best studied with **enterovirus** of the picornaviruses family (**PV**), which are nonenveloped, positive-stranded RNA viruses with an icosahedral capsid. The cellular receptor PVR (CD155) binds to a structural canyon on the surface of the virus capsid, leading to loss of the viral protein VP4, and exposure of the myristic acid group at the N-terminus of viral protein VP1. The virus is thought to sink into the bilayer and form a protein-lined channel through which the viral RNA can enter the cytosol.

Intracellular Transport

To move inside the cell, incoming viruses often exploit the cytoskeleton and cellular motor proteins, using two main ways:

- Allow endocytic vesicles to ferry the virus as passive luminal cargo
- Penetrated capsid itself can interact with the relevant motors (capsid protein binds and interacts with cellular factors)

-> Minus-end-directed microtubule-dependent motor dynein and dynactin (adaptor-protein) needed.

Actin can play a role during virus entry. The cortical cytoskeleton poses a barrier against the inward movement of capsids and viruses because of the high content of actin filaments. To overcome this barrier, viruses can activate tyrosine-kinase-induced signaling cascades that lead to the local dissociation of filamentous actin.

Signaling during Virus Entry

Two-way dialogue between cell and virus, signals induce changes that facilitate entry, invasion and neutralize host defense. Signals are usually generated at the cell surface through the virus binding to receptors that are signaling molecules or modulators and can be activated by virus binding (or virus-induced clustering). Adenovirus C members base their entry strategy entirely on signaling:

- Clustering entry receptors
- Activation of a variety of protein kinases, phosphatidylinositol-3-OH kinase
- Major changes in cell surface dynamics and microtubule-mediated transport
- Internalization, penetration and intracellular traffic of virus promoted (adenovirus 2)

Simian Virus-40 (SV40), polyomavirus family, activates its own internalization by activating tyrosine kinases in lipid rafts -> lipid raft endocytosis (normally dormant, ligand-inducible pathway).

Nuclear Import

Nucleus: DNA and RNA polymerases, RNA-splicing and modifying enzymes, perfect for virus. However, nucleus is difficult to enter and exit. In interphase cells, viral capsids enter via NPCs. For targeting, viruses use nuclear localization signals and cytosolic import receptors (ex. adenovirus binds importin 7, influenza bind importins α and β). Limit of diameter is 39nm, so smallest viral capsids (18-24 nm) and others (36nm) as well as helical and extended capsids can easily travel. Larger viruses and capsids must be deformed or disassembled. **Adenovirus 2** binds NUP214/CAN (base of filaments extending into the cytosol from NPC). Interaction of virus with histone H1 and importins β and 7 induces disassembly of the virus capsid. Viral DNA is liberated and imported. **Retroviruses** do not use the NPCs (except lentivirus class, e.g. HIV-1). Preintegration complexes can only enter the nucleus during mitosis when the nuclear envelope is temporarily absent, limiting their infectivity to dividing cells. Probably, three viral proteins are important for nuclear uptake of lentivirus preintegration complexes:

- Matrix protein
- Integrase enzyme
- Small accessory protein vpr
- Short triple-stranded overlap in the provirus DNA may be essential

Alternatives to Endocytosis: Cell-to-Cell Transfer with and without Infection

Infection can be transmitted from cell to cell directly. Extracellular **vaccinia virus**, a large enveloped double-stranded DNA virus belonging to the poxvirus family is practically pushed into an adjacent cell by

localized actin polymerization on the inside of the infected cell. Actin polymerization is triggered by a viral protein recruiting WASP, Arp2/3 and other cellular factors.

HIV-1 and other lentiviruses bud into endosome-like vacuoles. These particles may be directly transmitted from a macrophage to a T cell as part of the normal cell-to-cell interaction. There is also evidence that dendritic cells, without getting infected, may concentrate HIV-1 in regions of cell-to-cell contact and thus promote infection.

Animal viruses use the endomembrane system of mammalian cells for infection

Primary pathway	Virus family (DNA/RNA)	Replication nucleus or cytoplasm	Example virus envelope (±)	Low pH ± dependency	Receptor molecules	Comments
Viruses used to illistrate multiple aspects of vesicular trafficking in Modue II cellular infection						
CME	Alpha (ssRNA+)	Cyto	Semliki Forest (+)	+	GAGs (?)	**Example** for CCP formation, edosome maturation and Type II fusion; Induces CCPs de novo; transported to EEA1/Rab5 peripheral endosomes; fuses 5–8 min after internalization
CME	Rhabdo (ssRNA−)	Cyto	Vesicular stomatitis (+)	+ (<6.4)	?	**Example** for CCP, endosome maturation; Induces CCPs de novo; role of AP2 controversial; can fuse at EE; suggested fusion with ILVs
CME	Adeno (dsDNA)	Nuc	Adenovirus 2 and 5 (−)	+ (<6.0)	CAR, αV, integrins	**Example** for signaling during entry and penetration by membrane lysis; Uptake into CCVs in parallel with induction of macropinocytosis (required for infection); penetration in EE
Macro	Adeno (dsDNA)	Nuc	Adenovirus 3 (−)	+ (<6.0)	CD46, αV, integrins	**Example** for signaling during entry and macropinocytosis; Entry requires PAK1, CtBP1; cellular factors consistent with macropinocytosis
Macro	Pox (dsDNA)	Cyto	Vaccinia MV (+)	± (<5.0)	GAGs?	**Example** for signaling during entry, macropinocytosis and cell to cell spread; Entry linked to membrane blebbing; apoptotic mimicry strategy; cellular requirements vary with strain
Macro	Retro (ssRNA+)	Nuc	HIV-1 (+)	+	CD4, CCR5, CXCR4, CCR2	**Example** for attachment factors and receptors; and Cell type specific; Na+/H+ dependent; virions colocalize with fluid markers; needs further experimentation
CME/Novel pathway	Myxo (ssRNA−)	Nuc	Influenza A (+)	+ (<5.6)	Sialo-glycoproteins	**Example** for multiple entry pathways, endosome maturation and penetration by fusion; About 50% of particles or less use CME; induces CCPs de novo; epsin dependent, fuses at LEs. The rest enter clathrin independently
CAV1/Lipid raft	Polyoma (dsDNA)	Nuc	Simian virus 40 (−)	+	GM1	**Example** for lipid receptor usage, signaling during entry, CAV pathway, pentration by novel mechanism from ER; Parallel CAV1-dependent and –independent entry; and –independent entry; transport via EE and LE to ER where penetration occurs
Lipid raft	Polyoma (dsDNA)	Nuc	Mouse polyomavirus (−)	+	GD1a	**Example** for single particle studies, lipid receptor usage, lipid raft entry; Transport via EE and RE to ER, where penetration occurs
Viruses used to illistrate a single aspect of vesicular trafficking in Module II cellular infection						
CME	Flavi (ssRNA+)	Cyto	Dengue (+)	+	GAGs, DC-SIGN	**Example** for single particle studies of virus fusion in endosomes; Uses preformed CCPs; fuses at LE; entry similar to West Nile virus; in Vero cells clathrin, CAV1, and actin independent; dynamin dependent
Lipid raft (?)	Picorna (ssRNA+) Entro	Cyto	PV (−)	-	PVR (CD155)	**Example** for channel formation for membrane penetration; cellular receptor binds in virus surface sturctural canyon;
Macro	Picorna (ssRNA+)	Cyto	Coxsackievirus B (+)	±	CAR	**Example** for receptor shuffle for intracellualr access; Enters at tight junctions; does not require Rac1; requires Rab34
Novel pathway	Papilloma (dsDNA)	Nuc	Human papillomavirus type 16 (−)	+	GAGs (?)	**Example** for novel endocytosis pathway. Noncoated vesicles; clathrin, CAV1, flotillin, lipid-raft, and dynamin independent; escape likely in LE/lysosomes
Novel pathway	Reo (dsRNA)	Cyto	Rhesus rotavirus (−)	–	Sialic acid, integrins, Hsc70	**Example** for novel endocytosis pathway. Clathrin and CAV1 independent; cholesterol and dynamin dependent

Figure 5: Basic information about each virus and details of host cell entry of these viruses (Part A). Selected features illustrated by characteristic viruses (Part B). Know process associated with the virus, detailed knowledge of these 5 viruses or other aspects of their entry is not expected.

Chapter 2: Attachment Factors and Receptors

Introduction

Infection needs attachment of virus to the cell surface, binding a diverse collection of cellular proteins, carbohydrates and lipids, different from virus to virus, from abundant and ubiquitous to rare and cell specific. Some proteins serve as attachment factors concentrating the virus at the surface, others are true receptors (endocytic pathway induction, signaling). Receptors can also serve as cues inducing conformational changes leading to membrane fusion and penetration. Attachment factors and receptors determine which cell types, tissues and organisms a virus can infect.

