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Subversion of the actin cytoskeleton during viral infection

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Abstract

Viral infection converts the normal functions of a cell to optimize viral replication and virion production. One striking observation of this conversion is the reconfiguration and reorganization of cellular actin, affecting every stage of the viral life cycle, from entry through assembly to egress. The extent and degree of cytoskeletal reorganization varies among different viral infections, suggesting the evolution of myriad viral strategies. In this Review, we describe how the interaction of viral proteins with the cell modulates the structure and function of the actin cytoskeleton to initiate, sustain and spread infections. The molecular biology of such interactions continues to engage virologists in their quest to understand viral replication and informs cell biologists about the role of the cytoskeleton in the uninfected cell.

The shape and movement of cells, as well as phagocytosis, intercellular communication and the distribution of organelles, depend on actin[1] (for history, see BOX 1). Actin persists in the cell as two different forms: monomeric globular actin (G-actin) and polymeric filamentous actin (F-actin) (FIG. 1). F-actin is composed of two parallel strands of actin monomers. The directionality of the filament is determined by the orientation of the monomers, with the positive end being that opposite the end with the ATP-binding pocket. Polymerization begins with actin monomers being stabilized by an initiation complex, of which there are many. The initiation complex that is most often described as interacting with viruses is the ARP2/3 complex[2]. On its own, ARP2/3 has little polymerization-stimulating activity, but this activity is enhanced through interaction with multiple polymerization induction factors, such as members of the Wiskott–Aldrich syndrome protein (WASP) family and WASP-interacting proteins (WIPs)[3,4] (FIG. 2). Filament formation is promoted and stabilized through the action of proteins such as profilin and cortactin, and the filament is depolymerized through the action of proteins such as cofilin or gelsolin[5,6]. Actin filaments (called microfilaments) also bundle with other actin-interacting proteins, including fascins[7,8], forming more substantial structures. Alternatively, the filaments can be crosslinked by branching, which is initiated by actin-nucleating proteins[9], to form a meshwork such as cortical actin. F-actin fibres form the microfilament network inside the cell, varying from myosin-containing contractile stress fibres to the cortical actin network that resides beneath the plasma membrane and around intracellular organelles (FIG. 3). Actin fibres are also used to make: sheet-like extensions, such as lamellipodia, membrane ruffles and blebs; finger-like protrusions, such as microvilli and filopodia; or dot-like

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podosomes. These structures are modified by the action of several actin-binding and signalling proteins.

Box 1

A brief history of actin

Actin was first observed and isolated in 1887 by Halliburton *et al.* as a coagulating activity associated with extracts of muscle tissue[150]. It was not until the 1940s that the term ‘actin’ would become associated with the filamentous material that was isolated from muscle tissue[151]. Under the electron microscope, actin filaments had a consistent width and varied lengths. When mixed with myosin, the filament increased in width and became studded with “nodose structures which in the myofibril may be aligned” (REF. 152), indicative of the structures that are observed in muscle tissue. It was not until 1962 that people began to understand that actin was found in every eukaryotic cell[153] and not until 1973 that the first connection between actin and viruses was reported in the literature[16].

The actin cytoskeleton is highly dynamic and is mainly manipulated by members of the RHO-family GTPases that control signal transduction pathways linking membrane receptors to the cytoskeleton (FIG. 2). RHO-family GTPases regulate several cellular processes, including F-actin polymerization, assembly of intercellular junctions, cell polarity, cell migration and membrane trafficking (reviewed in REF. 10). More than twenty different RHO-family GTPases regulate cytoskeletal dynamics. Among these, the most ubiquitous members are: RHOA, which is responsible for the formation of stress fibres; RAC1, which induces membrane ruffles or lamellipodia; and cell division cycle 42 (CDC42), which regulates the formation of protrusive filopodia[11,12]. Many pathogens, including viruses, have evolved gene products to engage and subvert the actin cytoskeleton and, in particular, the RHO-family GTPase signalling system (reviewed in REFS 13–15).

In this Review, we highlight some of the interactions that are promoted by viral proteins which redirect the structure and function of the actin cytoskeleton. We attempt to connect the historic literature concerning actin with the current advances in the field. We discuss the role of actin rearrangement during the earliest known effect of viruses on cells: transformation. We continue by describing the ways in which actin is manipulated during entry, assembly and egress, the main stages of the viral life cycle.

It is important not to be anthropomorphic when discussing viruses. However, it is difficult not to be when viral infections seem to be so insidious and capable of thwarting or bypassing everything that blocks their replication and dissemination. The title of this Review uses the evocative word ‘subversion’ more to attract attention to the amazing cell biology engaged by these small entities than to ascribe any purpose to what is observed.

Viral infection and cell transformation

The first links between viral infection, cell morphology and changes in the actin cytoskeleton were realized in the early 1970s with the description of ‘transformation’ (BOX 2). The first-identified transforming viruses were: Rous sarcoma virus (RSV), an avian retrovirus[16]; simian virus 40 (SV40), a primate polyomavirus[17]; and adenoviruses[18]. The most prominent changes in transformed cells are the rounded cell shape, with motile blebs, bulging pseudopodia, loss of contact inhibition and lack of stress fibres. Indeed, transformed cells are immobile in cell culture and pile up on each other, with less pronounced adherens junctions, randomly distributed microtubules, diminished microfilaments and disrupted stress fibres[17–19]. Many of these changes also are seen in mitotic cells but are more

organized temporally[20]. At the onset of mitosis, cells round up, matrix adhesion is lost and a contractile actin–myosin ring is formed.

Box 2

Cell transformation

Virus-mediated cell transformation is a multistep process resulting in abnormally proliferating and morphologically altered cells *in vitro* which share the characteristics of tumour cells *in vivo*. Typically, transformed cells lack contact inhibition, are anchorage independent and form multiple layers in the culture dish, termed foci[17]. Key steps of virus-mediated transformation include deregulation of the cell cycle and inhibition of apoptotic pathways by the action of viral oncoproteins. To achieve such complex deregulation, oncoproteins of small DNA tumour viruses (for example, the small t- and large T-antigens of simian virus 40 (REF. 44), the E1A and E1B proteins of adenoviruses[20,47] or the E6 and E7 proteins of human papillomaviruses[61]) work in an orchestrated manner to interfere with several steps of signal transduction pathways. Incomplete action of these oncoproteins may result in immortalized cells, which do not form foci *in vitro* and are not tumorigenic when injected in animals[47]. In the case of retroviruses, viral RNA genomes contain aberrant forms of cellular proto-oncogenes (such as c-*SRC*, c-*FOS*, c-*JUN* and c-*MYC*) encoding proteins with abnormal functions (for example, the v-SRC protein of Rous sarcoma virus[25]) that cause deregulation of several signalling cascades, resulting in a completely transformed cell phenotype. Cells that are infected and transformed by these viruses do not produce infectious progeny virions and therefore are not lysed as a result of cytopathic effects. Transforming viruses rather stimulate unlimited cell proliferation and persist in these immortal cells as an evolutionary strategy.

These changes are accompanied by an increase in cortical rigidity, which involves actin remodelling mediated by a signalling pathway that involves RHO proteins and RHO-associated protein kinases (ROCKs)[21]. The main questions that emerged from these early studies focused on how viral infection induces dramatic cytoskeletal reorganization and whether these changes are related to the *in vivo* neoplastic properties of these viral infections. This connection seemed obvious because cells establish themselves in a stable state *in vivo* by contacting adjacent cells and the extracellular matrix. The transition from this normal state to an invasive, proliferating phenotype certainly requires drastic changes in the regulation of cell signalling, the cytoskeleton, transcription and the cell cycle, and these changes are probably caused by the specific actions of viral proteins.

