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Ari Helenius' Lecture Part 2:
Virus Internalization

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1. Keywords and Terms

Virus, host cell machinery, virus entry, receptor mediated endocytosis, pinocytosis, phagocytosis, early endosome, late endosome, lysosomes, penetration, cytoskeleton, GTPases, fusion machinery

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Introduction

Binding of viruses to the cell surface is in most cases followed by endocytosis and penetration of the viral capsids into the cytosol from an intracellular organelle such as an endosome. Once inside the cytosol, the viruses or their capsids move to their site of replication and uncoat. For most DNA viruses this is the nucleus. Most RNA viruses replicate in different cytoplasmic locations.

The entry program of a typical animal virus

The major stages in the entry of a typical virus are as follows:

- binding to cell surface
- lateral movement
- activation of signaling
- virus endocytosis
- penetration (the virus or the capsids released into cytosol)
- intra cellular transport
- genome uncoating

The entry of viruses thus involves multiple, consecutive steps. Note that while some viruses can penetrate directly through the plasma membrane, the endocytic entry is the rule.

Endocytosis

Endocytosis is a process by which fluid, solutes, membrane, and particles are internalized by invagination of the plasma membrane and formation of closed vesicles or vacuoles.

Receptor-mediated endocytosis

Receptor-mediated endocytosis is a special category of endocytic processes that depends on binding of the cargo (ligand) to cell surface molecules (receptors). The ligand/receptor complexes are then internalized together. This is very important because the receptors help to concentrate specific ligands on the cell surface even if these are present in very low concentrations, and to mediate and control the uptake process. The actual mechanisms by which endocytosis occurs can involve clathrin-coated pits, but other mechanisms are also possible.

Cellular endocytic pathways

Cells support many endocytic mechanisms. They are typically divided into phagocytosis (cell eating) and pinocytosis (cell drinking). Phagocytosis is the uptake of large particles into large tight-fitting vacuoles (ex. bacterial uptake by macrophages). It is restricted to specialized cell types such as certain immune cells. Pinocytosis includes a wide spectrum of mechanisms and cellular machinery. Clathrin-mediated endocytosis is the best characterized and is used by many viruses (see lecture 1 for examples). Some viruses can make use of different endocytic pathways in the same cell. Others use cell-type specific endocytic mechanisms.

Endosomes

After release from the plasma membrane, the cargo-containing endocytic vesicles and vacuoles usually fuse with endosomes. These are components of a highly complex and dynamic network of organelles forming the endocytic pathway. In addition to early endosomes, the pathway contains late endosomes, recycling endosomes, and lysosomes.

Early endosome: Almost all incoming-cargo is first delivered to these peripherally located complex organelles that serve as an important sorting station for incoming cargo. They have globular domains and long tubular extensions. Most of the incoming membrane components are recycled back from early endosomes to the plasma membrane either via the **recycling endosomes** or directly. A large fraction of the fluid, solutes, and particles (such as viruses) are directed to late endosomes.

Late endosome: These arise from the globular domains of early endosomes that detach and mature into late endosomes through loss/gain of factors, formation of internal vesicles, and movement into the perinuclear space.

Lysosome: Lysosomes are highly acidic vacuoles filled hydrolases. They fuse with late endosomes to form endolysosomes in which the incoming cargo is digested.

Importantly, the pH of each compartment in the pathway decreases as one progresses from early endosomes to lysosomes. In early endosomes the pH is 6-6.3, while in lysosomes it is lower than 5.0. The pH threshold for activation of the penetration

process varies between 6.5 and 5.2 or so depending on the virus type. This dictates in which organelle penetration occurs.

The primary endocytic organelles of macropinocytosis and phagocytosis (macropinosomes and phagosomes) are unique. However they too can feed into the classic endosomal system. The trafficking of these specialized organelles depends on the cell-type and cargo.

Which endocytic mechanisms are used?

It is important to realize that endocytosis displays great complexity with several different mechanisms working in parallel. Each endocytic mechanism relies on a distinct subset of cellular factors. As mentioned in lecture 1, there are a variety of biochemical and genetic tools that one can use to perturb the cell. By determining a perturbation sensitivity profile one can define the endocytic mechanism that a particular virus is using.

