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Systems biology of virus entry in mammalian cells

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Summary
In this article, we define systems biology of virus entry in mammalian cells as the discipline that combines several approaches to comprehensively understand the collective physical behaviour of virus entry routes, and to understand the coordinated operation of the functional modules and molecular machineries that lead to this physical behaviour. Clearly, these are extremely ambitious aims, but recent developments in different life science disciplines slowly allow us to set them as realistic, although very distant, goals. Besides classical approaches to obtain high-resolution information of the molecules, particles and machines involved, we require approaches that can monitor collective behaviour of many molecules, particles and machines simultaneously, in order to reveal design principles of the systems as a whole. Here we will discuss approaches that fall in the latter category, namely time-lapse imaging and single-particle tracking (SPT) combined with computational analysis and modelling, and genome-wide RNA interference approaches to reveal the host components required for virus entry. These techniques should in the future allow us to assign host genes to the systems’ functions and characteristics, and allow emergence-driven, in silico assembly of networks that include interactions with increasing hierarchy (molecules–multiprotein complexes–vesicles and organelles), and kinetics and subcellular spatiality, in order to allow realistic simulations of virus entry in real time.

Introduction
Virus infections are a major cause of death among mammalian organisms. Recent, unexpected outbreaks of Severe Acute Respiratory Syndrome (SARS) virus, a member of the coronavirus family, and scares that an avian form of Influenza virus may become a pandemic threat, strengthen the opinion that members of any virus family can in principle turn into a life- and population-threatening pathogen. Viruses are different from many other pathogens in that they fully depend on host cellular machineries for their replication and spread. The life cycle of a virus is therefore intricately intertwined with host cell biological processes at all stages, and these processes should not be seen separate from each other.

Classical virology has mainly focused on the virus itself, thereby ignoring many aspects of the much higher complexity of critically important processes from the host cells. It is clear that besides virus-receptor binding, virus envelope fusion or capsid membrane penetration, and viral genome replication, not much else can be predicted with classical models of viruses. The fact that viruses of similar families can enter via completely different pathways, or that viruses from different families can enter via similar pathways, can only be understood and predicted if information is available from systematic and comprehensive studies on the cell biology behind virus entry. We predict that this will be a very important focus of future virology research, as it will allow us to formulate new ways of classifying viruses, namely according to the entry pathways and host machineries that they hijack, and to focus on a new type of antiviral treatments that target crucial host cellular components instead of viral components. The latter may not only lead to broad-spectrum antiviral compounds, inhibiting the infectious entry of a range of ontologically different viruses that all rely on a similar entry route, but may also be less hampered by the development of viral resistance. Ideally, such research might in the future prepare us against all viruses by generating an arsenal of drugs that can inhibit all the potential entry routes. A quick analysis of the host genetic profile of a new emerging virus will immediately tell us which pathway is hijacked and which drug to take for antiviral treatment.
ings from these studies as summarized in Fig. 1 must be combined with findings from high-resolution techniques such as cryo-electron microscopy and X-ray crystallography to obtain a full understanding of virus particle physics.

**History of single-particle tracking (SPT)**

In the early 1980s, the first preliminary studies on low-density lipoprotein (LDL)-receptor mobility tracking fluorescent LDL particles bound to the cell surface using time-lapse imaging were reported (Gross and Webb, 1988). The first steps towards digital imaging and image analysis were then taken by tracking the receptor mobility of univalent and multivalent fluorescent particles on the cell surface: LDL that binds to a well-defined receptor, and influenza virus particles that bind to heterogenous sialic acid containing receptors. Analysis of the intensities of individual fluorescent spots permitted accurate determination of the particle position to within ±25 nm, revealing that LDL and influenza virus receptors displayed directed motion as well as motion restricted to a domain on the surface of dermal fibroblasts at 4°C (Anderson et al., 1992).