Functional Difference between Receptors and Attachment Factors

Attachment factors bind viruses and help to concentrate them on the cell surface. **Virus receptors** additionally serve to trigger changes in the virus, induce cellular signaling or trigger penetration. Many receptors promote endocytosis and accompany the virus into the cell. Binding to attachment factors -> associations with one or more receptors. In practice, these are difficult to differentiate (both contribute to effectiveness of infection).

Most common attachment factors are **glycosaminoglycan chains** (**GAG**), especially heparan sulphate, in proteoglycans. Binding to these negatively charged polysaccharides is electrostatic and nonspecific. Viruses evolve to use GAGs when adapting to growth in tissue culture. **Sialic acid** constitute another common group of carbohydrates viruses can bind to (polyomavirus, influenza), often highly specific, involving lectin domains or sites.

Binding specificity:

- Major factor in determining tropism and species specificity and thus the nature of viral diseases
- Determines the choice of endocytic pathway:
    - Ex. Transferrin binding virus, uptake via clathrin-mediated endocytosis, recycling to cell surface with its receptor
    - Ex. LDL binding virus, dissociation from receptor in EE, transport to LE

Some viruses use multiple attachment factors and receptors (in parallel or succession). **HIV-1** initially contacts cell surface attachment factors such as mannose binding C-type lectin receptor family members, the dendritic cell-specific intracellular adhesion molecule (ICAM)-3-grabbing nonintegrin (**DC-SIGN**) or the liver and lymphnode specific ICAM3-grabbing nonintegrin (**L-SIGN**). These bindings do not induce conformational changes. When glycoprotein 120 (**gp120**) subunit of the virus envelope binds to the outermost immunoglobulin G domain of **CD4**, it undergoes a conformational change that allows the

virus to associate with its co-receptors, the chemokine receptors **CXCR4** or **CCR5**. The interaction between gp120 and CXCR4 or CCR5 triggers the conversion of the **gp41** envelope subunit to the fusion-competent conformation. A second example occurs with **adenoviruses 2** and **5**, which have two receptors that induce conformational changes and promote endocytosis.

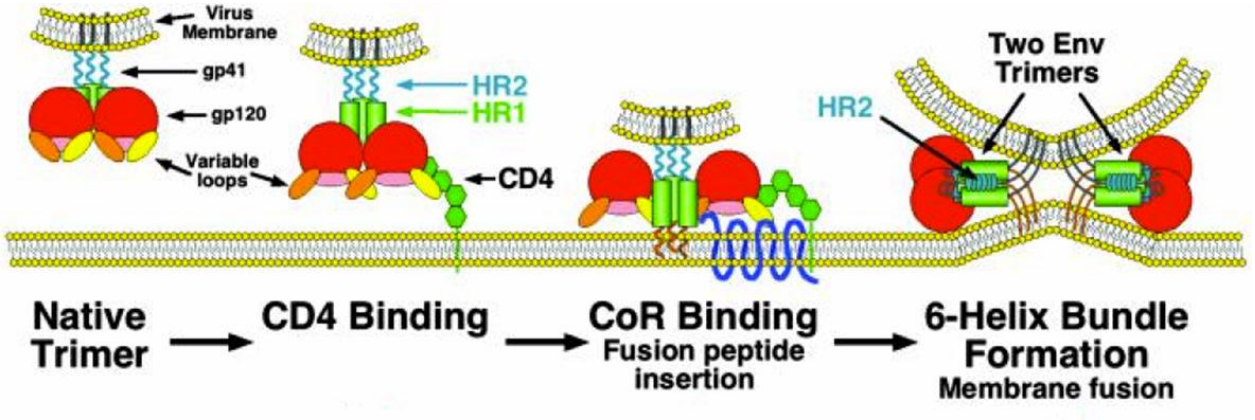


Figure 6: Model for HIV binding to receptors and co-receptors. Env protein (homotrimeric), each subunit containing surface gp120 and membrane-spanning gp41. Binding to CD4 mediated by gp120 induces conformational changes in gp120 -> exposure of conserved region important for co-receptor binding. gp120 binds to a seven-transmembrane domain co-receptors (CoR). Hydrophobic fusion peptide at the N terminus of gp41 becomes exposed and inserts into the membrane of the cell (not known whether from CD4 binding or from co-receptor binding). Co-receptor binding ultimately results in formation of a six-helix bundle in which the helical HR2 domains in each gp41 subunit fold back and pack into grooves on the outside of the triple-stranded HR1 domains, bringing the fusion peptide and transmembrane domain of gp41 into close proximity. It is likely that several Env trimers need to undergo this conformational change in order to form a fusion pore, although here only two trimers are depicted. It is not known whether pg120 remains associated during the fusion process or dissociates from gp41.

The use of different receptors often correlates with the need for a virus to overcome barriers existing in the cell type or tissue (need for shuttle function). **Coxsackievirus B**, a pathogenic single-stranded positive-sense RNA enterovirus, binds decay-accelerating factor (DAF) in the apical surface of epithelial cells, and subsequently to Coxsackievirus and adenovirus receptor (CAR), which is localized in the tight junction region. DAF brings virus to junctions and CAR induces conformational change, inducing endocytosis.

Receptor Binding Proteins

In enveloped viruses, spike glycoproteins bind to receptors (multifunctional proteins, serving additionally as membrane fusion factors and/or receptor-destroying enzymes). Structural data exist (HA bound to sialic acid -> influenza; gp120 and CD4 -> HIV-1). In nonenveloped viruses, the structures binding receptors are projections or indentations on the capsid surface. Adenovirus -> homotrimeric fibers with globular knobs. Crystal structure of 12 knob together with N-terminal domain of CAR -> large contact area on lateral side. Penton base of many adenovirus subfamilies contains an exposed RGD sequence

that associates with integrins. For some viruses, binding may cause destabilization -> first step toward uncoating.

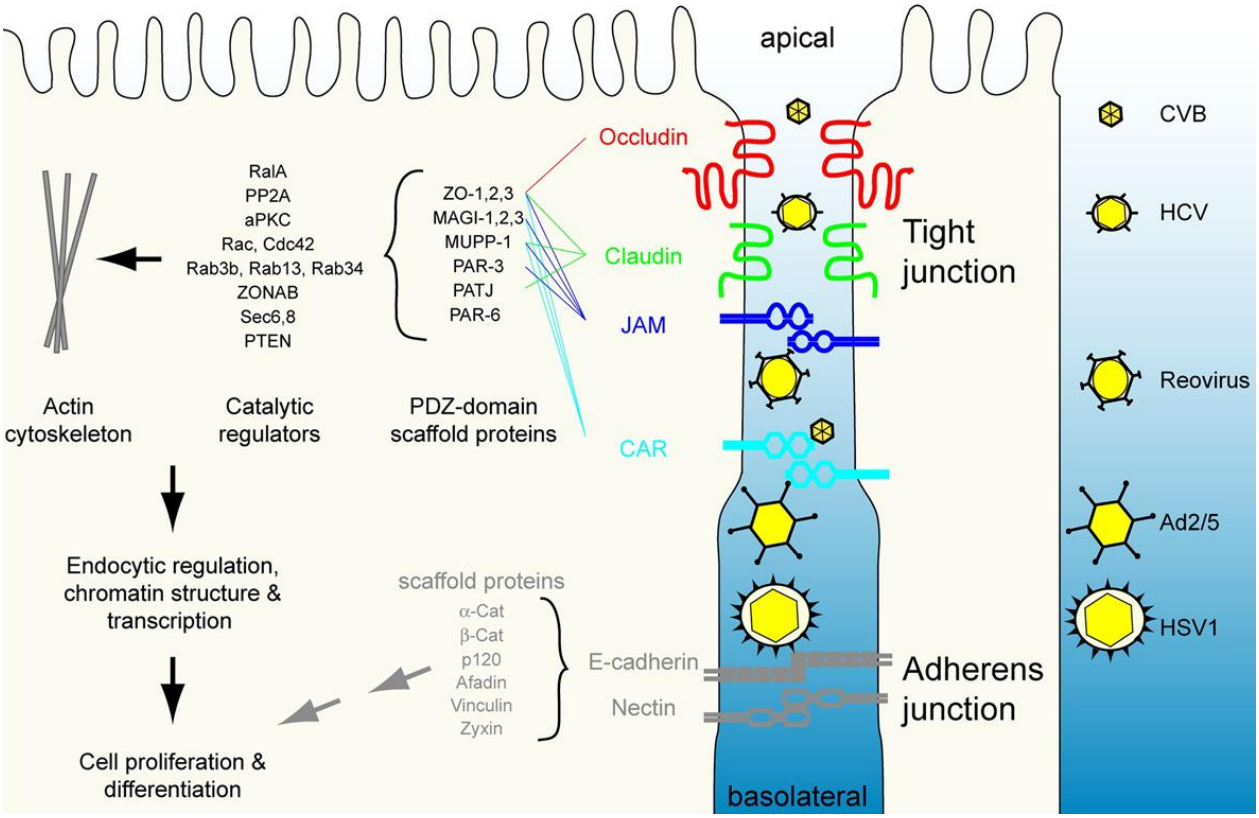


Figure 7: The TJ transmembrane proteins targeted by viral particles and their underlying regulatory network. Tight junction (TJ) and adherens junction (AJ) membrane proteins engage in hemophilic contacts with neighboring cells. Occludins, JAMs, and CAR have adhesive and/or signal-transducing function, while the claudins form ion-sensitive pores within the TJ strands. The AJ are largely made up of cadherins and nectins. The cytosolic domains of cell-cell adhesion molecules are linked to the actin cytoskeleton through PDZ-domain-binding proteins and scaffolding proteins, such as the membrane-associated guanylate kinase homologs Z0-1, Z0-2 and Z0-3, MAGI, MUPP, PATJ or PAR-3 and PAR-6 and α-/β-catenin, p120, afadin, vinculin or zyxin. The underlying scaffolding and regulatory network is important to control junctional dynamics. E.g. TJ proteins are controlled by GTPases, kinases and phosphatases, which often act through intermediate adaptor proteins linking the transmembrane proteins to the actin cytoskeleton. The evolutionary conserved atypical PKC (aPKC) of the PAR complex maintains epithelial polarity, Rac and Cdc42 GTPases regulate the underlying actin cytoskeleton and the PP2A phosphatase in complex with the RalA GTPase is involved in coupling exo- and endocytosis of junctional proteins.