The cellular factors used to define endocytic pathways include:

- Components of the endocytosis machinery (clathrin, caveolin, dynamin)
- Cytoskeleton (actin, microtubules)
- Signaling molecules (kinases, phosphatases)
- Regulator factors (Rho, Rab, and Arf GTPases)
- Ion channels (Na_+/H_+ exchangers, Ca_{++} channels)
- Inhibitors of endosomal acidification (vATPase inhibitors, Chloride channels)
- Lipids (cholesterol, phosphatidylinositides)

Example 1: Endocytosis of HPV16

HPV16 uses non-coated vesicles for endocytosis, and moves to late endosomes before penetration. With a half time of particle internalization of 3 hours, and a half time of acidification of 10 hours, entry of HPV16 is unusually slow. The cellular factors required were determined using the perturbation mechanisms described in lecture 1. The profile showed that this virus uses a novel endocytic mechanism; a non clathrin, non caveolin pathway. It is likely that other viruses utilize this mechanism as well.

Virus Penetration

After endocytosis, viruses are still separated from the cytosol by a membrane. To transfer their capsids into the cytosol they have to penetrate this membrane. What follows represents one of a few stages in the entry program where the virus actively participates. In response to low pH or other cues, the spike glycoproteins of enveloped viruses undergo a conformational change and become membrane fusion-active. They then mediate the fusion of the viral envelope with the limiting membrane of the organelle in the lumen of which they are located. Non-enveloped viruses respond to the cues by lysing the membrane or by generating a pore through which the genome can be translocated into the cytosol.

The site of virus penetration varies depending on the virus

- Plasma membrane (Herpesviruses, some retroviruses, paramyxoviruses)
- Early endosome (Alphaviruses, rhabdoviruses)
- Late endosome (Influenza A, flaviviruses)
- Lysosome (possibly parvoviruses)
- Endoplasmic reticulum (polyoma viruses)

Example 1: Non-enveloped virus penetration by endosome lysis

Adenoviruses 2 and 5 are non-enveloped viruses that cause common cold symptoms. The viruses are endocytosed by a clathrin-mediated process. Low endosomal pH causes a change in the virus particle. This makes components of the capsids capable of inducing lysis of the endosomal membrane. The virus then escapes from the ruptured endosome, and moves to the nuclear pore complexes where it is uncoated. As the lysis is compartmentalized and transient, it apparently does not damage the cell.

Example 2: Non-enveloped virus penetration by pore formation

Poliovirus uses a pore forming mechanism to deliver its genome into the host cell cytosol. Upon activation via receptor binding, capsid components insert into the endosomal membrane forming a pore through which the RNA genome passes. The empty viral capsid remains in the endosomal compartment.

Example 3: Enveloped virus fusion at the plasma membrane

In the case of HIV, the fusion activity of the surface spike glycoproteins is activated upon consecutive binding to two receptors. This triggers a major conformational change that results in fusion of the virus envelope with the plasma membrane. HIV can also enter by endocytosis and fuse in endosomes.

Example 4: Enveloped virus fusion in endosomes

The Influenza virus hemagglutinin, a spike glycoprotein, is responsible for both virus binding to cell surface receptors and for penetration by membrane fusion. Influenza is endocytosed by a clathrin-mediated and by a non-clathrin mediated endocytosis mechanism. Upon reaching the late endosome, the hemagglutinin undergoes an acid-triggered conformational change (pH threshold about 5.5). This leads to insertion of the fusion peptide of the glycoprotein into the endosomal membrane, approach of the two membranes, and eventually to a membrane fusion reaction. The viral capsids are delivered into the cytosol, and the viral membrane becomes part of the endosomal membrane.

Viral glycoproteins: Complex membrane fusion machines

Several viral glycoproteins have been crystallized. The structures show that while they share the overall functional principles, they are structurally different. Importantly, the energy necessary to mediate fusion is provided by the conformational change, and therefore does not requires energy in the form of ATP. The reactions are, as a rule, irreversible, which means that unlike cellular fusion machines, viral fusogens can only induce a single fusion event.