Nowadays, the combination of high-resolution laser-scanning time-lapse microscopy and analysis of trajectories obtained by tracking single fluorescent particles on the surface of living cells allows us to study the movement of particles, and the molecules attached to them, with submicron spatial resolution (Kusumi and Sako, 1996; Saxton and Jacobson, 1997). By this technique, so-called transient confinement zones (TCZ) were resolved in the plasma membrane of cells, zones of ~200–300 nm in diameter in which particles, preferentially those attached to GPI-anchored proteins and the glycosphingolipid GM1 were trapped for ~5–10 s (Simson et al., 1995; 1998; Sheets et al., 1997). On the basis of SPT, several groups have postulated that actin filaments on the cytosolic surface partition the plasma membrane into subcompartments, allowing rapid local diffusion of proteins and lipids.
Several lines of evidence led to the conclusion that TCZs, as detected by the particle trajectories, reflected the presence of lipid microdomains. First, they were dependent on appropriate levels of cholesterol. Second, the tendency to reside in TCZs depended on the membrane molecule investigated: the GPI-anchored protein Thy-1 and the glycosphingolipid GM1 had a significantly higher tendency to reside in TCZs as compared with non-raft phospholipid analogues. Third, the mobility of particles was reduced as it entered a TCZ, likely caused by the diffusional restriction that the liquid-ordered, cholesterol-enriched phase imposed on them (Dietrich et al., 2002). Based on the finding that TCZs encompass cholesterol-dependent liquid ordered regions of ~200 nm in dimension, a model has evolved in which the membrane-apposed cytoskeleton provides a cytoskeletal barrier with transmembrane proteins linked to the underlying cytoskeleton constituting a picket (‘picket model’). This barrier restricts the diffusion of GPI-APs residing in the outer monolayer of the plasma membrane (Sheets et al., 1997). One alternative scenario involves the partitioning of the cytosolic surface of the plasma membrane by the apposed actin filaments either actively through actin- and lipid-binding proteins or passively by forming a grid on the inside surface of the plasma membrane, and thus preventing free diffusion of bulky proteins and complexes (‘fence’ or ‘corral’ model) (Sako and Kusumi, 1994; Kusumi et al., 2005). Indeed, cytoskeletal components including actin, annexin and filamin were found associated with rafts and/or caveolae (Oliferenko et al., 1999; Stahlhut and van Deurs, 2000; Rodgers and Zavzavadjian, 2001).

Monitoring events on the cell surface

In a recent study Ewers et al. provided experimental evidence that the model described above also holds true for virus particles bound to their cell-surface receptors of live cells and on artificial membranes. A combination of TIRF microscopy and single fluorescent particle tracking revealed different modes of motion of individual murine polyoma virus-like particles (VLPs). Immediately after binding, the VLP-receptor complex displayed 5–10 s of rapid, random diffusion, followed by its confinement to areas of 30–60 nm in diameter. Consistent with the ‘picket’ or the ‘fence’ model outlined above, this process was strictly dependent on a functional actin-filament cytoskeleton. Thus, particle trajectories can provide information about the lateral mobility of membrane components and about constraints imposed on them by cytoskeletal elements, cholesterol-rich microdomains or other structures that entail heterogeneity to the plasma membrane (Edidin et al., 1991; Sako and Kusumi, 1994; Dietrich et al., 2002; Ewers et al., 2005). However, it should be noted that viruses have multivalent binding sites and it is currently not understood how the valency of ligands influences their lateral mobility with respect to their partitioning properties into cholesterol-rich microdomains.

Monitoring individual internalization events

In addition to studying the behaviour of virus particles bound to their receptors on the plasma membrane, the ability to monitor individual viruses in real time also provides previously unavailable information about the dynamics of endocytic processes and how viruses are targeted to the endocytic machinery. As such, digital time-lapse fluorescence microscopy was used to simultaneously track individual influenza viruses and endocytic structures in living cells. Tracking of individual viruses and single clathrin-coated pits (CCP) showed that the fraction of influenza viruses that exploited the clathrin-mediated pathway was internalized through de novo formation of CCPs, which formed around the viruses 2–3 min after viral binding. Notably, the CCP formation rate was much higher at the sites of bound viruses than elsewhere on the cell surface, suggesting that the formation of CCP or the stabilization of tiny undetectable clathrin patches which may be randomly initiated, was a virus-induced mechanism (Rust et al., 2004).