Virus Binding: Carbohydrate/Protein Interactions

Carbohydrate/protein interactions have long been known to play an important role in viral invasion. Some viruses specifically bind to sialic-acid containing groups, others bind GAGs or glycolipids. Heparan sulfate has been identified as an attachment factor for numerous viruses (degree of sulfation or presence of sulfotransferase added groups important for some viruses). Mostly, carbohydrates serve as attachment factors that do not trigger conformational changes.

SV40 (and its murine-targeted cousin, mouse polyoma virus, mPy) uses gangliosides (GM1, GD1a and GT1b). In mPy, the disaccharide sialic acid α-2,3-galactose present in these gangiosides binds to a shallow pocket in the major capsid protein, VP1.

In some virus systems, the lectin is located on the cell surface and the carbohydrate ligand is located on the virus. HIV-1 binds to cell surface lectins such as DC-SIGN and L-SIGN through high mannose N-linked glycans in their envelope glycoproteins.

Chapter 3: Mechanisms of Virus Endocytosis I

Introduction

Viruses make use of various pinocytic mechanisms of endocytosis that serve the cell by promoting the uptake of fluid, solutes, and small particles. Two well-studied endocytic pathways used by viruses are **clathrin-mediated endocytosis** (**CME**) and **macropinocytosis**. A large number of different cellular factors are involved in these pathways (CME of influenza A can be constitutive or triggered whereas macropinocytosis of vaccinia virus requires activation of kinases and GTPases that regulate changes in actin dynamics).

Endocytosis in Mammalian Cells

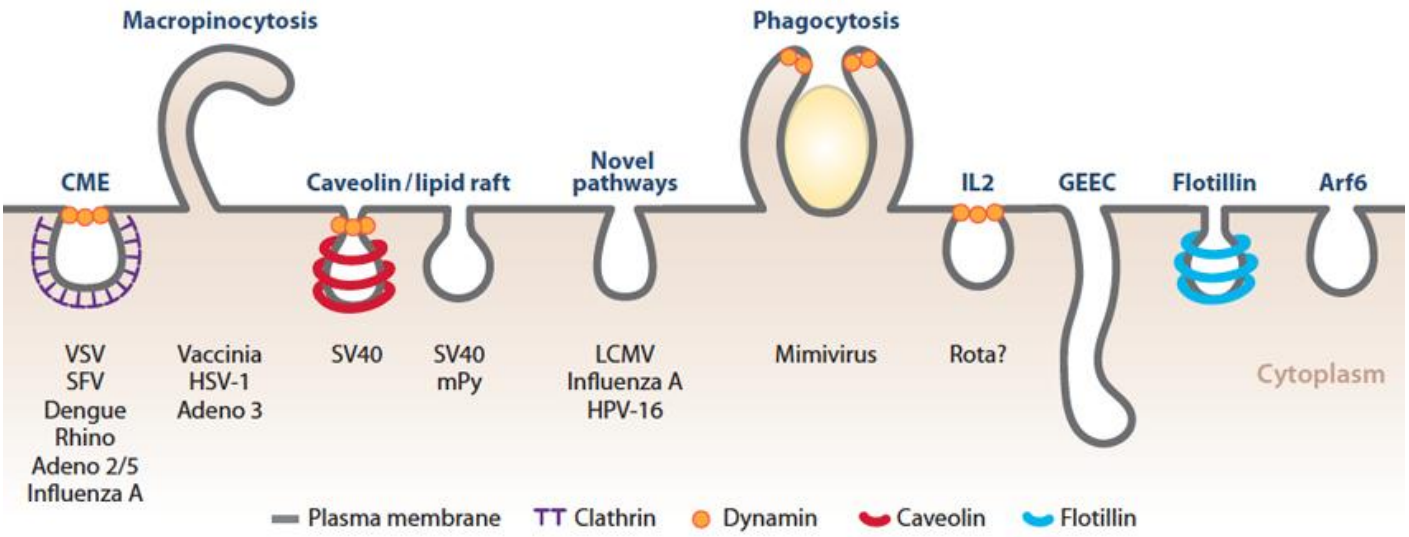


Figure 8: Endocytosis in animal cells can occur via several different mechanisms. Multiple mechanisms are defined as pinocytic, i.e. they involve the uptake of fluid, solutes, and small particles. These include CME, macropinocytosis, caveolar/raft-mediated mechanisms, as well as several novel mechanisms. Some of these pathways involve dynamin-2 as indicated by the beads around the neck of the endocytic indentations. Large particles are taken up by phagocytosis, a process restricted to a few cell types. In addition, there are pathways such as IL-2, the so-called GEEC pathway, and the flotillin- and ADP-ribosylation factor 6 (ARF6)-dependent pathways that carry specific cellular cargo but are not yet indicated for virus entry.

Examples of Mammalian Cellular Factors in Endocytosis Pathways

Endocytosis:

- Formation of a structural coat
- Adaptor protein recruitment
- Scission
- Regulation by cellular factors
- Cytoskeletal activation
- Intracellular trafficking

To identify cellular organelles and factors involved, fluorescent cellular marker proteins and cargos were used.

Cellular factors of endocytosis pathways

Function	Clathrin-mediated	Macropinocytosis	CAV1 pathway	Lipid raft
Coat proteins	Clathrin heavy chain, clathrin light chain	None	Caveolin-1	None (clathrin and caveolin independent)
Adaptors	AP2*, eps15*, epsin1*	None	Unknown	None
Scission factors	Dynamin-2	Unknown	Dynamin-2	Unknown (dynamin-2 independent)
Regulatory factors	PI(3,4)P, PI(4,5)P2, cholesterol*, cortactin*, Arp2/3*	Tyrosine kinases, PAK1, PI(3)K, PKC, Ras, Rac1, Cdc42*, Rab34*, CtBP1, Na+/H+ exchange, cholesterol	Tyrosine kinases, phosphatases, PKC, RhoA, cholesterol	Tyrosine kinases, Rho A, cholesterol
Cytoskeleton	Actin*, microtubules*	Actin, microtubules*, myosins*	Actin, microtubules	Actin
Trafficking	Rab5, Rab7*, Rab4*, Rab11*, Rab22*	Rab5*, Rab7*, Arf6*	Rab5	Unknown

* Factors that are required in a cell type or system-dependant fashion.

Table 3. Markers of specific cellular organelles/processes

Process or organelle	Marker	Cargo
Clathrin-mediated uptake	Clathrin light chain-XFP; AP-2-XFP; Eps-15-XFP; Dynamin-2-XFP	transferrin, SFV, EGFR
Macropinocytosis	Actin-XFP	Vaccania virus
Caveolar endocytosis	Caveolin-1-XFP; Cavin-XFP	SV40,
Clathrin & caveolar-independent endocytosis	GPI-anchored-XFP (some)	Ctx (some)
Dynamin mediated processes	Dynamin-2-XFP	SV40, SFV
early endosome	Rab5-XFP:	SFV, VSV
recycling endosome	Rab4-XFP; Rab11-XFP	transferrin
maturing endosomes	Rab7-XFP	Influenza A
late endosome and Golgi	Rab9-XFP	
Golgi traffic	COPI-XFP	
Lysosomes	LAMP-1-XFP	EGFR
Dependance on acid activation	Cypher-5 activation	SFV, Influenza A
Actin mediated transport	Actin-XFP	SV40, Vaccania virus
Microtubuli mediated transport	Tubulin-XFP	SV40, SFV, Adenovirus

Viruses that use the Clathrin Pathway

Adenovirus 2 and 5 and vesicular stomatitis virus (VSV), a membrane-bound, negative-sense RNA arbovirus of the family Rhabdoviridae, were present in thickened regions of the plasma membrane. Now,

these regions are known as **clathrin-coated pits** (**CCPs**). In SFV, CME was first shown to be part of the infectious pathway. Today, it is the most commonly observed virus entry pathway.