Mechanism of class II viral fusion

The fusion reaction mediated by a so-called class II viral fusion protein is discussed here as an example. It involves a multistep process beginning with the low pH-triggered conformational change in the SFV spike glycoprotein complex that leads to a change in oligomeric structure of the fusion protein E1 subunits. In the homo-trimeric structure generated, each E1 subunit exposes a fusion peptide, a hydrophobic loop previously hidden in the structure. The fusion peptides insert into the target membrane, and several trimers together then undergo a cooperative change pulling the viral and host

membranes into close proximity, which results in the fusion of the outer bilayer leaflets. This is known as hemifusion. Fusion between the two remaining leaflets occurs next, and in the final step the fusion pore expands to let the viral capsid pass through.

Example: Visualization of influenza virus fusion in a live cell

To visualize the fusion of an incoming influenza virus in live cells, investigators used a self-quenching fluorescent dye to label the envelope. After endocytosis, the virus could be seen to enter the perinuclear region of a cell. Fusion was seen as a “flash” of fluorescence caused by de-quenching the dye as it is diluted out in the late endosome membrane.

Intracellular transport of viral capsids

After penetration, viral capsids delivered into the cytosol need to move to the site of genome uncoating and replication. For many viruses this means moving long distances through the cytosol to the nucleus. Once again, viruses take advantage of existing cellular machinery to achieve this. In many cases, microtubules and dynein-based motors (Discussed in Ron Vale’s Lecture 1: Introduction to Motor Proteins) are hijacked to move the capsids to the nuclear pore complex.

Nuclear import of viral genomes

To transport their genome into the nucleus, most viruses use the nuclear pore complexes (NPCs). There are several different ways in which viruses ensure genome delivery into the nucleoplasm.

- Influenza virus: The genome is composed of 8 separate RNAs individually packaged as ribonucleoproteins (vRNPs) in small enough particles to be able to move through the NPC using existing cellular transport machinery.
- Herpes simple virus: Capsids are too big to enter through the NPC. They dock on the cytoplasmic side of the NPC, open up one of the vertices, and releases the DNA into the nucleoplasm. The empty capsid remains associated with NPC for some time.
- Adenovirus: Capsids associate with NPC and then disassemble. The DNA is released and transported into the nucleus.

- Parvovirus: Viruses are very small, and it is likely that the entire virus is transported through the NPC

Words you should be able to define

Virus, virus entry, virus uncoating, virus replication, virus genome, capsid, spike glycoprotein, endocytosis, early endosome, late endosome, lysosomes, macropinosome, phagosome, fusion, fusion peptide, hemifusion, penetration, intracellular transport, nuclear pore complex.

3. Review Questions

1. Describe the process of receptor-mediated endocytosis and the role played by the receptors in this process.
2. Endocytosis involves either pinocytosis or phagocytosis. What are some of the major differences between these?
3. What “classical” endocytic organelles does a typical virus pass through after endocytosis?
4. How does endosomal pH dictate when and where virus penetration occurs?
5. Name 5 different cellular parameters one can use to define a virus endocytic pathway.
6. Name two common features of viral fusion proteins presented in this lecture.
7. What are the steps of class II viral fusion?
8. Which cytoskeletal elements support intra cytosolic transport of herpes simplex virus 1 capsids?
9. In this lecture several mechanisms of nuclear import of virus genomes are presented. Name two of these mechanisms.

4. Answers to Review Questions

1. Receptor-mediated endocytosis occurs when the cargo internalized constitutes a receptor-bound ligand. The receptors first help to concentrate the ligand on the cell

surface, and then mediate the endocytic internalization of the ligand into the cell. This is how nutrients, growth hormones, and extra cellular proteins are selectively internalized by cells. The receptors are usually recycled back to the cell surface while the ligands are degraded in lysosomes. Sometimes this type of endocytosis depends on the activation of signal transduction cascade through the occupied receptor. Receptor-mediated endocytosis often involves clathrin-coated pits but other mechanisms are also used.