In another recent report on the real-time visualization of cargo sorting and endocytosis by CCP, the authors found the clathrin-based endocytic machinery to be of an exploratory character that initiates coated pits at randomly distributed points within active domains in the plasma membrane. A proportion of clathrin nucleation events were found to be short-lived and collapsed unless they were stabilized as a consequence of cargo capture. Upon cargo capture, the intensity of a cargo-loaded clathrin-cluster grew at a steady state, and its size and the time required to complete clathrin assembly was determined by the size of the cargo (Ehrlich et al., 2004).

Monitoring virus particle dynamics after internalization

After clathrin-mediated endocytosis, it is crucial for many viruses to reach early and/or late endosomes where they undergo acid-activated fusion with the endosomal membrane to release the viral genome (Marsh and Helenius, 1989; 2006). However, the mechanism for transport from one endosomal compartment to another is still unsolved. Here, the vesicular transport model opposes the compartment maturation model in which the membrane composition of the cargo-enclosing membrane progressively changes its composition (Griffiths and Gruenberg, 1991; Murphy, 1991; Schekman and Orci, 1996). Rink et al. 2005).
combined fast live cell imaging techniques with new software tools to visualize and quantify the dynamics of membrane bound rab5 and rab7 during the transport of LDL from rab5-positive early to rab7-positive late endosomes. By tracking early endosomes over long periods of time and combining high-speed 4D imaging with novel tracking algorithms the conversion from rab5 to rab7 was found to be the key mechanism for the remodelling of membrane identity and cargo progression between early and late endosomes. At the same time, degradative cargo became enriched in larger endosomes by repetitive fusion events that migrated from the cell periphery to the centre (Rink et al., 2005). However, in another study addressing the sorting of Semliki Forest Virus (SFV) from early to late endosomes using triple-colour, video-enhanced fluorescence microscopy in live cells, SFV progressed from rab5-positive early endosomes to a population of early endosomes that contained rab5 and rab7. Sequestration of SFV in the rab7-positive domain and subsequent detachment of the virus-loaded rab7-positive carrier vesicle targeted the cargo to late endosomes, favouring the vesicular transport model (Vonderheit and Helenius, 2005). More recently, Zhuang and colleagues proposed another model in which early endosomes are not comprised of a uniform pool of endocytic vesicles but instead constitute two populations that are characterized by distinct mobility and maturation kinetics. By a pre-early endosome sorting mechanism at the plasma membrane, ligands destined for degradation are preferentially targeted to the smaller population of dynamic rapidly maturing early endosomes whereas ligands of the recycling pathway are non-selectively targeted to all endosomes and effectively enriched in the larger population of static, slowly maturing early endosomes (Lakadamyali et al., 2006).

After internalization, one of the obstacles viruses encounter before they can target their genome to the nucleus is the diffusional barrier imposed by the cytoplasm, which because of its high viscosity does not allow viral movement by diffusion (Luby-Phelps et al., 1986; Kao et al., 1993). Greber and colleagues were the first to show that SPT is a powerful technique to investigate the nuclear trafficking of fluorophore-tagged virus particles in living cells by time-lapse microscopy. They showed that after penetration of the endosomal membrane, Adenovirus particles engaged plus- and minus end-directed motor activities that supported alternating minus- and plus end-directed movements along microtubules, finally resulting in Ad2 accumulation near the microtubule-organizing centre (MTOC) (Suomalainen et al., 1999). By a similar mechanism, the reverse transcription complex of HIV particles remained its interaction with microtubules after capsid dissociation and ultimately reached the MTOC from where it then could enter through nuclear pore complexes and integrate into the host DNA (McDonald et al., 2002).

Other viruses that modulate bidirectional capsid transport to favour either retrograde (minus-end) or anterograde (plus-end) motion are herpesviruses. Association with a bidirectional transport apparatus allows them to travel either to sensory ganglia in order to establish latency or to peripheral tissue for reactivation from latency. A collection of recombinant pseudorabies viruses, an alphaherpesvirus of swine, expressing mRFP fused to a capsid protein, and GFP fused to one of five different viral tegument proteins, was tracked in axons of primary sensory neurons. The correlated motion between red and green fluorescence revealed that distinct composition of the capsid-transport complex during entry and egress phases of infection controlled bidirectional capsid transport in axons. The tegument proteins VP1/2 and UL37 remained associated with capsids as they traveled toward the nucleus and may be responsible for the interaction with the host's transport machinery. Three other tegument proteins, VP16, VP13/14 and VP22 were found to be removed from the capsid surface before retrograde transport and their association with progeny capsids may direct viral egress to the periphery late in infection (Luxton et al., 2005). In another recent study an in vitro assay was developed that allows the analysis of the direct interaction between the viral capsid of herpes simplex virus type I with microtubules. This study showed that capsids exposing inner tegument proteins can recruit dynein and dyanin whereas capsids covered by outer tegument proteins or capsids devoid of tegument do not provide good substrates for transport along microtubules (Wolfstein et al., 2006).