RSV, SV40 and adenoviruses

Work with RSV, the causative agent of chicken tumours, revealed the first viral protein that was sufficient to transform cells[19]. Using a temperature-sensitive RSV mutant, investigators proved that the viral *src* (v-*src*) gene product (v-SRC; also known as $pp60^{v-src}$) was responsible for the actin filament reorganization and altered surface topography observed during infection. RSV v-SRC-induced morphological changes occurred in three steps. In the first 3 hours of temperature shift, membrane alterations were obvious, with the appearance of actin ruffles[22]. At later times after the temperature shift, cells rounded up with retraction of the peripheral cytoplasm before the final step, which was marked by the production of microvilli, microspikes and blebs[19,23]. Subsequent investigations revealed that v-SRC was able to ‘dissolve’ actin stress fibres when microinjected into the cytosol of 3T3 cells, confirming that the cytoskeleton and cell motility system were among the main targets of the viral oncoprotein[24]. In the following years, v-SRC was shown to be a membrane-associated phosphoprotein with tyrosine kinase activity[25]. In transformed cells, v-SRC concentrates in

podosomes and rosettes, where it colocalizes with actin-modifying proteins such as vinculin, talin, α-actinin[26,27] and cortactin[28]. Cytoskeletal targets that are phosphorylated by v-SRC include vinculin[29] and cofilin[30]. In normal cells, cellular SRC kinases (c-SRC proteins) regulate the formation of integrin-mediated focal adhesions. Initially, researchers thought that v-SRC changed normal focal adhesions into unique dot-like podosomes in transformed cells. However, similar podosome structures were identified in normal motile cells such as macrophages, osteoclasts[22] and dendritic cells[31], as well as in smooth muscle cells[32] and endothelial cells[33]. Although podosomes are often referred to as 'actin dots', they are in fact complex structures containing an actin core that is surrounded by several cytoskeletal proteins and matrix metalloproteinases[34] that, together with signalling molecules, remodel the cytoskeleton and extracellular matrix as necessary for tissue invasion[35]. Myosin was observed close to the actin core of v-SRC-induced podosomes or naturally occurring podosomes of osteoclasts, but the podosomes of cancerous or transformed cells are usually not contractile (reviewed in REF. 36). Given these observations, these podosomes of cancerous or transformed cells were renamed invadopodia, referring to the invasive properties of those cells[35].

v-SRC induces the loss of stress fibres, not by depolymerizing F-actin but rather by reorganizing actin polymers so that canonical stress fibres are not formed[37]. These F-actin aggregates, located at the adhesion areas of transformed cells, are more resistant to the cytoskeleton-disrupting drug cytochalasin B (TABLE 1) than normal F-actin, confirming that altered polymerization occurs during transformation[38]. This phenotype is linked to the reduction of caldesmon expression in transformed cells, which results in a reduction in calcium-regulated actin reorganization[39]. In addition, other calcium-dependent actin-interacting proteins — gelsolin and α-actinin — are localized at the regions of small stress fibres in RSV-transformed rat fibroblasts[40]. In addition to these changes in calcium-regulated, actin-binding proteins, RSV- or SV40-transformed cells exhibit a substantial decrease in their synthesis of a subset of the tropomyosin family of proteins[8,41]. Tropomyosin in non-muscle cells modulates actin binding to actin-severing, actin-capping, actin-crosslinking and actin-nucleating proteins[42]. Tropomyosin binding to F-actin stabilizes the stress fibres and protects them from disassembly[43]. Reduction of not only tropomyosin but also extracellular matrix proteins (for example, collagen and fibronectin) may contribute to the disruption of both cytoskeletal and extracellular architecture in cells transformed with RNA and DNA viruses[41].

The small t-antigen of the small DNA tumour virus SV40 also engages the actin cytoskeleton during cell transformation. This viral protein alone is responsible for the loss of actin filaments in rat cells[44]. In 2003, it was shown that the SV40 small t-antigen affects protein phosphatase 2A (PP2A), resulting in the loss of tight junctions as well as the stimulated rearrangement of F-actin networks in epithelial cells[45]. These rearrangements include RAC-induced membrane ruffling and formation of lamellipodia, CDC42-initiated formation of filopodia, and loss of RHO-dependent stress fibres. In epithelial cells expressing small t-antigen, levels of RAC1 and CDC42 increased while RHOA levels decreased.

Some types of adenovirus efficiently transform primary rodent cells in culture (reviewed in REF. 46). Adenovirus infection stimulates the cell cycle through the action of the viral E1A proteins (both the 13S and 12S RNA-encoded isoforms)[47]. In addition, E1A proteins also disrupt actin stress fibres[48] and interact with the RAC–CDC42 pathway in transformed rodent cells. These interactions lead to a disorganization of the actin cytoskeleton with increased filopodial and lamellipodial production, thus enhancing cellular motility and contributing to the loss of contact inhibition[49], as seen in cells transformed with RSV and SV40.

EBV, HBV and HPV

Epstein–Barr virus (EBV), a member of the family *Herpesviridae*, infects a large proportion of the human population worldwide and establishes a long-term, quiescent infection in virally transformed B cells. EBV infections induce efficient proliferation and transformation of B cells and are associated with several types of cancer. The first indication that cells transformed with EBV have an altered actin cytoskeleton was noted in the late 1970s. Analysis of EBV-transformed lymphocytes[50] and the sera from patients with Burkitt's lymphoma or nasopharyngeal carcinoma[51] revealed substantial increases in the levels of actin and tubulin in these cells compared with levels in untransformed cells and in the sera of healthy individuals. Surprisingly, actin and tubulin accumulated on the surface of EBV-transformed cells in large quantities[52]. Such an extracellular presence of cytoskeletal proteins has also been observed by other groups and has attracted much attention (reviewed in REFS 53,54). However, the transport route and the extracellular function of these normally intracellular molecules still await elucidation. Moreover, in EBV-transformed B cells, the expression of fascins, which are actin-bundling proteins, is increased by more than 200-fold[55]. This overexpression might result in the induction of membrane protrusions, such as lamellipodia, through the reorganization of F-actin at the cell periphery[56] and possibly increases cytoskeletal rigidity, as evidenced by *in vitro* studies[57]. The changes in EBV-transformed B cells reflect the action of EBV nuclear antigen 2 (EBNA2), a transcription factor that induces several cellular changes which lead to cell proliferation. EBNA2 activates a signalling pathway through the induction of one of the regulatory subunits of phosphoinositide 3-kinase (PI3K), p55α (an isoform encoded by the *PI3KR1* gene), presumably interfering with signalling of the RHO-family GTPases[58].

Hepatitis B virus (HBV) is a hepadnavirus that causes serious liver diseases, including hepatocellular carcinoma, in humans. HBV replication in HepG2 cells activates RAC1 and induces lamellipodia formation and membrane ruffling[59]. The viral protein X interacts directly with RAC1 and enhances virus replication. The cytoskeletal rearrangements that are induced by protein X (for example, the redistribution of F-actin-binding proteins at pseudopodia tips) may contribute to the prominent increased migratory phenotype that is observed in HBV-infected hepatocellular carcinoma cells[60].