Various steps:

- Clathrin coat assembly
- Induction of membrane curvature
- Cargo recruitment
- Vesicle fission
- Coat disassembly

Composition of CCPs in the plasma membrane is not always the same. The adaptor complex **AP2** has turned out not to be an absolute requirement for the formation of coated pits and internalization of cargo. Some viruses are dependent on AP2, others are not.

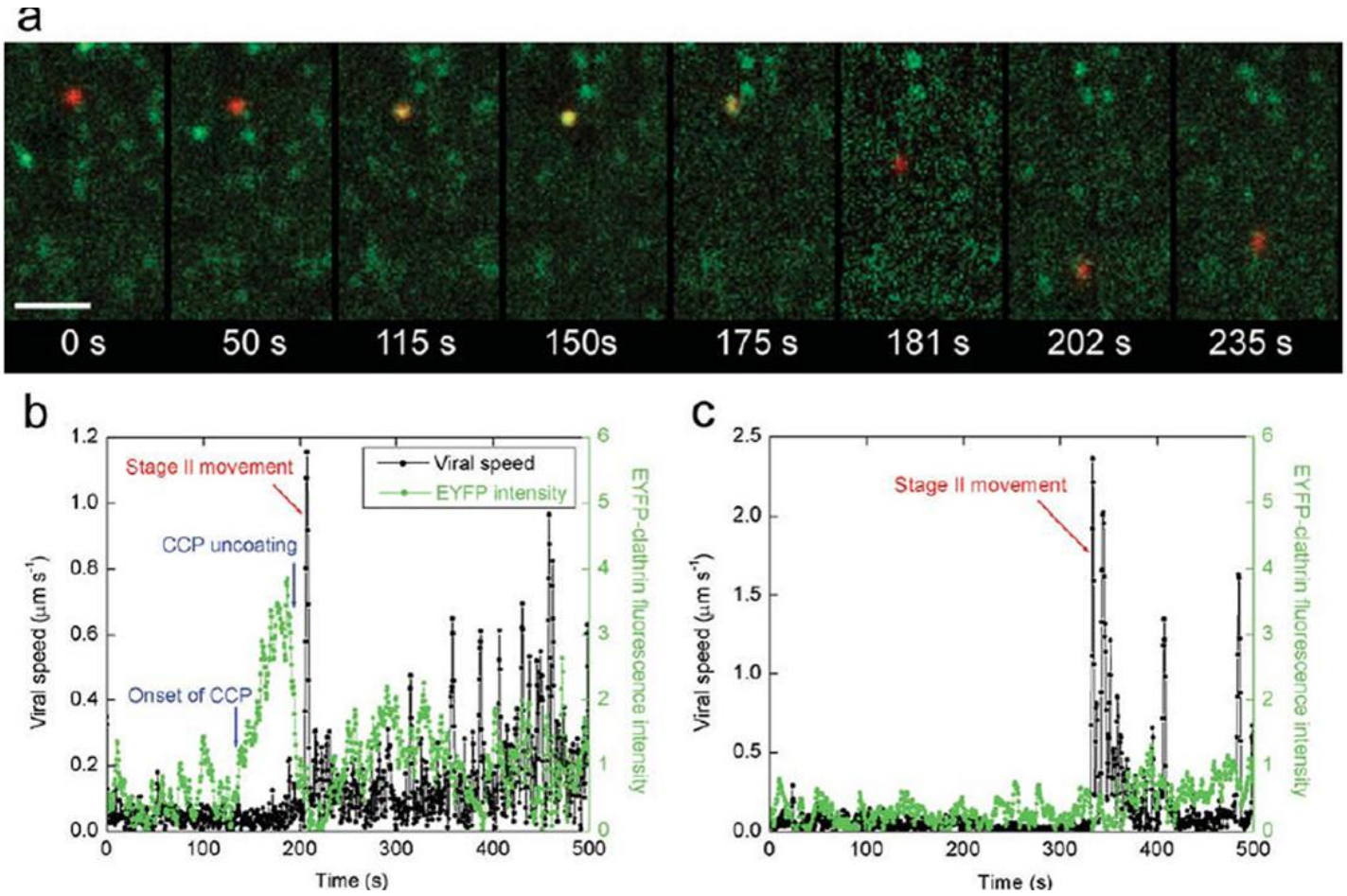


Figure 9: (a) Snapshots of a virus internalized via CCP. Scale bar 10 µm. t=0 s: the virus (red) binds to the cell. t=50 s: the virus is undergoing stage I movement. t=115 s: a CCP labeled with EYFP (green) begins to form at the virus site. t=150 s: the clathrin coat reaches its peak fluorescence intensity. t=175 s: the clathrin coat rapidly disassembles. t=181 s: the virus is transported towards the nucleus on a microtubule (stage II movement). t=202 s: the virus enters stage III transport involving both plus- and minus-ended directed motilities on microtubules. t=235 s: the virus continues stage III movement. (b) Time-trajectories of a virues internalized via de novo formation of CCP. Black symbols are the velocity, stage II movement identified as the rapid unidirectional translocation from the cell periphery to the perinuclear region (red arrows). Green symbols are the integrated florescence intensity of EYFP-clathrin associated with the virus. (c) Time-trajectories of a virus internalized without association with a clathrin-coated structure.

Cargo can actively initiate the formation of CCPs, e.g. VSV and influenza A. 94% of clathrin-internalized influenza virus particles on BSC1 cells induced de novo assembly of CCPs. The required adaptor protein in this case is epsin-1. Close to surface-bound VSV particles, clathrin, dynamin-2 and AP2 are accumulated. This assembly phase lasts about 110s (normal coated pits 50s). If particle is fixed to the substrate, frustrated CCP formation can be observed. Coat assembly might be induced by receptor clustering (-> microdomain formation).

CME is generally a rapid process, where particles enter within minutes after attachment. Delivery to EEs follows within 1-2 minutes. For VSV and SFV (high fusion pH, EE), acid-activation occurs within 1-5 minutes after internalization. For viruses with a fusion pH in the LE range, fusion happens 10-20 minutes after internalization.

Single-particle tracking study dengue virus (serotype 2, strain 1, member of Flaviviridae, transmitted through mosquito bite): Visualization of various steps in clathrin-mediated entry. After binding, virus particles move randomly along the plasma membrane (about 110s) before associating with preexisting, clathrin-containing domains (movement constrained). Chlorpromazine (inhibitor of clathrin assembly) causes that the movement continues without stopping. Delivery to endosomes (86% to Rab5-positive EE, 14% to Rab5- and Rab7-positive ME), majority (80%) fuse within an average of 12.5 minutes after virus addition and 5.5 minutes after entry into Rab7-positive LEs. Fusion events majorly occur in peripheral endosomes.

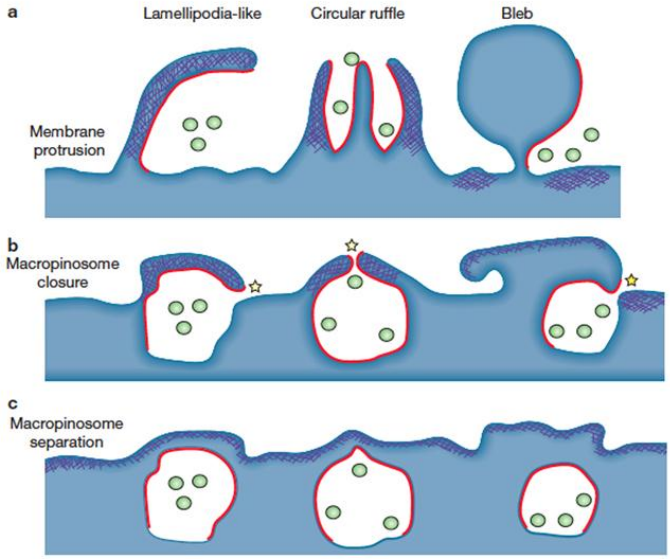
Macropinocytosis and Virus Entry

Macropinocytosis: Transient, growth factor-induced, actin-dependent endocytic process, leading to the internalization of fluid and membrane in large vacuoles. Unique, involves cell-wide plasma membrane ruffling (lamellipodia, filopodia, blebs). Macropinosomes are formed when membrane ruffles fold back on the the plasma-membrane (-> fluid-filled cavities).

Macropinosomes: 10µm, large, 5-10fold increase in cellular fluid uptake. After formation, they move into cytoplasm, where acidification and homo- and heterotypic fusions occur. Then recycling or feeding into endosomes (maturation with the gain and loss of classic EE and LE markers) before lysosome fusion. Induced by growth factors, apoptotic bodies, necrotic cells, bacteria and viruses (inducing ruffling, and promoting their internalization together with fluid).