Single-particle tracking experiments can also provide new insights into the regulation mechanisms for the nuclear import of viral ribonucleoproteins (vRNPs), which direct viral replication and expression in the host's nucleus. The time trajectories revealed that single influenza vRNPs were transported by diffusion, both in the cytoplasm and in the nucleus and directly interacted with the nuclear envelope via nuclear pore complexes, with dissociation rate constants ranging from 0.01 to 1 s⁻¹. In the late stage of infection, when the regulatory influenza M1 protein is expressed, the interactions between the vRNPs and the nuclear envelope were significantly inhibited, suggesting that M1 downregulated the nuclear import of vRNPs by directly inhibiting its binding to NPCs (Babcock et al., 2004).

The examples outlined above demonstrate that single virus particle tracking is a powerful tool to obtain information on the lateral mobility of viruses bound to their receptors and also to get insights into the interplay of virus particles with their host cell components. Thus, this technique provides valuable information not only on the virus-
host interactions but also on the cell biology behind it. Our next challenge will be to analyse the collective behaviour of all virus particles, and not just a few as has been done until now, and to identify the host components that govern the emerging physical properties of virus entry. This will generate a situation where interaction networks of genes and proteins can be integrated with kinetic, physical networks of particles and organelles.

**Host genetic profiling of infectious virus entry**

The physical behaviour of virus particles is not only dictated by the proteins, sugars, lipids, metabolites and ions that interact directly with virus particles, but by the whole machinery that underlies the cellular mechanisms that virus particles hijack. This implies that we have to consider all the signalling cascades, the cytoskeleton, and physiological processes that impinge on the cellular mechanisms that are being hijacked during virus entry. For instance, we found that nutrient sensing controls the machinery behind clathrin-mediated endocytosis (Pelkmans et al., 2005), and thus nutrient sensing is also involved in the infectious entry of vesicular stomatitis virus (VSV). It may be clear that models that describe virus particles only, can certainly not make such predictions.

The advent of RNA interference (RNAi) in mammalian cells (Elbashir et al., 2001) allows us to systematically eliminate each gene of the host cell's genome (Carpenter and Sabatini, 2004), and to study how this affects infectious entry of viruses (Pelkmans et al., 2005). Such a measurement is a direct reflection of the function of a host gene in the infectious entry process, and is in strong contrast to mRNA profiling using cDNA microarrays, which can only record activity changes in host gene transcription during infectious virus entry, and does not generate direct functional insights of a gene. Collectively, the measurements of the function of all host genes are a direct reflection of the cellular processes involved in the entry process, including signalling cascades, the cellular cytoskeleton and the endocytic machinery. Host functional genetic profiling with RNAi screens is thus the only means to systematically obtain a comprehensive picture on which cellular pathway each virus utilizes.

**Comparing two virus entry routes: simian virus 40 (SV40) and VSV**

We recently presented a proof-of-principle to the approach outlined above (Pelkmans et al., 2005). By comparing the involvement of the human complement of protein, lipid and carbohydrate kinases (the kinome) in the infectious entry of SV40 and VSV, we could reveal the host complexity behind these two entry routes. A remarkable finding was the limited overlap of kinases controlling both pathways. A unique set of 92 kinases specifically regulated VSV infectious entry, while a set of 80 kinases specifically regulated SV40 infectious entry. Moreover, of the 36 kinases that are involved in both infectious entry pathways, 23 (two-third) have opposite effects, being required for one pathway, while suppressing another. We knew from previous studies that these viruses hijack different endocytic routes to enter their host cells (Pelkmans et al., 2001; Sieczkarski and Whittaker, 2003), but not to what extent the regulatory circuitry of the two routes would be different. Therefore, these observations not only strengthen our view of the existence of multiple endocytic routes operating in parallel, but also indicate that these routes are linked to very different physiological processes.