The early oncoproteins E6 and E7 of human papillomaviruses (HPVs) trigger malignant transformation by interfering with several cell cycle and survival pathways. E7 affects both cell cycle and RHO-family GTPase signalling cascades. E7 also enhances the cytoplasmic accumulation of p27 (also known as KIP1) (a positive regulator of cell motility and tumour invasion), which in turn inhibits RHOA activity and induces cell migration[61]. RAC1 is also overexpressed in papilloma cells, leading to misregulation of the RHO-family GTPase signalling pathway and thus resulting in elevated cyclooxygenase 2 expression[62].

Actin and the viral life cycle: entry

The first steps of every viral infection involve virions engaging the cell surface, with subsequent penetration of the cell membrane and entry into the cytoplasm. The physical barrier imposed by the cortical actin meshwork must be overcome, and this process often involves the stimulation of actin cytoskeleton remodelling. Virion binding can initiate signalling events that change the cell surface and activate endocytosis, as we discuss below.

Virion surfing

Virion surfing is the physical movement of virions on the surface of cells or cell projections. Remarkably, interactions between viral and cellular proteins stimulate virion movement to previously masked entry sites, where fusion or endocytosis occurs. Viral proteins located on the virion surface bind with high specificity and affinity to cellular receptors that are

associated with actin filaments just beneath the cell surface. The virion movement (surfing) towards entry sites is mediated by internal myosin motors, which are located in the cortical actin or at the base of filopodia and which contract the actin filaments[63] (FIG. 4a). In effect, the movement of the actin cytoskeleton drags the cellular receptor–virion complexes to sites of high endocytic activity. Viruses that have been observed surfing across the cell surface include murine leukemia virus, avian leukosis virus, HIV, vesicular stomatitis virus (VSV)[64], HPV[65] and vaccinia virus (VV)[66]. Surfing is sensitive to cytochalasin D and the myosin II inhibitor blebbistatin (TABLE 1). Treatment with these drugs results in the undirected wandering of virions on the cell surface, with a corresponding reduction in viral infectivity. The virus–cell interaction that initiates such motion is largely uncharacterized. For VV, surface phosphatidylserine is crucial for virion movement and subsequent endocytosis. This unusual lipid, which is normally found on extracellular apoptotic vesicles, stimulates rapid endocytosis. Virions bind and transit to the base of filopodia, where the phosphatidylserine induces signalling mediated by serine/threonine-protein kinases PAK1 and PAK2 to initiate virion engulfment in a ‘bleb’ of plasma membrane. Active actin remodelling is necessary for both steps in this process, as VV infection is inhibited by the above-mentioned drugs[66].

Some non-enveloped virions also move on the cell surface and induce actin remodelling for entry. Cellular entry of coxsackie viruses (which are picornaviruses) and adenoviruses relies on interactions with the same cellular receptor. Coxsackie virus and adenovirus receptor (CAR) is located at tight junctions between epithelial cells[67], where it is effectively hidden from extracellular virions. To gain entry via this receptor, virions first bind the glycophosphatidylinositol (GPI)-anchored complement decay-accelerating factor (DAF), which is easily accessible on the apical cell surface. This interaction activates the tyrosine protein kinase ABL, which promotes reorganization of the cytoskeleton through the activity of RAC GTPases. DAF-bound virions move laterally to tight junctions to engage CAR[68]. Subsequent internalization of adenoviruses and of some coxsackievirus serotypes requires clathrin-mediated endocytosis[69] (FIG. 4b).

Virion endocytosis

The engagement of endocytosis for virion entry is a subject of research and debate. Evidence for the endocytosis of infecting virions is widespread and is often actin dependent or actin stimulated (for a comprehensive review, see REF. 70). For example, entry of Kaposi’s sarcoma-associated virus, a herpesvirus, is sensitive to the action of drugs that disrupt actin filaments[71] (for example, cytochalasin D, latrunculin B and jasplakinolide (TABLE 1)). Similarly, entry of Ebola virus particles and VSV pseudovirions is stimulated when RHOB and RHOC are overexpressed and decreased when RHOA is overexpressed[72]. These results are consistent with actinmediated macropinocytosis. Entry of VSV virions via clathrin-coated pit-mediated endocytosis is enhanced by the formation of actin filaments[73] (FIG. 4b). Entry of poliovirus, an unenveloped picornavirus, is dependent on actin[74] and, following entry, the rapid transit of poliovirus particles to replication centres is also dependent on an intact actin network[75]. In general, actin-enhanced endocytic pathways, which have previously been associated with uptake of bacteria, are often stimulated by small virus particles[76].

Fusion of virion membranes and cell membranes at the cell surface

Virions that do not engage the endocytic machinery at the cell surface may gain entry by direct fusion with the cell’s plasma membrane. The involvement of actin in this process is not well understood. Viruses of the family *Paramyxoviridae*, such as human respiratory syncytial virus (HRSV) and human parainfluenza virus 3 (HPIV-3), encode an attachment glycoprotein G (a haemagglutinin–neuraminidase) and a fusion protein (F), both of which

are required for virion entry. F protein mediates fusion of the viral membrane with the cellular membrane (FIG. 4c), subsequent transfer of the viral genome and the formation of syncytia between infected and adjacent cells. Modulation of actin filaments by cytochalasin D reduces HRSV entry as well as subsequent synctium formation, suggesting that actin filaments are involved in both processes[77]. The fusion and synctium-forming activity of the HPIV-3 F protein are dependent on a dynamic actin filament network and RHOA[78,79]. The expression of RAC1 and CDC42 promotes glycoprotein G-mediated cell–cell fusion, whereas the expression of RHOA decreases this activity[80].

Receptor clustering

While some virions move on the cell surface to find an entry site, HIV virion attachment induces clustering and enrichment of the receptors CD4 and CXC-cytokine receptor 4 (CXCR4) at points of virion contact[81]. Receptor clustering is an actin-dependent process that uses RHO-family GTPase signalling and the actin-remodelling proteins filamin and cofilin[82,83]. Env-mediated fusion of virions with the plasma membrane activates RAC1, which signals to the WASP-family verprolin-homologous protein 2 (WAVE2) complex and the kinase ABL1, resulting in the initiation of ARP2/3-mediated actin polymerization and capsid entry[84]. Inhibition of any of these functions blocks entry at the step of fusion initiation or fusion pore expansion.

Extension induction

Virion attachment to cells can stimulate an increased uptake of virions by several mechanisms, including the extension of cell surface protrusions. Herpes simplex virus (HSV) virions engage the cytoskeleton during entry by activating RHOA-induced phagocytosis. This process involves the production of cell surface extensions and clustering of viral-entry receptors[85]. Formation of the extensions requires long actin filaments and is sensitive to treatment with cytochalasin D and latrunculin B. Stimulating an endophagocytic pathway through the activation of RHO-family GTPases encourages uptake of multiple virions, perhaps ensuring robust viral replication. HPV infection induces stress fibre dissolution and rapid filopodia formation within 10 minutes of infection. This activity is blocked by genistein or wortmannin treatment, suggesting that tyrosine phosphorylation and PI3K activity are behind the signalling pathways that induce these transient structures[86]. The production of filopodia not only allows entry of already engaged virions but also acquires virions that are near, but not in direct contact with, the target cell.