Involvement of various cellular components. Actin-based process, and activation involves cellular lipids, kinases, GTPases, Na^+/H^+ exchangers, adaptor molecules, actin, actin modulatory factors, myosins and fusion and fission factors.

Best characterized example **vaccinia virus**. The association of mature virions with cells triggers the formation of large transient plasma membrane blebs. As part of the bleb retraction, vaccinia and fluid are taken up. Additionally, the membrane of vaccinia is enriched in phosphatidylserine, a phospholipid required for macropinocytic clearance of apoptotic debris -> mimicking apoptotic bodies.



Actin-associated factors	Lamellipodia	Circular	Retracting blebs
Nucleation			
Arp2/3	Yes	Yes	No
WASP	Yes	Yes	
WAVE	Yes	Yes	
VASP	Yes	Yes	No
Polymerization			
Cortactin	Yes	Yes	Yes
Coronin	Yes	Yes	Yes
Profilin	Yes	Yes	
Depolymerization			
Cofilin	Yes		
Crosslinking			
Hsp27	Yes	Yes	Yes
α-Actinin	Yes	Yes	Yes
Fimbrin	Yes	Yes	Yes
Fascin	Yes	Yes	No
Filamin	Yes	Yes	Yes
Membrane tethering			
ERMs	Yes	Yes	Yes

Figure 10: Macropinosome formation is an actin-based process. (a) The plasma membrane protrusions involved can take the form of planar lamellipodia, circular ruffles or blebs. Lamellipodia and ruffles require polymerization of actin. Bleb formation is probably the result of local actin cortex destabilization. The blue lines represent actin and the red lines highlight the portion of the protrusion that will constitute the membrane of the macropinosome when it is formed. (b) To form a macropinosome, lamellipodia probably fold back on the plasma membrane, forming a cave-like invagination, and undergo a membrane fission event that separates the lumen of the macropinosome from the extracellular space. The individual protrusions in circular ruffles are likely to combine and then together close off the macropinosome by membrane fission. The closure of bleb-associated macropinosomes may involve the formation of a vacuole next to retracting bleb followed by membrane fission. The star represents the site of closure. (c) After closure, the macropinosomes move further into the cytoplasm. (d) Actin nucleation, polymerization, depolymerization, crosslinking and membrane tethering factors recruited to lamellipodial ruffles and collapsing blebs largely overlap, with blebs as an exception, they do not seem to contain Arp2/3, WASP or WAVE. Green -> localized to protrusions, red -> factors not localized to protrusions, grey -> no data.

Evolution of macropinocytosis:

- Particle size (large viruses too large for uptake by most other forms of endocytosis)
- Broaden host range or tissue specificity
- Acidification of macropinosomes (many viruses are acid activated)

Species C human adenoviruses 2 and 5 require macropinocytosis for infectivity but not as direct internalization pathway. Internalized by CME, but induction of macropinocytosis and rupture of newly formed macropinosomes are required for escape of the virus from EEs (induction or formation of macropinosomes or release of macropinosomal components upon rupture is required for productive penetration and infection, somehow).

Table 4. Macropinocytosis and vaccinia entry

Hallmarks of macropinocytosis (1)	Tools (2)	Vaccinia virus infection (3)
Markers		
Ruffling/blebbing	Phase contrast microscopy, fluorescent actin	Yes
Fluid uptake	Fluorescent dextrans, lucifer yellow, horseradish peroxidase	Yes
Requirements	**Perturbants**	**Vaccinia virus infection**
Cytoskeleton		
Actin	Cytochalasin B/D, Latrunculin, Taxol	Yes
Microtubules	Nocodozole, Colcemide	No
Myosin II	Blebbistatin, ML-7	Yes
Rac1 and Cdc42	Toxin B, D/N and C/A Rac1 and Cdc42mutants	Yes
Na+/H+ exchange	Amiloride, ethylisopropyl amiloride (EIPA)	Yes
Kinases		
Pak1	IPA-3, PAK1 autoinhibitory domain	Yes
PI(3)K	Wortmannin, LY294002	Yes/No
PKC	Bisindolylmaleimide	Yes
Other factors		
RhoA	CT04, D/N and C/A RhoA mutant	No
Dynamin-2	Dynasore, D/N dynamin mutant	No
Arf6	C/A Arf6 mutant	Yes
Cholesterol	Methyl-beta-cyclodextrin (M CD), filipin, nystatin	Yes
pH dependence	Bafilomycin A1, monensin, NH4Cl	Yes/No

(1) The known hallmarks and cellular requirements of macropinocytosis.

(2) Markers/perturbants of the required cellular factors used to test for macropinocytosis as an endocytic mechanism.

(3) The macropinocytic factors associated with and required for vaccinia virus infection.

(Mercer and Helenius. 2010. Ann. N.Y. Acad. Sci.)

Chapter 4: Mechanisms of Virus Endocytosis II

Introduction

Some viruses do not enter via clathrin-mediated endocytosis. Instead, they make e.g. use of caveolae, flask-shaped indentations of the plasma membrane enriched in cholesterol and sphingolipids, caveolins, and signaling factors. Caveolae are generally immobile, but support internalization of certain ligands. Additionally, cells have other clathrin-independent pathways, serving as primary or alternative entry routes for viruses and bacterial toxins. These noncaveolar, lipid raft-dependent pathways are still poorly understood.

Cellular Clathrin Independent/Lipid Raft Pathways

Feature	Caveolae	CLIC/GEEC
Major regulator	Cav1/Cav3	Cdc42/Arf1 [7,43] RhoA [5]
Carrier	50–80 nm smooth,flask-shaped (chemically fixed)	100–200 nm ring-shaped Clathrin-independent carrier (CLIC)
Dynamin	Dependent	Independent
Cargo	Lipid-rafts, SV40 (minor fraction)	GPI-AP, Ctx-beta, SV40, mPy

Viruses that Use Caveolar/Raft-Dependent Entry

Formation of vesicles depends on cholesterol, lipid rafts, and a complex signaling pathway involving tyrosine kinases and phosphatases. The process is ligand triggered. Viruses use glycosphingolipids as their receptors. Uptake may involve caveolae but does not have to. Upon internalization, cargo passes through EEs and LEs, followed by transport to the ER. To initiate uncoating, some viruses exploit specific ER thiol oxidoreductases; they use components of the ER-associated protein degradation pathway for penetration. Uptake and forward movement are slow and asynchronous. Penetration can occur as late as 6-12 h after internalization.

Best studied examples are **Polyomavirus family members** (structural components are 72 homopentamers of VP1, icosahedrally arranged, each VP1 has one binding site for carbohydrate moiety of gangliosides); SV40, mPy, BK and JC viruses. All have different gangliosides as receptors. Association with glycan moieties is highly specific but of relatively low affinity. Multiple receptors -> high avidity. Use of gangliosides as receptors is of high significance, as it determines their behaviors on the cell surface, their mechanism of endocytosis and the intracellular pathway from endosomes to the ER:

- SV40 needs GM1 for binding, internalization and infection
- mPy needs GD1α only for guidance from LEs to the ER

Viruses associate with detergent-resistant microdomains in the plasma membrane and with liquid-ordered phases in giant unilamellar vesicles. After binding, mPy undergoes rapid, random, lateral movement for 5-10 s before stopping or changing to a slow drift. Cell-associated viruses enter uncoated, tight-fitting indentations and pits of variable depth. SV40 induces membrane curvature and promotes formation of primary endocytic vesicles. Final fission generating the tight-fitting small vesicle depends on cellular energy sources and factors.

The majority of surface-bound SV40 and mPy do not associate with caveolae. Colocalization with caveolin-1 occurs later when most of the viruses already have been internalized. Evidence for a second, closely related mechanism of SV40 uptake has emerged from studies with a caveolin-1 deficient cell line and fibroblasts from caveolin-1 knockout mice. These cells are efficiently infected by SV40 and mPy, and viruses enter via virus-containing invaginations (morphologically indistinguishable from caveolin-1 invaginations). This mechanism is also cholesterol and tyrosine kinase dependent. The fraction of virus using caveolar entry may vary with cell type and virus because expression of CAV1 in caveolin-deficient Jurkat cells enhanced infection.

