Microreview

Cell entry by human pathogenic arenaviruses

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Summary

The arenaviruses Lassa virus (LASV) in Africa and Machupo (MACV), Guanarito (GTOV) and Junin viruses (JUNV) in South America cause severe haemorrhagic fevers in humans with fatality rates of 15–35%. The present review focuses on the first steps of infection with human pathogenic arenaviruses, the interaction with their cellular receptor molecules and subsequent entry into the host cell. While similarities exist in genomic organization, structure and clinical disease caused by pathogenic Old World and New World arenaviruses these pathogens use different primary receptors. The Old World arenaviruses employ α-dystroglycan, a cellular receptor for proteins of the extracellular matrix, and the human pathogenic New World arenaviruses use the cellular cargo receptor transferrin receptor 1. While the New World arenavirus JUNV enters cells via clathrin-dependent endocytosis, evidence occurred for clathrin-independent entry of the prototypic Old World arenavirus lymphocytic choriomeningitis virus. Upon internalization, arenaviruses are delivered to the endosome, where pH-dependent membrane fusion is mediated by the envelope glycoprotein (GP). While arenavirus GPs share characteristics with class I fusion GPs of other enveloped viruses, unusual mechanistic features of GP-mediated membrane fusion have recently been discovered for arenaviruses with important implications for viral entry.

Arenaviruses are important human pathogens

The Arenaviridae represent a large group of viruses, which is subdivided into two major subgroups, Old World and New World arenaviruses (Clegg, 2002). Arenaviruses cause persistent infections in their natural rodent hosts in Europe, Africa and the Americas. Infection is usually asymptomatic in the natural host, and transmission to humans occurs by contact with infected rodents (Buchmeier et al., 2007).

Infection of the prototypic Old World arenavirus lymphocytic choriomeningitis virus (LCMV) in its natural host, the mouse, provided numerous fundamental concepts in virology and immunology that have been extended to other viruses and pathogens (Oldstone, 2002). LCMV infections in humans are common and are of considerable concern in human paediatric medicine (Jamieson et al., 2006) and immunocompromised individuals, as tragically illustrated by recent fatal transplant-associated LCMV infections (Fischer et al., 2006).

The Old World arenavirus Lassa virus (LASV) is the causative agent of a severe viral haemorrhagic fever (VHF) in humans and is the most prevalent human pathogen among arenaviruses (McCormick and Fisher-Hoch, 2002). In its endemic region in Western Africa, LASV infects several hundred thousand individuals yearly, resulting in significant mortality and high morbidity. There is currently no licensed vaccine available and therapeutic options are limited, resulting in high fatality among hospitalized Lassa fever patients of 15–30%.

The human pathogenic South American haemorrhagic fever (HF) viruses Junin (JUNV), Machupo (MACV), Guanarito (GTOV) and Sabia (SABV) belong to Clade B of the New World arenaviruses. JUNV causes Argentine haemorrhagic fever (AHF), a severe illness with haemorrhagic and neurological manifestations and a case fatality of 15–35% (Weissenbacher et al., 1987). MACV, GTOV and SABV emerged as causative agents of severe VHF in Bolivia, Venezuela and Brazil respectively (Peters, 2002). Apart from the severe humanitarian burden in endemic regions, haemorrhagic arenaviruses are regularly imported into metropolitan areas around the globe placing local populations at risk (Isaacson, 2001; Schmitz et al., 2002).

Recent excellent reviews cover the structural organization and replication of arenaviruses (Meyer et al., 2002; Buchmeier et al., 2007) and only a brief summary will be given here. Arenaviruses are enveloped viruses with a bi-segmented negative strand RNA genome (Fig. 1) and a non-lytic life cycle restricted to the cytoplasm. Each genomic RNA segment, L (c. 7.3 kb) and S (c. 3.5 kb),
uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientation, separated by a non-coding intergenic region (Fig. 1B). The L RNA encodes the viral RNA-dependent RNA polymerase (RdRp, or L), and a small RING finger protein Z, which acts as a bona fide matrix protein (Perez et al., 2003). The S RNA encodes the viral glycoprotein precursor (GPC) and the nucleoprotein, NP. GPC is post-translationally cleaved by the cellular protease site 1 protease (S1P), and with the nucleoprotein, NP. GPC is post-translationally cleaved by the cellular protease site 1 protease (S1P), also known as subtilisin-kexin-isozyme-1, to yield the mature virion glycoproteins GP1 and GP2 (Lenz et al., 2001; Beyer et al., 2003; Kunz et al., 2003). Oligomers of GP1/GP2 form the spikes that decorate the virus surface (Fig. 1A). GP1 is located at the top of the spike and mediates virus interaction with host cell surface receptors (Borrow and Oldstone, 1992) and GP2 is similar to membrane proximal fusion-active parts of other viral membrane proteins (Gallaher et al., 2001; York et al., 2005; Eschli et al., 2006). In contrast to other viral GPs, arenavirus GPC contains a stable signal peptide (SSP) of unusual length that is retained as an essential component of the mature GP complex (Eichler et al., 2003a,b; Froeschke et al., 2003; York et al., 2004). During biosynthesis, the SSP is essential for proper transport and maturation of the GP (Eichler et al., 2003b; York et al., 2004; Aghiometer et al., 2006; Saunders et al., 2007). Recent studies revealed a crucial role for SSP in the pH-dependent fusion activity of arenavirus GPs (York and Nunberg, 2006; 2007a,b; Saunders et al., 2007) suggesting an unusual mechanism for arenavirus GP-mediated membrane fusion discussed in detail below.