Viral replication and assembly

Viral replication requires the coordinated production, processing and assembly of viral proteins and nucleic acids to produce progeny virions that will be used to infect new cells. Depending on the cellular compartment in which replication and assembly occur, the cytoskeleton has interesting and sometimes surprising roles that are not always well understood. For example, it is known that many enveloped virions contain cellular actin[87], but whether this actin has a function (for example, whether it is required for assembly or egress) or is an incidental passenger largely remains an unanswered question. Some insights have come from studying the role of actin during viral replication and assembly.

Enhancement of viral replication

Actin enhances paramyxovirus replication, but cytochalasin D has little effect on virus production[77]. *In vitro* reconstitution of HRSV RNA replication required actin (FIG. 5a) and could be further enhanced by the addition of profilin[88]. Furthermore, the matrix (M) protein of the related canine distemper virus does not associate with actin, but replication is sensitive to actin depolymerization[89]. By contrast, the M protein of influenza viruses, which

are orthomyxoviruses, does associate with actin filaments[90]. Monomeric G-actin and profilin may enhance the replication and assembly of negative-strand RNA viruses in general, either by acting as a transcription factor or by stabilizing nucleic acids, although no clear mechanism has yet been elucidated[91].

Movement of intracellular assemblies

The actin cytoskeleton has been suggested to have a role in moving viral genomes and proteins to cytoplasmic assembly sites. After entry, the phosphorylated HIV Gag–MA complex associates with F-actin before microtubule-based transport of virions to the nucleus[92]. Disruption of actin filaments with cytochalasin D not only inhibits entry of retroviral capsids but also inhibits reverse transcription. Inhibition of reverse transcription by cytochalasin D indicates that actin may regulate or enhance HIV genome synthesis, similar to the role of actin in paramyxovirus replication. Nuclear localization of Gag–MA was reduced, as was HIV infectivity, when inhibitors of myosin light chain kinases were used to inhibit myosin-dependent movement on actin filaments[93]. Taken together, these results show that actin affects many early steps of HIV infection, from entry through to nuclear import.

Actin may have a role in the final assembly and budding of infectious HIV virions at the cell surface[94]. Assembly usually occurs at the plasma membrane, where Gag assembles and buds into plasma membrane vesicles containing Env. HIV budding assemblies are associated with Triton-X100-insoluble lipid rafts, but the driving process for interactions between Gag and Env is believed to be the actin network[95]. Actin-depolymerizing agents such as mycalolide B (TABLE 1) inhibit HIV assembly[96]. HIV Gag interacts with actin filaments, and equine infectious anaemia virus Gag multimers associate with F-actin assemblies during infection[97].

The interaction of Newcastle disease virus and Sendai virus M proteins with actin filaments is important during virion maturation[98,99]. For Sendai virus, expression of M protein is essential for the actin restructuring that is observed during infection and virion release[100]. The nucleocapsids of measles virus, a related paramyxovirus, associate with actin filaments in small villus-like structures at the cell periphery[101] (FIG. 5b). Cytochalasin D inhibits the release of both measles virus[102] and Sendai virus, implicating the formation of new actin filaments in viral assembly and release.

Actin has also been implicated in the nuclear assembly and egress of DNA viruses. Alphaherpesviruses, such as HSV and pseudorabies virus (PRV), assemble capsids in the nucleus, following which the capsids move to sites of nuclear egress along the inner nuclear membrane. The intranuclear movement of HSV capsids is sensitive to temperature, inhibition of ATP hydrolysis by sodium azide treatment, and inhibition of actin polymerization by latrunculin B treatment but not by cytochalasin D treatment[103]. Infection by both HSV and PRV induces nuclear actin filaments (FIG. 5c) and the accumulation of nuclear myosin V that may be necessary for movement and nuclear egress[104]. Further, latrunculin B and cytochalasin D treatment affects the nuclear size changes and chromatin marginalization that are prominent after HSV infection[105]. The mechanism that stimulates the formation of nuclear actin filaments is unknown but may involve the actin-modifying activities of the viral kinase US3 (REF. 106), as discussed below. Actin filaments in the nucleus are not unique to herpesvirus infections and may be a requirement for other viruses that replicate in the nucleus, such as baculoviruses[107]. After release from the nucleus, completely assembled HSV-1 virions navigate out of the cell in a process that requires the action of myosin Va motors. When infected Vero cells express a dominant-negative form of myosin Va, virion secretion is inhibited with a concomitant increase in intracellular virions[108]. Myosin Va may interact with virion transport vesicles to mediate intracellular

movement, or may modify the cortical actin network to facilitate access of intracellular virions to the plasma membrane for subsequent release.

Viral egress and spread

Newly assembled virions leaving the cell face many of the same barriers that are encountered during virus entry, including the need to move past the cortical actin meshwork under the plasma membrane, to cover large spatial distances and to engage a new, viable host cell. Considerable evidence suggests that some viruses require the actin cytoskeleton for successful egress and spread[109].

Viral budding

The first connection between virion egress and the actin cytoskeleton was made for mouse mammary tumour virus (MMTV), a retrovirus. Mammary tumour epithelial cells were observed to release virus particles from the ends of long microvilli[110]. This observation was extended by immunogold staining of actin in egress sites and MMTV particles[111], and for other retroviruses, including HIV[112] (FIG. 5d), raising the question of whether viral proteins interact with the actin cytoskeleton for the final steps of assembly and release. Disrupting actin filaments with cytochalasin D dramatically blocked virus budding and reduced virus yield by 80%[113].

Extension production

Efficient retroviral spread depends on cell-to-cell contacts and does not occur readily by free virion release and serial infection. Recent evidence reveals the existence of thin, actin-based, filopodial extensions — termed cytonemes or tunnelling nanotubes — that act as bridges for the transfer of virus particles between infected and uninfected cells. Infected macrophages produce large numbers of these extensions during infection[114]. In MMTV-infected cells, engagement of Env and receptors on the uninfected cell filopodia results in stabilization and lengthening of the actin-based bridge[115]. Virus particles bud from the infected cell and move in a directed manner across the extension before entry at the base of the filopodium, similar to the viral surfing described earlier for the entry of free particles[64].

The HIV Nef protein is involved in the formation of filopodial extensions in Jurkat T cells. Nef affects actin filaments by altering neural WASP (N-WASP) activity[116]. In addition, Nef also inhibits membrane ruffling and cell motility. The modifications of the actin network and GTPase signalling have long-term effects on cell adhesion and T cell motility in chemokine gradients[117]. Nef also modulates PAK2 activity, despite there being a lack of detectable interaction between these two proteins by immunoprecipitation experiments[118]; this modulation of PAK2 activity results in the inactivation of cofilin by hyper-phosphorylation[119], and the inhibition of actin remodelling. The F195A point mutation in Nef blocks its interaction with PAK1 and PAK2, as well as the majority of its actin-modulating activity[120].

Other viruses also induce actin extensions to disseminate infection over large distances in cultured cells. HSV and PRV produce filopodia or nanotube-like extensions through the activity of the viral kinase US3 (REFS 121,122). These extensions are necessary for efficient cell-to-cell spread of virions *in vitro*[123]. The activity of US3 may modify actin kinase signalling directly via phosphorylation of PAK1 and PAK2. PAK1 is necessary for the PRV-induced formation of actin projections, whereas PAK2 is necessary for the disassembly of stress fibres[124]. US3-induced extensions and their function in *in vitro* viral dissemination is also observed in cells infected with other alphaherpesvirus species[125].