- Only a small fraction of SV40, mPy and other polyomaviruses enter via caveolae
- Majority use related, caveolin-independent mechanism

- Genistein (tyrosine kinase inhibitor), nystatin and progesterone (depletion of cholesterol), brefeldin A (inhibitor of Arf1, a guanine nucleotide binding protein), bafilomycin A (inhibitor of vacuolar ATPase), lactrunculin A or jaspakinolide (inhibitors of actin dynamids) and temperature reduction (20°) reduce entry of SV40 and mPy
- Internalization is accelerated by okadaic acid and orthovanadate (inhibitors of Ser and Thr or Tyr phosphatases)
- Internalization through caveolae is generally slower and dependent on dynamin-2 and also more dependent on actin dynamics that the noncaveolar pathway

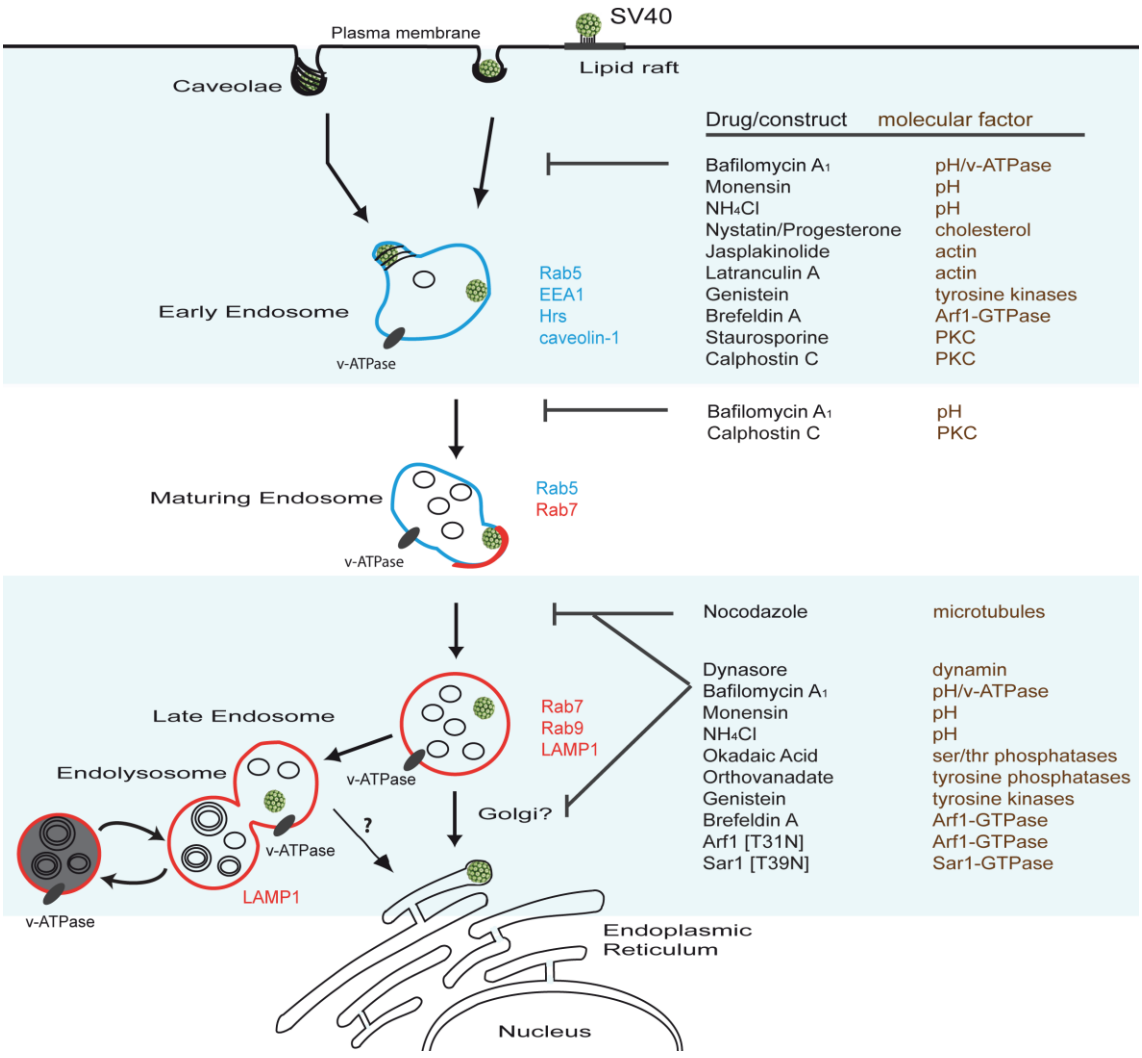


Figure 11: Model of infectious SV40 entry into CV-1 cells. Binding to GM1 receptor, partitioning into lipid rafts, inducing uptake from plasma membrane (caveolae-mediated or caveolin-1-independent endocytosis), entering EE, LE and endolysosomes before reaching the ER, transport to Rab5-, EEA1-, Hrs-, and caveolin-1-positive EEs. Upon requiring Rab7, SV40 associates the Rab7-positive domain and becomes a luminal component of LAMP1-, Rab9-, and Rab7-positive LEs and eventually endolysosomes. Virus transport to the ER occurs from the late compartments of the endocytic pathway by an unknown mechanism. The v-ATPase is responsible for acidification of endosomes and lysosomes, and acidification is required for SV40 internalization and subsequent transport steps. Early and late events in the entry pathway can be blocked by various inhibitors and other perturbants. SV40 is slowly transported to the ER where partial uncoating and penetration into the cytosol occurs.

Mechanism for lipid raft-dependent, caveolar and caveolin-1 independent endocytic pathways are fundamentally similar -> Basic pathway probably a lipid raft-dependent, cargo-activated process, where caveolin-1 and dynamin are an additional level of regulation (inhibitory effect, CAV1 expression suppresses lipid raft-mediated endocytosis).

SV40 and mPy spend most of their time in LEs. 15-20% of virus particles colocalize with EE markers 1-2 h after cell warming. After about 60 min, virus begins colocalizing with LE markers and after 2-4 h, SV40 enters tubular, vesicular extensions that detach from LEs and move along microtubules and accumulates in smooth ER. For mPy, the step after formation of vesicular extensions probably needs GD1α receptor.

There seem to be other mechanisms of virus entry in which no coats were visualized so far and that lack clathrin and caveolin dependency and transport of viruses to the endosomal network. Viruses using these pathways come from several families (influenza A, Rotavirus, HPV-16).

Rotavirus is a nonenveloped RNA virus of the reovirus family, leading cause of severe diarrhea among infants and children. Particles sequentially interact on the cell surface with sialic acid containing molecules, integrin α2β1, Hsc70, and finally integrins α5β3 and αXβ2. These interactions trigger conformational changes in the capsid preparing virus for uncoating and penetration. The mechanism is dynamin-2 dependent and somewhat sensitive to cholesterol depletion, suggesting a role for lipid rafts. Possible relationship with uptake of interleukin 2, which is internalized by clathrin- and caveolin-independent pathways.

Membrane penetration of **rotavirus** occurs by lysis or pore formation in endosomes, mediated by trypsin-activated VP4, independent of acid activation (no block by lysosomotropic weak bases). Bafilomycin A blocks infection, possibly through decrease in Ca^{2+} concentration.

HPV-16 is a nonenveloped virus of the papillomavirus family infecting mucosal epithelia and causing warts and cervical and anogenital tumors. Particles contain two structural proteins (L1 and L2) that form icosahedron (T=7) of 55nm diameter. Entry involves initial interaction with heparan sulfate proteogylcans (HSPGs), followed by a sequence of structural changes caused by interaction with sugar moiety of HSPGs, by cyclophilin B, and by activation by furin, a proprotein convertase.

Endocytosis occurs by a clathrin-, caveolin-, flotillin-, lipid raft-, dynamin-independent mechanism distinct from macropinocytosis and phagocytosis. Uptake depends on actin polymerization and is independent of Rho-like GTPases. Endocytosis via small tubular pits requires PI3-kinase and protein kinase C activities, as well as a sodium-proton exchanger. Intracellular trafficking occurs through the endosomal netweok, where HPV-16 seems to follow the canonical transport from EEs and LEs to lysosomes. The virus is acid acrivated. Taken together, the requirements for infectious endocytosis and

the morphology of vesicular carriers suggest an endocytic mechanism that combines features of macropinocytosis and a novel clathrin independent/lipid raft pathway.

Pathways in literature with which no viruses have been associated yet:

- GEEC pathway (endocytosis of GPI-anchored proteins)
- Flotillin pathway (GPI-anchored proteins and proteoglycans)
- Arf6 pathway (internalization of major histocompatibility antigens)
- IL-2 pathway (internalization of cytokine receptors)

Chapter 5: Penetration from Endosomes and the ER

Introduction

Once internalized, the intracellular pathways followed by incoming viruses are the same as those used by physiological ligands (nutrient, hormones, growth factors, lipids…). The ultimate goal of the virus journey through the endosomal system is the release of viral genome in a replication-competent form through penetration and uncoating. Penetration mechanisms:

- Membrane bound viruses, membrane fusion (influenza A, via Type I fusion apparatus, HA)
- Membrane fusion (Type II, SFV, variation in fusion mechanism)
- Nonenveloped viruses, lysis or pore formation (Adenovirus, viral capsid penton base cebomes lytic at low pH, enterovirus PV creates pore for release of RNA genome)
- New: SV40 penetration from the ER

The Endosome Network

Main organelle classes:

- EEs
- LEs (often in the form of multivesicular bodies)
- Maturing endosomes (ME, containing both Rab5 and Rab7, LE precursors)
- Trans Golgi network (TGN)
- Plasma membrane

Time frame after internalization:

- 2min -> EE
- 10-12 min -> ME and LE (perinuclear region)
- 30-60 min -> Lysosomes

The different classes of endosomes are very heterogeneous, and functions and transit highly asynchronous. There seem to be at least two populations of EE, highly motile and rapidly maturing and more static and slowly maturing ones.