**Linking virus entry pathways to cellular physiology**

While we are currently still exploring which computational approaches and bioinformatics sources are most useful to interpret such data, and which new tools to develop, we did attempt an interpretation at the systems-level using information from human genome annotation databases and hierarchical clustering of phenotypes (Pelkmans et al., 2005). It should be noted that the annotation of the human genome is currently very poor. Most human genes (approx. 70%) have no known function, while a few genes have been studied extensively, resulting in conflicting functional annotations for those genes. Nevertheless, we asked the question whether a group of kinases acting together in a particular signalling system, would have a similar effect on the activity of a particular virus entry pathway. Surprisingly, such links did indeed emerge from the large data sets. For instance, nutrient sensing specifically controls clathrin-mediated endocytosis, and thus infectious entry of VSV, while cell adhesion signalling specifically controls caveolae/raft-mediated endocytosis, and thus infectious entry of SV40. The systems of Ca\(^{2+}\)-signalling and the assembly/disassembly of the cortical actin cytoskeleton were found to act oppositely on the two entry pathways. Cell cycle signalling mainly regulated clathrin-mediated endocytosis and VSV infectious entry, with little effect on caveolae/raft-mediated endocytosis. See Pelkmans et al. (Pelkmans et al., 2005), for detailed information. In summary, this was the first time that a systems level view on the regulation of endocytosis and infectious virus entry was obtained, and illustrated how intricately these pathways are linked to various specific cellular processes.

**Towards a new virus family tree based on host requirements**

As one can group genes together with similar RNAi phe-
Hierarchical clustering is used to statistically correlate different viruses according to the phenotypic RNAi profiles of all genes silenced. The more HOST genes are equally important for the infectious entry of a set of viruses, the more likely it is that these hijack similar entry routes. Thus, clustered ‘functional host genetic profiles’ generates groups of viruses that hijack similar pathways, according to ‘host requirement ontology’. Secondary RNAi screens of the identified components with quantitative light microscopy-based assays are used to map the pathway in detail (right). The assays measure a series of physical parameters of the pathway, which generate quantitative information for the RNAi phenotype of each gene. Hierarchical clustering can also be used to statistically correlate different genes according to how their RNAi phenotype changes the different physical parameters. The more the combined changes across the whole series of physical parameters are similar between a set of genes, the more likely it is that these genes function together as so-called ‘functional modules’. These functional modules will reflect machineries and signal cascades that underlie and control different parameters such as entry, membrane traffic, or membrane penetration.

Previously, the real complexity of infectious virus entry comes from the host components and machineries that are hijacked, which cannot be predicted from viral ontology. Setting up a system to group viruses according to ‘host requirement ontology’ would reveal which viruses hijack which entry pathways, and might be able to reveal the total number of virus entry pathways existing in mammalian cells. Furthermore, those host genes that are similarly involved in the infectious entry of all viruses of one group can be expected to represent the core machinery of the entry pathway, while those host genes that are uniquely or differently involved in the infectious entry of this group represent upstream and downstream variations of the commonly hijacked pathway. Upstream variations may represent virus receptors and specific signalling downstream from these receptors, while downstream var-
A new class of broad-spectrum antivirals, also useful against new emerging viruses

Systems biology of virus entry will also be a very promising approach to find a new class of antiviral treatments. Almost all current antiviral strategies, consisting either of vaccines, drugs, or a combination of both, target the virus, or virus-host receptor interactions. The advantage of this approach is obvious: Targeting the pathogen will have little side-effects in the host organism. However, a big disadvantage has also become clear: Because especially viruses have in most cases the ability to mutate quickly – their evolution is many orders of magnitude faster than that of the host organism – they become quickly resistant to the antiviral treatment. This is especially true for drugs that target the activities of viral enzymes, such as polymerases or proteases, but also for molecules that inhibit fusogenic activities of envelope glycoproteins.