Rotaviruses also induce filopodial extensions for disseminated spread. The expression of rotavirus protein NSP4 and rotavirus infection both result in loss of phosphorylated cofilin in a calcium-dependent manner, whereas extended expression of NSP4 induces long, actin-based extensions from cells[126]. The spike protein, VP4, associates with actin filaments during infection and ectopic expression. VP4 immunoprecipitates with actin and localizes to the apical surface of polarized Caco-2 cells. Long-term VP4 expression results in actin aggregation, similar to that observed after cytochalasin D treatment[127]. Furthermore, VP4 modulates the polarized spread of infection, using actin treadmilling to promote virus release from apical surfaces in polarized cells[128]. Together, these findings suggest that two viral proteins may synergistically alter the actin architecture to promote the directional, long-distance transport of virions from the infected cell. The necessity of filopodia formation for rotavirus spread *in vivo* remains to be determined.

Comet tails and long-distance spread

VV infections reveal extensive interactions between the virus and the actin cytoskeleton. The first interactions involve the assembly and transport of intracellular virions. The viral protein F11 (coded by the gene *F11L*) blocks the regulation of RHO-family GTPase GTP cycling by binding RHOA[129], thus preventing RHOA interactions with ROCK proteins and Diaphanous-related formins (mDIA proteins; also known as DRF or DIAPH proteins), and therefore resulting in a loss of actin stress fibres. Further, microtubule and actin dynamics are required for efficient viral transport and release. An *F11L* deletion or the expression of a mutated F11 protein that cannot bind RHOA results in the accumulation of virions beneath the plasma membrane[130]. Latrunculin B- or cytochalasin D-mediated dissolution of cortical actin restores viral movement to the cell periphery in the absence of functional F11, supporting the conclusion that F11 reorganizes the cortical actin network to allow virions access to the plasma membrane. Interestingly, the activity of F11 also is needed for VV-induced actin-mediated cell motility via lamellipodial extensions[131] and for viral spread *in vivo*[132].

VV-infected cells exhibit a unique phenotype involving changes in the actin cytoskeleton that are required for the spread of the infection. Hiller first reported actin extensions with considerable accumulations of actin filaments, reminiscent of the ‘comet tails’ that had been seen underneath extracellular virions[133]. These actin comet tails induced by VV (FIG. 5e) propel extracellular virions quickly, and for long distances, away from the infected cell[134]. The viral protein A36 (REF. 135) is an integral membrane protein of the outer viral envelope. When intracellular enveloped virions fuse with the plasma membrane, becoming cell-associated extracellular virions, A36 remains in the plasma membrane underneath the virion, where it undergoes c-SRC-mediated tyrosine phosphorylation[136]. Phosphorylated A36 recruits NCK proteins and growth factor receptor-bound protein 2 (GRB2)[137], which in turn recruit N-WASP and WIPs[3] to stimulate ARP2/3-mediated actin polymerization. In the absence of A36, or of other accessory viral proteins or the cellular factors required for actin polymerization, viral spread is substantially reduced (most often observed as a small plaque on cell monolayers). The association between actin tail formation and rapid plaque growth pointed directly at the process of cell-to-cell spread. On cell monolayers, cell-to-cell spread of VV is rapid — much faster than the replication cycle in a single cell. Remarkably, the proteins that mediate actin tail formation also reduce superinfection by mediating active repulsion through a similar mechanism[138]. Under these circumstances, A36 expressed by the newly infected cells is recruited to subsequently attaching virions, thus repelling the virion away from the cell body using actin tail polymerization. In this manner, infection travels much faster than one cell distance per replication cycle, resulting in rapid plaque formation.

Actin tails on egressing virions are a hallmark of poxvirus infections[139], even when obvious A36 homologues are not present. Yaba-like disease virus, another member of the family

Poxviridae, has short-lived actin tails that are dependent on the protein YL126 and on the recruitment of NCK1 and NCK2 through the tyrosine phosphorylation of YL126, similar to NCK protein recruitment by A36 (REF. 140). Further, actin-based extensions have been observed in a divergent number of large, cytoplasmically replicating DNA viruses (LCDV), including the iridovirus frog virus 3 (REF. 141).

Infection of the asfarvirus African swine fever virus (ASFV), another LCDV, induces actin-based projections, but these resemble filopodia in that they are linear actin filaments bound together with fascins. These extensions are similar to those formed during VV egress in that actin filament polymerization is induced to extend virions to distances away from the cell[142], but the mechanism of viral transport in these ASFV-induced extentions is radically different from that in the VV-induced extensions. Importantly, ASFV virions remain inside the cell at the tip of the actin-based extension.

Cell disruption

In some circumstances, virions are released by destruction of the infected cell. Little is known about the mechanisms of cell disruption after viral infection in eukaryotic cells. The most informative studies have been carried out with adenovirus-infected permissive cells *in vitro*. Here, the expression of early region ORF4 protein (E4ORF4) leads to altered RHO-family GTPase signalling, which results in cell death[143]. This early viral protein triggers a p53-independent cell death pathway by accumulating on cellular membranes and the cytoskeleton, and by interacting with c-SRC family kinases as well as with PP2A[143]. E4ORF4 interaction with c-SRCs deregulates c-SRC signalling and activates the JUN kinase signalling pathway, resulting in dramatic changes in the actin cytoskeleton and in cell blebbing. In particular, E4ORF4 triggers *de novo* actin polymerization through complex interactions with CDC42, RAC1 and RHOA, which in turn promote the assembly of a contractile juxtanuclear actin–myosin network[144]. As a consequence, endosomes are recruited to the sites of actin assembly, leading to impaired membrane trafficking, which results in caspase-independent cell death.

Actin as an antiviral target

Cell biologists have used small-molecule modulators of actin to influence the cytoskeleton for years (TABLE 1). These same compounds also alter the outcome of viral infections, implying a function for the cytoskeleton. Such an interpretation is complicated by the involvement of actin in essential cellular processes, including cell division, polarity, motility and uptake of nutrients. The interpretation of these experiments is further complicated by the fact that actin-modulating compounds often have pleiotropic effects, disrupting more cellular processes than just those involving the actin cytoskeleton. Despite these complications, current research indicates that viral interactions with the actin cytoskeleton can be successfully targeted for new antiviral therapies and to treat virally induced disease. The small molecule Y-27632, a RHO–ROCK pathway inhibitor, has been demonstrated to block metastasis of HPV-transformed cervical cancer cells and could be developed as a novel antimetastatic therapy[145]. Antiviral screens using RNA interference and traditional small-molecule inhibitors will undoubtedly uncover new regulatory nodes and other potential therapeutic targets[146].

Conclusion

Actin and actin-like molecules are ubiquitous in cells, from microorganisms to eukaryotes. These ancient proteins have fundamental roles in cell architecture as well as in many cell functions. It is noteworthy that every virus system studied to date has revealed interactions with the actin cytoskeleton. However, viral genomes do not usually encode actin

cytoskeleton or myosin components. One exception is the reported presence of actin-like genes in the ASFV genome[147], although it is unknown whether these virally encoded homologues function as cytoskeletal proteins. Therefore, the modification and modulation of actin structures in infected cells must result from the action of viral proteins that activate cell signalling pathways or directly recruit actin-remodelling proteins. The molecular biology of such interactions continues to both engage virologists in their quest to understand viral replication, and inform cell biologists about the role of the cytoskeleton in the uninfected cell.