EEs have a complex structure with vacuolar elements and many long, narrow, often-branched tubes. The membrane is composed of a patchwork of functionally different domains. These differ in composition, location, and structure (tubular, vacuolar), and EEs are often defined by different Rabs and their effectors. The domains are responsible for selective vesicular transport to distinct targets:

- Rab4 -> Plasma membrane
- Rab7 -> LEs
- Rab22 -> REs
- Rab9 and retromer complex -> TGN

Each endosome class corresponds to a heterogeneous collection of organelles and goes through a program of changes with time. The conversion from EEs to LEs is a particularly complex process: Maturation or vesicle transport? Both models apply; the process involves formation of an ME, a hybrid endosome with Rab5 and Rab7 domains. The ME mainly contains vacuolar components of EEs and ILVs.

The repertoire of changes during maturation includes:

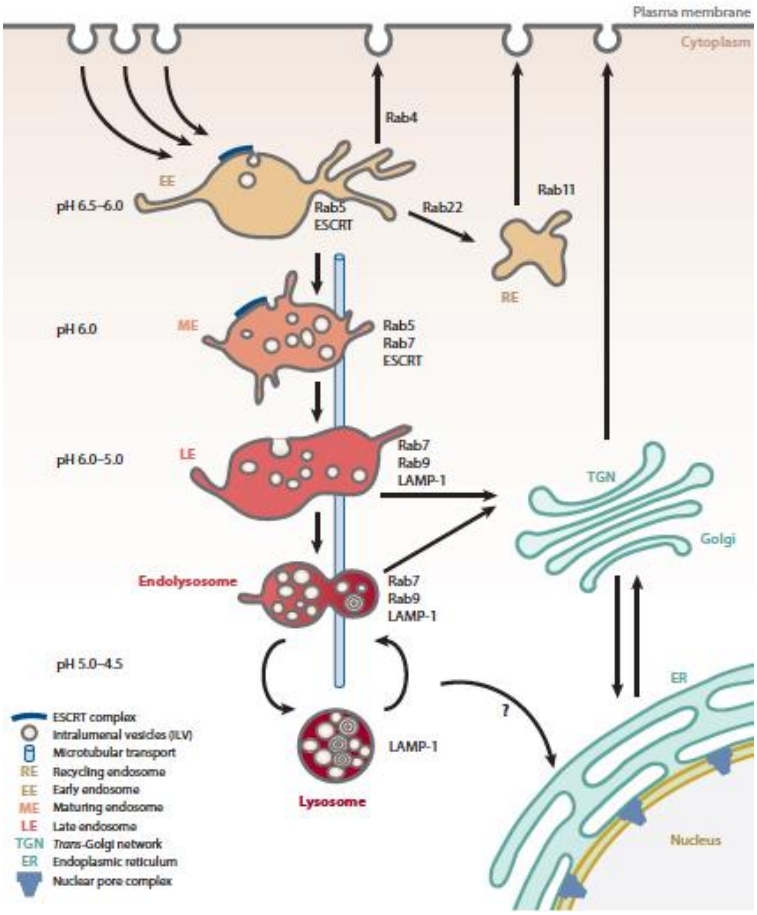


Figure 12: Endosome Network: EE, ME, LE, recycling endosomes (RE), lysosomes. EEs located in the periphery, complex, several domains, tubular (50-90nm diameter, up to 4 µm length), contain most of the membrane; give rise to recycling vesicles and vesicles for transport to REs and the TGN. Vacuolar domains, 200-1000nm diameter, contain most of the volume, ILVs and larger endocytosed particles. Vacuolar domains dissociate and undergo MT-mediated, dynein-dependent movement to the perinuclear region.These MEs contain Rab5 and Rab7, further acidification and conversion to mature LEs, which can fuse with each other and eventually with lysosomes, generating endolysosomes in which active degradation takes place. The dense core lysosomes correspond to the end points of such degradation processes; they serve as a depository for lysosomal enzymes and membrane proteins awaiting fusion with incoming LEs. The majority of incoming membrane components undergo recycling to the plasma membrane. However, membrane proteins destined for degradation are first tagged with monoubiquitin-binding components of the endosomal sorting complex required for transport (ESCRT) machinery, and finally sequestered into ILVs. ILVs are formed by inward budding of EEs and LEs. They fill the lumen of the vacuolar domains, forming MVBs, and are eventually degraded in lysosomes.

- Loss of recycling receptors and other membrane proteins and lipids (targeted for recycling); these leave EE by direct vesicle transport or REs
- Gradual drop in internal pH (mild acidity, pH 6-6.5 in EEs till value below 5); caused by variation in subunit composition and concentration of vATPase as well as different isoforms of Cl-channels
- Formation of ILVs; carried out by ESCRT complexes associated with EEs and MEs; monoubiquitinated membrane proteins are selectively included in ILVs; results in formation of LEs filled with vesicles (MVB) destined for lysosomal degradation
- Switch of Rab subsets (Rab4 and Rab5 -> Rab9 and Rab7) and their effectors, changes in predominant phosphatidylinositides from PI(3)P to PI(3,5)P_2
- Change in interaction with cytoskeletal elements (mainly MT and their motors) -> endosome migration from the peripheral to the perinuclear area of the cell -> fusion with lysosomes

Perturbations lead to retardation of the whole program (e.g. overexpressing Rabs and mutants, prevent ILV formation, inhibit endosome movement, inhibit PI(3) kinases, prevent acidification, block maturation with temperature drop etc.).

- Inhibition of v-ATPase with bafilomycin A -> blocks ME formation
- Nocodazole -> blocks LE formation and transport of cargo to REs
- Wortmannin (PI(3) kinase inhibitor) -> Delay in EE to LE transport
- 20° incubation -> blocks LE fusion with lysosomes

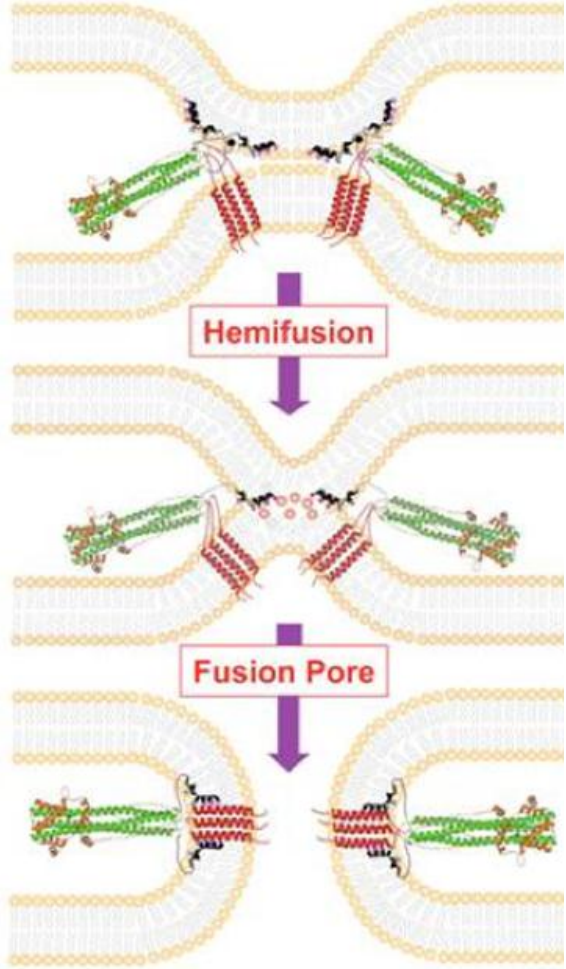


Figure 13: HA mediated fusion of viral cell membranes. HA mediates a local point fusion. Hemifusion is an intermediate on the pathway to complete fusion in which the outer but not the inner leaflets of two fusing membranes have merged and there is not yet mixing of aqueous contents. After hemifusion, initial fusion pores mediated by HA have been recorded electrophysiologically, and the pore diameter is estimated to be 1-2 nm. After the initial fusion pore is stabilized, the opening of the pore dilates.

Viral Penetration of Cellular Membranes by Fusion Mechanisms

Influenza A virus uptake results in trafficking to acidic LEs, the site of viral uncoating and membrane fusion,

which is initiated by the Type I viral glycoprotein HA (haemagglutinin) undergoing a conformational change upon exposure to low pH. HA are homotrimeric spike glycoproteins in which the subunits are joined by long coiled-coils. In transit through the secretory pathway, they undergo postsynthetic proteolytic cleavages that render them conformationally metastable and fusion competent. This allows cooperative conversion into a lower energy conformation. When triggered, the resulting conformation exposes hydrophobic fusion sequences that insert into the target membrane. The released free energy is used to force the membranes closer together in a focal site, resulting in fusion.