Targeting host components will not be hampered by a rapid development of viral resistance, because it is very unlikely that a point mutation in the viral genome will be sufficient to hijack a completely new entry pathway. For instance, SV40 requires a special redox environment and the presence of chaperones that participate in capsid uncoating, and transmembrane channels that dispose of incorrectly folded proteins (Schelhaas et al., in preparation), which can only be found in the endoplasmic reticulum. If we targeted a key host component crucial for trafficking to the endoplasmic reticulum, SV40 would have to develop a completely new strategy, relying on different cues and factors for uncoating and membrane penetration. If unsuccessful, SV40 particles will likely end up in lysosomes (Pelkmans et al., 2004), where they are degraded. Such a drastic change of entry strategy cannot be brought about by a couple of point mutations.

Clearly, targeting host components will result in side-effects due to the interference with cellular processes. While it remains to be shown how disadvantageous this will be, it might be less a problem than often feared. The reason is that the robustness of many systems in a cell allows the temporary inactivation of one component. Many viruses however, due to their simplicity, do not have backup strategies in place to bypass an entry pathway block. The robustness/simplicity argument may well imply that where single host targets are not very suitable to fight complex host-derived diseases (such as cancer), they might be very suitable to fight infections of simple pathogens (such as viruses), without causing major, unacceptable side-effects in the host.

It should be mentioned that the above might not hold for viruses that are able to hijack different entry routes depending on the tissue culture cell type that is infected (such as HIV or HSV1). For such viruses, it may be beneficial to determine the functional host genetic profile for infectious virus entry in several different cell types to determine those components that are commonly used. For instance, we observed that SV40 infectious entry in cells devoid of caveolin-1, which occurs via a caveolae-independent endocytic route (Damm et al., 2005), is equally regulated by a same subset of kinases as in cells expressing caveolin-1 (Pelkmans et al., 2005). It may well be that while entry routes in different cell types do seem to differ in the dependence on some proteins or in their kinetics, they might actually not be that different when examined on a more global, or genome-wide scale.

Kalman and colleagues have recently presented a proof-or-principle that a strategy to target the host instead of the virus does indeed work in a whole organism (Reeves et al., 2005). They found that Vaccinia Virus infection of tissue culture cells is dependent on the host kinase c-Abl, which is essential for the formation of actin comets that are used by vaccinia virus particles for intercellular spread. Treatment of mice with Gleevec, a specific c-Abl inhibitor that is on the market as an anticancer drug, drastically reduced Vaccinia Virus infection, without causing major side-effects in the organism.

Virus family trees according to ‘host requirement ontology’ will also reveal host targets crucial for a group of viruses entering via one pathway. These common host requirements would be ideal targets for the development of broad-spectrum antiviral drugs. All viruses, ontologically related or not, that enter via that pathway will be inhibited in their infectious entry using such a drug. Even more, if sets of drugs can be developed that can target all the possible entry pathways, we will be prepared in the rare case a virus can hijack an alternative route or is able to mutate drastically enough. And, we will be prepared for any new emerging virus. Once the functional host genetic profile of that virus is determined, this can become a routine diagnostic approach in the future, we know which entry pathway it takes, and thus which antiviral drug to use.

A look into the future

In recent years, many technologies that were originally developed in other disciplines have found their way into research on the cell biology of virus entry. Several of them were discussed here, quantitative light microscopy and SPT, and functional genomics based on RNAi. These
technologies, combined with computational image processing and analysis (Sbalzarini and Koumoutsakos, 2005), bioinformatics, network biology (Ideker et al., 2001) and mathematical modelling (Dinh et al., 2006), are also the most promising to make cell biology a systems discipline. This is where the main challenges lie for the future. We can automatically track some virus particles, and obtain some quantitative parameters from their movement, but we are far from monitoring collective behaviour, and even further away from discovering the physical or mathematical principles that underlie collective behaviour. The same holds true for the collective behaviour of cellular membrane domains, vesicles and organelles (Dinh et al., 2006), which we need to understand in order to know the behaviour of virus particles that hijack these systems. In addition, we must obtain a comprehensive understanding of all the influences to which a system of moving particles and vesicles is exposed. Knowing the proteins, and understanding the molecular machineries, and their interactions to form functional modules (e.g. membrane microdomains, vesicle coats, organelle subdomains), will be a major step forward. Ultimately however, this understanding must be translated into the physical properties of the systems observed. A systematic annotation of the components with particular physical characteristics of a virus entry pathway will be very helpful to reach that goal, requiring a full integration of the main technologies that we have described here.

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