Historically, studies of the transforming viruses provided the first appreciation that viral infection alters the host cytoskeleton. Virally transformed cells round up and cell adhesions are altered, leading to uncontrolled cell division and invasive or metastatic phenotypes. These changes are mediated by viral proteins binding to and modifying cellular signalling pathways that involve proteins such as RAC1 and CDC42. Subsequently, it was found that non-transforming viral infections alter the same pathways to promote replication and dissemination. In both cases, infection induces the disruption of densely packed actin stress fibres, resulting in the production of new actin-based structures within the cell or extending far from the cell body. Many similarities exist between transforming and non-transforming viruses, such as the effects of RHOA inhibition by VV protein F11 (R EF. 129) and the SV40 small t-antigen[45]. In both cases, the viral proteins cause a decrease in cell adherence to substrates and an increase in cellular motility.

Both infected and uninfected cells require a dynamic actin cytoskeleton to carry out their functions. An important caveat is that most of our current knowledge about virus–cytoskeleton interactions is derived from *in vitro* studies. How these findings relate to infected tissues and animals is not known. For example, stress fibre assemblies (a common feature of tissue culture cells) are often altered during viral infection of cultured cells. *In vivo*, these structures have been observed only in endothelial cells[148,149]. There are reports of novel actin structures in tissues after infection of animals. One study used serial block face scanning electron microscopy of infected rodent peripheral nervous system tissue to provide high-resolution, three-dimensional images of nuclear actin filaments that were stimulated by herpesvirus infection[104]. Another study demonstrated that VV dissemination in animals requires actin-modulating machinery[132]. Certainly, observing and manipulating the three-dimensional architecture of infected tissues is challenging experimentally but will yield large rewards. Multiphoton imaging of infected tissue and animals at the single-cell and single-molecule level is but one of many new approaches for *in vivo* studies. The systems biology tools of proteomics, metabolomics and lipidomics will provide much needed insights into the interface between viral proteins and the actin cytoskeleton in cells and tissues. Although studies of virus-infected cultured cells have provided many basic ideas about the molecular and cellular biology of the actin cytoskeleton, *in vivo* studies will undoubtedly reveal even more interactions of viruses with the cytoskeleton.

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Glossary

Gelsolin A calcium-activated protein that severs actin filaments

Lamellipodia	Wide, thin sheets of membrane extending from the cell body; lamellipodia are often associated with the leading edge of motile cells.
Membrane ruffles	Membrane-enclosed, densely packed actin bundles that increase during cell migration or transformation. Ruffles localize to the leading edge of the lamellipodia, giving these structures a flower-like appearance
Filopodia	Long, thin membranous extensions of the cell with a core of actin filaments
Podosomes	Dot-like extracellular matrix attachment sites in motile cells. In transformed cells, podosomes aggregate in the presence of serum to form ring-or crescent-shaped rosettes
Pseudopodia	Large membranous protrusions that are used to promote the movement of highly motile cells; the name is derived from the Greek for ‘false-footed’.
Contact inhibition	The inhibition of uncontrolled cell division by cell–cell contact through mitogen-activated protein kinase signalling. Contact inhibition is deregulated in transformed cell populations
Adherens junctions	Epithelial cell-to-cell junctions that connect the actin cytoskeleton of one cell to the cytoplasm of the neighbouring cell via cadherins and catenins
Vinculin	A focal-adhesion plaque protein that is associated with the microfilament ends and talin
Talin	An actin-binding protein associated with adherens junctions, ruffling membranes and other sites of actin–membrane interaction
α-actinin	A large family of proteins that crosslink and bundle actin filaments in a calcium-dependent manner in non-muscle cells
Focal adhesions	Macromolecular complexes that connect the actin cytoskeleton to the extracellular matrix by the association of transmembrane integrins with extracellular proteins such as fibronectin
Caldesmon	A calmodulin-binding and F-actin-binding protein that regulates the function of actin filaments in a calcium-dependent manner
Clathrin-mediated endocytosis	The process of enveloping extracellular material and bringing it into the cellular cytoplasm within a membranous vesicle. Invagination of the plasma membrane and stabilization of the vesicle is carried out by triskelions of clathrin that polymerize at the membrane surface to induce curvature
Macropinocytosis	A specialized form of endocytosis that is used by the cell to obtain soluble materials from the extracellular environment
Actin treadmilling	The act of moving a specific actin monomer, or its position, along an actin filament using active polymerization and depolymerization processes
Comet tails	Dense structures of actin filaments that are used to propel objects through the cytoplasm or long distances away from the cell

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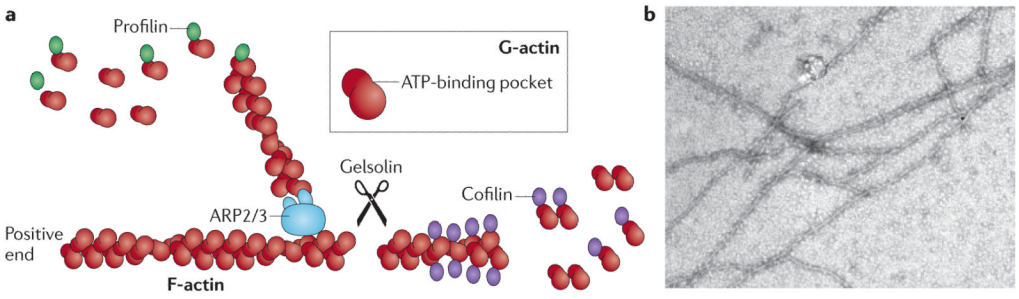

Figure 1. Actin filament dynamics

a | Actin filaments (known as filamentous actin (F-actin)) are formed by two parallel strands of head–tail polymers of actin monomers (globular actin (G-actin)). Actin polymerization is initiated by the ARP2/3 complex and stimulated by cofactors such as profilin. The direction of actin filaments is determined by the orientation of the monomers, with the positive end being defined as opposite the ATP-binding pocket. Actin depolymerization can occur at either end of the filament. Cofilin interacts with actin dimers to promote disassembly, which can be initiated by the activity of gelsolin. **b** | Actin filaments that were polymerized *in vitro* and visualized under an electron microscope. Image is reproduced, with permission, from REF. 154 © (2009) American Society for Biochemistry and Molecular Biology.