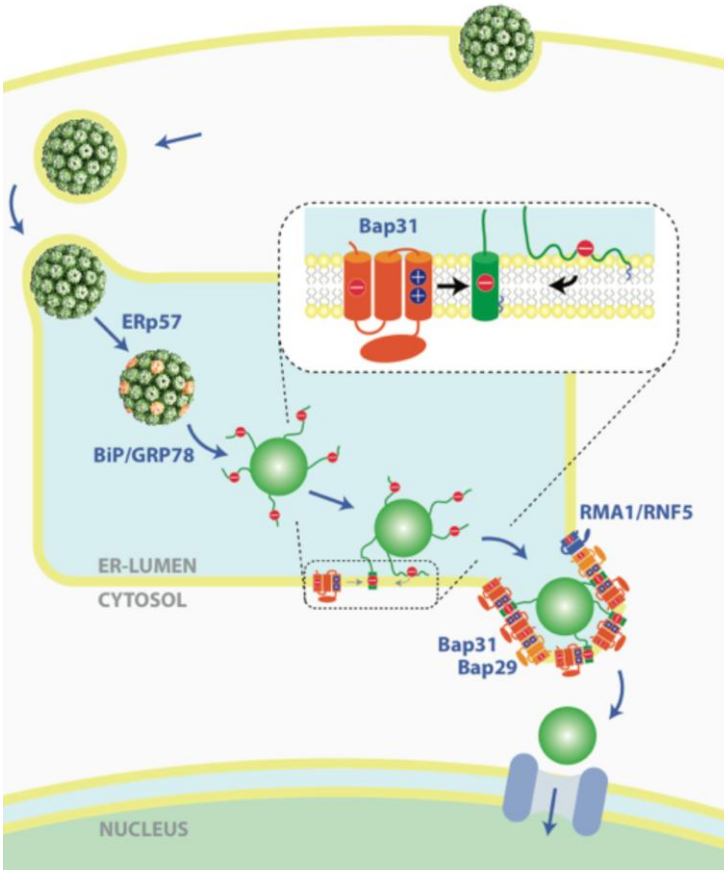


Figure 14: SV40 penetration from the ER. After arrival in the ER, luminal factors including ERp57 and BiP induce a major conformational change in the capsid. Of the 72 VP1 pentamers, a fraction (the 12 vertex pentamers) is disconnected from the disulfide-bonded network of VP1 pentamers, and the particle collapses to a diameter of about 34nm. The hydrophobic and myristylated N-terminal sequences of the previously hidden VP2 proteins are exposed. They insert into the ER membrane in an α-helical conformation, thus providing multiple extensions that anchor the virus particles to the membrane. Inside the membrane, the VP2 peptides attract Bap31 (and probably Bap29 and RMA1) through an interaction with the TM domain of Bap31. Recognition is specific and critically dependent on the exposed glutamic acid residue in position 17 of VP2. This residue forms a charge pair with positively charged residues, Arg117 and/or Arg118, in the TM domain of Bap31. The interaction between Bap31 and VP2 induces an accumulation of viruses and Bap31 complexes to specialized smooth ER subdomains associated with ER quality control functions and dislocation of misfolded TM proteins.

Viral Penetration of Cellular Membranes by Non-Fusion Mechanisms

Polyomavirus: Site of penetration is the ER. Viruses accumulate in tubular, smooth membrane networks with complex geometry enriched for the smooth ER markers syntaxin 17 and BiP. Polyomaviruses pass through the ER because they require ER-resident proteins for initial uncoating of their stable capsid -> visible structural changes in virus particle.

ERp29, a protein disulfide isomerase (PDI), induces conformational change for uncoating in the mPy capsid. It leads to exposure of the inner structural proteins generating a hydrophobic particle binding to membranes. ERp57 was shown to be crucial for SV40 infection. It mediates isomerization of

interpentameric to intrapentameric disulfide bonds. This uncouples the 12 vertex pentamers from the capsid initiating uncoating.

After uncoating, polyomavirus most likely penetrate the ER membrane by a poorly understood process. Viruses seem to hijack a cellular system dedicated to the transport of misfolded proteins from the ER to the cytosol. This system is referred to as ER-associated degradation (ERAD). It was shown that cellular factors involved in ERAD affect mPy, SV40 and BK infection. SV40 probably undergoes conformational change in the ER leading to exposure and membrane insertion of the N-terminus of VP2. ERAD factors recognize this peptide and assist tranlocation of the viral particle from the ER to the cytosol.

Chapter 6: Approaches to Study Virus Entry

Introduction

- Viruses are threats -> Inhibit entry and/or uncoating as targets for antivirals
- Viruses as vectors for expression of recombinant proteins (protein production)
- Viruses as vectors used in gene therapy
- Powerful tools in different areas of research

Virus Infection and Experimental Perturbation of the Endomembrane System

Early studies -> electron microscopy, **EM**, continued importance, cryo-EM, tomography, focused ion beam-scanning EM (FIB-EM). New techniques -> better organelle preservation, 3D imaging, and correlative analysis with light microscopy. **Light microscopy** important -> enhanced resolution; follow **fluorescent** viruses in live cells. Tagging of host components (GFP) -> track fate of individual particles, determine which cell proteins are involved. **Single-particle tracking** -> enhanced understanding, define center of virus particle (within less than 5 nm).

Perturbations using inhibitors, dominant negative (DN) or constitutively active (CA) constructs of cellular proteins, siRNA silencing, and cell mutants provide another general approach commonly used. Use adequate controls, and specific, quantitative assays for infection, endocytosis, and penetration, these perturbations are quite powerful. Pitfalls:

- Toxicity
- Cell type variability
- Off-target effects
- Unwanted side effects of protein tagging
- Poor transfection or silencing efficiency

- Compensatory activation of alternative pathways when one pathway is closed down

Chemical inhibitors span a wide array of effects, including signaling, regulation, endocytic machinery, cytoskeleton function, lipid composition of membranes and others. The following are used for determining whether viruses require low pH for infection:

- Lysomotropic weak bases (ammonium chloride, chlorquinone, methylamine)
- Carboxylic ionophores (monesin, nigericin)
- v-ATPase inhibitors (bafilomycin A1 and concanamycin)

Inhibition of infection does not necessarily mean that the virus undergoes a pH-induced conformational change. Inhibition can also be caused by secondary effects:

- Defective receptor recycling
- Inhibition of endosome maturation
- Inhibition of enzymes with low pH optimum
- Inhibition of Ca^{2+} efflux from endosomes
- pH neutralization of nonendocytic compartments (TGN)

Weak bases raise the pH almost instantly after addition -> useful for time course of acidic activation. They only raise endosomal and lysosomal pH and inhibit penetration if the pH in the medium remains above 7.0 (use of well buffered media necessary).

Cholesterol depletion is used to test whether viruses enter by caveolar/raft mediated pathways. Methyl-β-cyclodextran extracts the cholesterol from the plasma membrane rapidly and efficiently and inhibits not only caveolar/raft mediated pathways but clathrin-mediated pathways as well. The effects of nystatin, an agent binding cholesterol, often used together with progesterone, a cholesterol synthesis inhibitor, are generally milder.

Transfection with **DN and CA constructs and siRNA** allows targeted analysis of individual host cell proteins and pathways. Use of controls is critical, and poor cell viability can be a problem. Validation of siRNA happens via immunoblotting or other techniques (quantify amount of target protein remaining), moreover use multiple siRNAs (avoid off-target effects). Negative data only interpretable with controls (knockdown, never complete knockout).

Proper storage of virus: Acid-activated viruses never in phosphate-buffered media such as MEM without Hepes or Tris buffer, because the drop in pH during freezing may preacidify the virus, leading to losses in infectivity.

Table 6. Perturbants of specific cellular activities

Target	Mode of Inhibition
Clathrin-mediated endocytosis	Chlorpromazine; Expression of dominant-negative adaptor proteins such as Eps15, AP-2, AP-180; Expression of clathrin hub domain (dominant-negative regulation)
Caveolar endocytosis	Expression of dominant-negative caveolin-1 mutant; Cholesterol depletion by progesterone/nystatin
Macropinocytosis	Amiloride
Lipid-raft dependance	Cholesterol depletion by progesterone/nystatin
Dynamin dependance	Expression of dominant-negative dynamin-2; Dynasore
Kinases	Staurosporin
Tyrosine kinases	Genistein
Phosphatases	Okadaic acid
Tyrosine phosphatases	Vanadate
Rho-like GTPase signalling	Expression dominant-active and negative Cdc42, Rac1, RhoA
Endosomal traffic	Rab mutants (Rab4, Rab11: recycling endosome; Rab5: early endosome; Rab7: maturation of endosomes; Rab9: communication between late endosome and Golgi)
Dependance on acid activation	Chloroquine; Ammonium chloride; Bafilomycin A1; Monensin
Actin dependance	Cytochalasin D; Latrunculin A; Jasplakinolide
Microtubuli dependance	Nocodazole