2.
    a. Pinocytic mechanisms are used for the uptake of fluids, solutes, and small particles while phagocytic mechanisms involve uptake of large particles (such as bacteria).
    b. In phagocytosis, the membrane of the endocytic vacuole binds to the surface of the particle in zipper like fashion and wraps the membrane tightly around it. In pinocytosis, that cargo does not define the size and shape of the endocytic vacuole.
    c. All cells are capable of pinocytosis while phagocytosis is restricted to specialized cell types such as macrophages and amoeba.

3. During endocytosis almost all viruses first enter early endosomes. Although many penetrate here, some must continue on to the late endosome for penetration. In some cases, although rare, viruses may even require the harsh conditions of the lysosomes to promote penetration.

4. The pH environment of endosomes becomes more acidic the deeper in the pathway the virus moves. Early endosome have pH 6.0-6.3, late endosomes pH 5.5-5.7, and lysosomes lower than pH 5.0. The majority of animal virus penetration requires acidic pH, but the pH-threshold differs. Therefore endosomal pH dictates where and when and where penetration of a specific virus occurs.

5.
    a. Acid dependence
    b. Endocytosis factors
    c. Cytoskeletal requirement
    d. Signaling molecules
    e. Regulator factors
    f. Lon channels
    g. Lipids

6. All of the viral fusion proteins presented in this lecture are acid-activated, undergo conformational changes to become fusion competent, contain a buried fusion peptide, and operate in an ATP-independent fashion.

7.
    a. **Low pH trigger**: Low pH triggers conformational change or dissociation of the fusion complex exposing the fusion peptides.
    b. **Fusion peptide insertion**: Once exposed the fusion peptides are inserted into the target membrane.
    c. **Pull or Fold back**: The stem regions of the fusion machinery fold, distorting the target and virus membranes to bring the outer leaflets together. Alternatively the cluster of fusion proteins undergo a cooperative change in configuration that brings the membranes together.
    d. **Hemifusion:** The outer leaflets fuse, resulting in the formation of an intermediate stalk-like structure between the virus and target membranes.
    e. **Fusion pore formation**: In the final step of the fusion reaction, the two remaining inner leaflets of the membranes mix, forming a fusion pore through which the virus capsids can pass.

8. Transport of HSV capsids occurs on microtubules with the help of the dynein motor.

9.
    a. Subgenomic vRNP particles small enough to pass through the NPC (Influenza)
    b. Release of genome at NPC (Herpes simplex virus)
    c. Capsid disassembly at NPC (Adenoviruses)
    d. Entire virus or capsids moves into nucleus (Parvovirus)

5. Discussion Questions

1. Why do cells have such a wide array of pinocytic mechanisms?

2. It was mentioned in the lecture that several viruses use more than one endocytic mechanism. How can a virus do this?

3. In the lecture two mechanisms were discussed for the penetration of non-enveloped viruses: endosome lysis and pore formation. Which mechanism do you think is more advantageous and why?

4. One major difference between the classical early and late endosomes is pH, what other differences are there? (This will require additional background reading)

5. What mechanisms could a virus use to associate with and move along microtubules?

6. What advantages could using viruses to study endocytosis have over the use of other classical endocytosis ligands?

6. Answers to Discussion Questions

1. One reason is that the cargo to be internalized comes in many sizes and forms: Large particles and small soluble substances. Solutes that are present in very low concentrations and have to be concentrated on the cell surface, i.e., for which the plasma membrane and its receptors serve as a sort of antenna. Substances that trigger signaling pathways have to be cleared from the surface. Antigens to be digested and presented on the cell surface bound to receptors. Membrane proteins that have to be internalized to change cell surface properties, or to retrieve components of secretory vesicles. Lipids to modify membrane micro domains. Extracellular matrix components. The list can go on….

   It is important to keep in mind that endocytosis represents a highly complex, flexible, and interconnected system. It is the major mechanism by which a cell can sample its environment and respond to it in an appropriate fashion. Endocytosis regulates many processes, including nutrient uptake, cell adhesion, motility, signaling, receptor down regulation, cell-cell recognition, and antigen presentation. Endocytosis influences cell polarity, mitosis, growth, and differentiation.