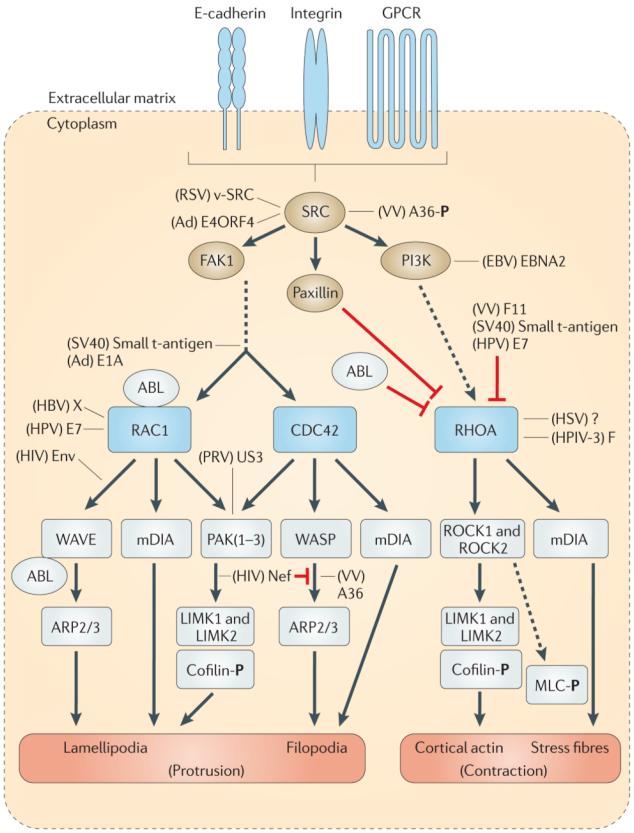

Figure 2. RHO-family GTPase-mediated modelling of the actin cytoskeleton

E-cadherins, integrins or guanylyl-nucleotide-binding protein (G protein)-coupled receptors (GPCRs) activate SRC, which phosphorylates focal adhesion kinase 1 (FAK1). FAK1 promotes the formation of protrusive actin structures by activating RHO-family GTPases such as RAC1 and cell division cycle 42 (CDC42). The downstream effectors of RHO-family GTPases are: Wiscott–Aldrich syndrome protein (WASP)–WASP-family verprolin-homologous protein (WAVE) proteins, Diaphanous-related formins (mDIA proteins; also known as DRF or DIAPH proteins) and kinases such as PAKs and RHO-associated protein kinases (ROCKs). WASP–WAVE proteins stimulate the activation of the ARP2/3 complex. PAKs and ROCKs contribute to the formation of actin filaments by inactivating cofilin via phosphorylation of LIM domain kinases (LIMKs). mDIA stimulates the nucleation and extension of parallel actin filaments. RHOA is initially inactivated by integrin signalling via paxillin. Phosphoinositide 3-kinase (PI3K) is phosphorylated by SRC and then activates RHOA, leading to the formation of stress fibres. ABL tyrosine kinases negatively regulate the RHO–ROCK signalling pathway while activating RAC1 and WASP–WAVE (for reviews, see REFS 10,11). ROCK proteins also stimulate the phosphorylation of myosin regulatory light chain (MLC), thus contributing to the contractility of actin–myosin. Several viral proteins interact with this signalling machinery at multiple levels (see main text for details). Black arrows indicate direct phosphorylation or stimulation of the downstream molecule. Inhibitory or indirect interactions are shown as red or dotted lines, respectively. Stimulatory or modulatory interactions of viral proteins are indicated by black lines. Ad, adenoviruses; E4ORF4, early region ORF4 protein; EBNA2, EBV nuclear antigen 2; EBV, Epstein–Barr virus; F, fusion protein; HBV, hepatitis B virus; HPV, human papillomavirus; HSV, herpes simplex virus; HPIV-3, human parainfluenza virus 3; PRV, pseudorabies virus; RSV, Rous sarcoma virus; SV40, simian virus 40; VV, vaccinia virus;

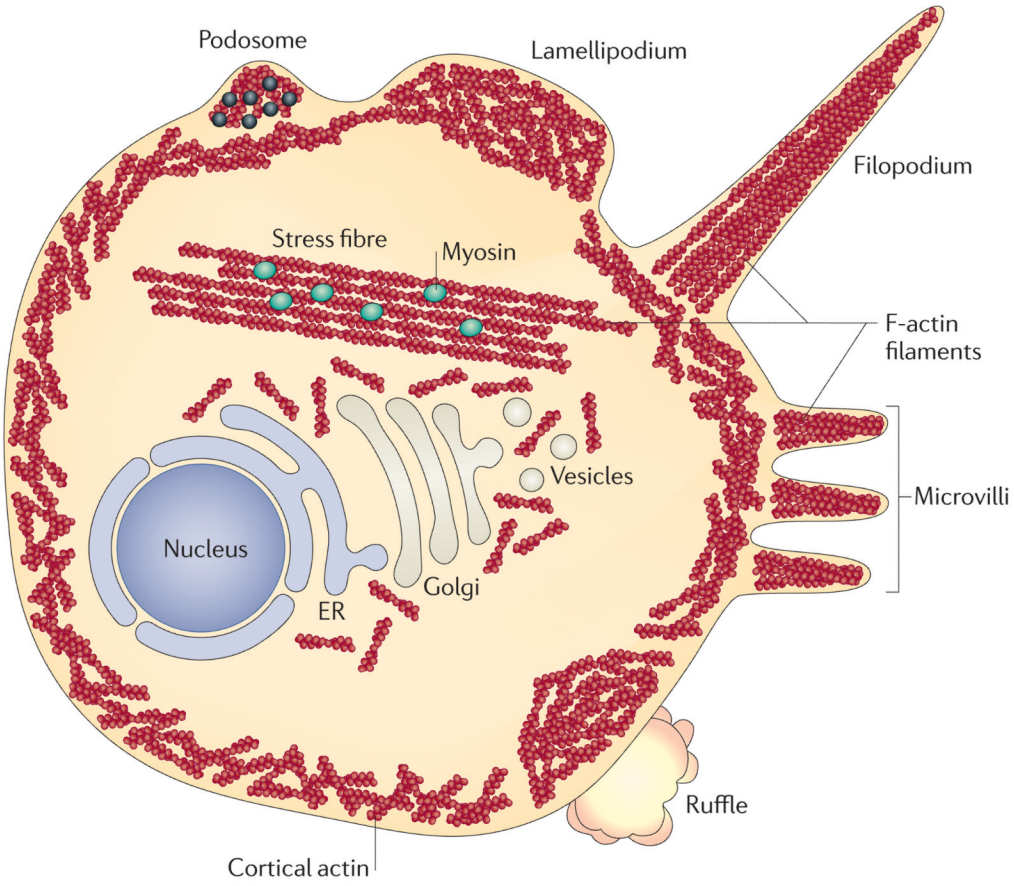

Figure 3. Manifestations of actin rearrangement

Within the cell, actin filaments can be arranged to form multiple structures. Stress fibres are large assemblies of actin filaments that can span the length of the cell. The presence of myosin in stress fibres enables contractility. Underneath the plasma membrane is the loosely organized network of actin filaments that is termed cortical actin. Actin filaments also can be organized to produce a range of cellular extensions, including podosomes, lamellipodia, filopodia, microvilli and large membrane ruffles. Podosomes contain several actin-binding proteins, signalling molecules and metalloproteinases (black dots). ER, endoplasmic reticulum.

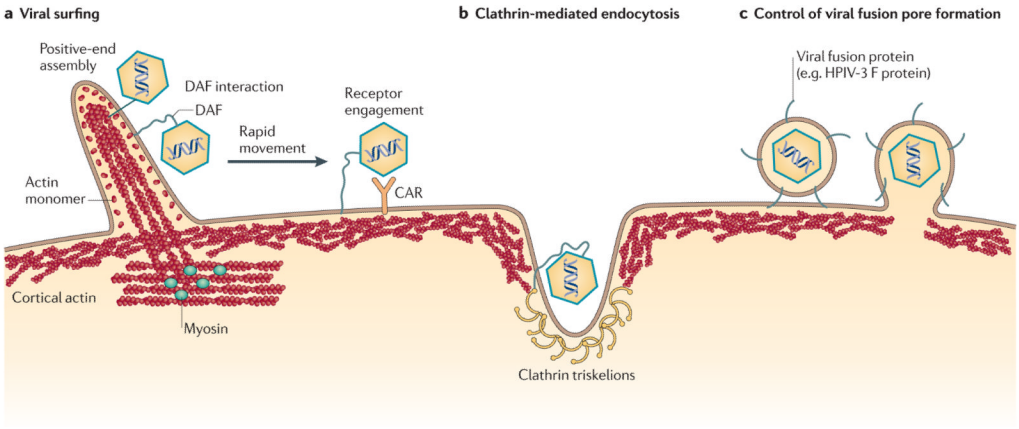

Figure 4. Models of entry

Virions enter cells through a range of pathways, some of which are depicted here. **a** | Viral surfing. Myosin II at the base of the filopodium pulls the actin filaments, while the constant actin turnover at the tip pushes them. This movement makes virions 'surf' down the filopodium to the sites of entry. In some cases, virions bind glycophosphatidylinositol (GPI)-anchored complement decay-accelerating factor (DAF), resulting in cytoskeletal reorganization that enables the virions to rapidly reach their specific receptors. **b** | Actin-enhanced clathrin-mediated endocytosis. Clathrin initiates endocytosis, and actin filaments (possibly cortical actin) are recruited to increase the size of the endocytic structure. **c** | The suggested involvement of cortical actin in regulating formation of the fusion pore; this pore formation is mediated by viral fusion proteins such as the human parainfluenza virus 3 (HPIV-3) fusion protein (F). CAR, coxsackie virus and adenovirus receptor.

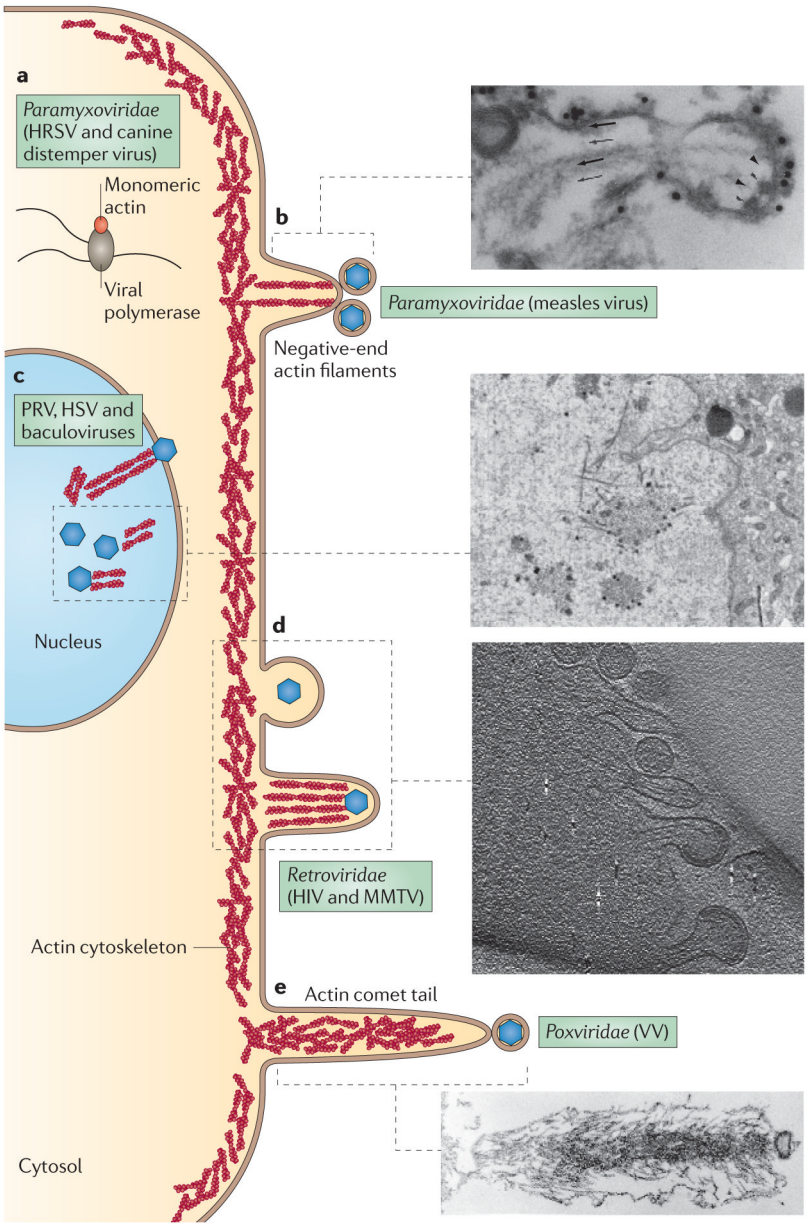

Figure 5. Actin involvement in viral replication and egress

a | The cytoplasmic replication complexes of some negative-strand RNA viruses associate with actin. A putative replication complex of a paramyxovirus is shown, with globular actin stimulating RNA polymerase activity. **b** | Measles virions bud off the plasma membrane at the ends of microvillus-like structures, often associating with the ends of negative-oriented actin filaments. As seen in the myosin-labelled electron micrograph, budding virions are indicated by arrowheads, extracellular virions are dense structures at the membrane, and the directionality of the microfilament (arrows) is determined by the orientation of myosin barbs. **c** | Actin fibres interact with assemblies of DNA virus capsids within the nucleus. An electron micrograph of a pseudorabies virus (PRV)-infected nucleus is shown. The high-contrast spots are capsids, and the long, fibrous projections are actin fibres. **d** | Retroviruses also bud off the plasma membrane, presumably by stimulating the cortical actin network. As virion budding progresses, small actin-filled microvilli form underneath the virions, as

observed under cryoelectron microscopy. **e** | Vaccinia virus (VV)-infected cells produce dense actin comet tails underneath virions. These comet tails push the virus long distances away from the cell to enhance viral dissemination and spread. HRSV, human respiratory syncytial virus; HSV, herpes simplex virus; MMTV, mouse mammary tumour virus. Part **b** electron micrograph is reproduced, with permission, from REF. 101 © (1986) Elsevier. Part **c** electron micrograph is reproduced from REF. 104. Part **d** electron micrograph is reproduced from REF. 112. Part **e** electron micrograph is reproduced, with permission, from REF. 155 © (1996) The Company of Biologists.

Table 1

Modulation of actin filaments

Drug	Mechanisms of action and additional notes	Reference
Latrunculin A and B	• Promote filamentous actin (F-actin) disassembly by binding and inhibiting the polymerization of monomers into filaments • Block the polymerization step without stopping depolymerization	156
Cytochalasin A, B, C and D	• Inhibit actin polymerization, blocking the addition of new monomers to actin filaments by binding the positive end of actin filaments (as a competitive inhibitor) • Promote nucleation and polymerization of short filaments • Pleiotropic effects of cytochalasin A and B include inhibition of monosaccharide uptake and transport	157
Mycalolide B	• Binds and stabilizes actin monomers and induces the cleavage and breakdown of actin filaments • A complete loss of phalloidin staining is observed after most treatments	158
Phalloidin	• Binds and stabilizes actin dimers (phalloidin is used to visualize actin filaments when it is fluorescently labelled) • Alters the kinetics of actin polymerization and directly competes with cytochalasins for dimer binding	157
Jasplakinolide	• Binds and stabilizes F-actin assemblies, promoting actin polymerization • Prolonged treatment leads to loss of cortical actin and stress fibre assemblies	159
Blebbistatin	• Inhibits myosin II	160
Tat–C3	• The *Clostridium botulinum* C3 exoenzyme (which inactivates RHOA, RHOB and RHOC) fused to the HIV Tat leader sequence (which enables the protein to cross the plasma membrane)	161
Y-27632	• Inhibits ROCKs	162
IPA-3	• Inhibits group I PAKs (PAK1 and PAK2)	163