2. The most obvious possibility is that viruses can use multiple receptors for internalization. To add to this complexity, some viruses such as herpes viruses vary their endocytic mechanism depending on the host cell type

3. There is no textbook answer to this question. In the case of pore formation, however, penetration into the cytosol and uncoating occurs in one common step. The genome is released into the cytosol through the transmembrane pore in an uncoated and presumably decondensed form. In this unprotected and extended state it cannot be moved long distances.

Therefore it is postulated that the pore mechanism is used by simple positive stranded RNA viruses such as polio virus, which replicates in the cytosol. To transport a DNA genome through the cytosol to the nucleus, it has to remain condensed at least until passage through the nuclear pore complex. This may be why DNA viruses do not seem to use the pore mechanism. For them uncoating occurs in the nucleus or at the NPC.

4. There are many differences between the endosomes including morphology, cytoplasmic location, associated molecular factors, and function. Early endosomes tend to be branched organelles that “compartmentalize” their recycling and transport functions. Late endosomes contain additional intra-luminal vesicles thought to be a component of their degradative capacity. Most early endosomes localize to the cell periphery close to the plasma membrane and late endosomes to the perinuclear region. Early and late endosomes contain different Rab GTPases that are important for their trafficking and sorting functions. Early endosomes contain Rab5 and late endosomes Rab7. These two endosome populations also differ with regards to their lipid content with early endosomes containing PI(3)P and late endosomes PI(3,5)P2. These factors are thought to contribute to the differences in recruitment of various cytosolic factors to these two endosome populations. There are many more differences that can be discussed including the fate of these organelles, the fate of their cargo, from what cellular compartment they originate, etc…

5. Microtubules are cytoskeletal filaments that are used to transport various cargoes, membranous organelles, and proteins to specific cytosolic locations. Movement along microtubules is directed by the motor proteins kinesin and dynein. Dynein is responsible for movement from the cell periphery to the cell center. It is important to realize that while they are in endosomes, viruses are already taking advantage of microtubule transport. In this case the endosome serves as a carrier organelle equipped with the machinery needed to move the virus to the appropriate location for uncoating. Once released viral capsids can associate with the microtubule network using different virus-specific mechanisms. Many of the viruses analyzed for microtubule association directly interact with dynein. Some viruses take advantage of host cargo adaptors to indirectly link up with motor molecules. It is also possible that virus capsids are mistaken by the cells as cytoplasmic protein aggregates, which under normal cellular conditions are transported to the perinuclear region of the cell on microtubules.

6. The advantages viruses offer over traditional endocytic ligands include: Infection of the cell as detected by expression of viral proteins provides a clear quantitative endpoint assay. The virus load can be extremely low with less than one particle added per cell. When combined with different perturbation regimens described throughout the lecture (inhibitors, small interfering RNAs [siRNAs], dominant negative/active constructs) and high-throughput screening, infection assays can be used to define endocytic factors in different endocytic pathways. Viral particles are, moreover, often identifiable by electron microscopy without labeling, and fluorescently tagged virions allow single particle tracking in live cells. In addition, a variety of antibodies, mutants, and other tools are generally available.

7. Explain or Teach These Concepts to a Friend

1. Explain the process of receptor-mediated endocytosis.
2. Explain how endosomal pH dictates the timing and site of virus penetration.
3. Explain the mechanism used by poliovirus for penetration.
4. Explain the stages of class II fusion.
5. Explain how adenoviruses deliver their genome to the nucleus.

8. Research the Literature on Your Own

1. The large GTPase dynamin was shown as a major branch point of pinocytic pathways in this lecture. What is the role of this cellular factor in endocytosis?
2. This lecture highlights the dependence of virus endocytosis on cellular factors. What is known about the development of HIV antiviral agents that block virus entry? (Hint: check the literature for HIV entry and antiviral agents).
3. What defense mechanisms do cells have to prevent virus entry and replication?