The interaction of a virus with its receptor molecules on the cell membrane and its subsequent entry into the host cell are the first steps of every virus infection and are an important determinant for the cellular tropism, host-range and pathogenesis of a virus. Virus-receptor binding and entry also represent important targets for the development of antiviral drugs. Research over the past decade has illuminated many aspects of these initial steps of arenavirus infection and will be covered by the present review.

The Old World arena virus receptor α-dystroglycan and the role of post-translational modifications for virus receptor function

In the early 1990s, characterization of the cellular receptor for the prototypic Old World arenavirus LCMV revealed the presence of a 120–150 kDa virus-binding membrane glycoprotein in permissive cells, which was absent from resistant cells (Borrow and Oldstone, 1992). Subsequent studies identified this virus-binding protein as α-dystroglycan (α-DG), a highly conserved and ubiquitously expressed cell surface receptor for extracellular matrix (ECM) proteins (Cao et al., 1998).

Dystroglycan (DG) is encoded as a single polypeptide that is cleaved into the extracellular α-DG and membrane-anchored β-DG (Barresi and Campbell, 2006). α-DG has a central, highly glycosylated mucin-type domain that connects the globular N- and C-terminal domains. At the extracellular site, α-DG undergoes high-affinity interactions with the ECM proteins laminin, agrin, perlecan, and with neurexins. α-DG is non-covalently associated with β-DG, which binds intracellular to the cytoskeletal adaptor proteins dystrophin and utrophin, and signalling molecules. DG is expressed in most cells of developing and adult tissues and provides a molecular link between the ECM and the actin-based cytoskeleton.

Within the arenavirus family, α-DG is a primary receptor for most isolates of LCMV, LASV, the related African arenaviruses Mopeia (MOPV) and Mobala (MOBV), as well as the Clade C New World arenaviruses Latino (LATV) and Oliveros (OLIV) (Cao et al., 1998; Sevilla et al., 2000; Kunz et al., 2001; 2004; 2005a; Smelt et al., 2001; Spiropoulou et al., 2002; Reignier et al., 2006).

In the host cell, α-DG is subject to a remarkably complex pattern of post-translational modifications, including unusual O-glycan modifications of the mucin-type domain, which are crucial for α-DG’s function as an ECM receptor (Barresi and Campbell, 2006). These modifications involve the known and putative glycosyltransferases protein O-mannosyltransferase 1/2 (POMT1/2), protein O-mannose β1,2-N-GlcNAc transferase 1 (POMGnT1), LARGE, LARGE2, fukutin and fukutin-related protein (FKRP). The genes of these enzymes are affected in a number of human congenital neuromuscular diseases called ‘dystroglycanopathies’ that are caused primarily by defects in α-DG glycosylation and a consequent loss of function as an ECM receptor (Cohn, 2005; Barresi and Campbell, 2006; Kanagawa and Toda, 2006). Interestingly, protein O-mannosylation and LARGE-dependent modifications are also crucial for α-DG’s func-

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tion as a receptor for Old World and Clade C New World arenaviruses (Imperiali et al., 2005; Kunz et al., 2005b; Rojek et al., 2007a), indicating recognition of similar α-DG-derived glycan structures by ECM proteins and arenaviruses. Considering the high conservation and functional importance of the α-DG-derived glycans involved, their recognition by the viruses may have been advantageous during host–pathogen co-evolution. As a consequence, pathogenic arenaviruses like LASV may have created selective pressure on the human population that contributed to the genetic and biochemical complexity of α-DG glycosylation and possibly to the relatively high frequency of defective alleles of α-DG modification enzymes in the human population. A recent genome-wide analysis of positive selection in human populations revealed indeed a remarkably strong selection for an allele of the LARGE gene within an African population from Nigeria (Sabeti et al., 2007), where LASV is endemic (Richmond and Baglole, 2003). It will be of great interest to investigate the correlation between the presence of the selected LARGE allele in Lassa patients and disease outcome.

While a primary role of virus receptors is to bind and thus concentrate virus particles at the cell membrane, some viruses modulate expression of their receptors during replication. The best-studied example of this is the human immunodeficiency virus type 1 (HIV-1), which utilizes three of its gene products, Vpu, Env and Nef, to downregulate its primary receptor CD4 and the principal co-receptors CCR5 and CXCR4 (Doms and Trono, 2000; Michel et al., 2005; Venzke et al., 2006; Wildum et al., 2006). Such virus-induced changes in receptor expression are likely critical for the host–virus interaction and viral pathogenesis. Recent studies investigated the impact of Old World arenavirus infection on α-DG expression. In infected cells, expression of the viral GP interfered specifically with the post-translational modification of α-DG, resulting in marked downregulation of functional receptor, without affecting other cell surface glycoproteins (Rojek et al., 2007b). This virus-mediated perturbation of receptor modification resulted in a loss of α-DG’s function as a cellular receptor for ECM proteins, affecting cell–matrix interactions (Rojek et al., 2007b). The implications of this type of ‘virus-receptor interference’ for the virus–cell interaction and virus-induced host-cell pathology are clearly of great interest.

Old World arenaviruses use an unknown endocytotic pathway for entry

Upon receptor binding, arenaviruses are internalized by endocytosis into a low-pH environment providing the trigger for a pH-dependent membrane fusion step that releases the viral ribonucleoprotein (RNP) complex into the cytoplasm, where unpacking of the viral RNA occurs, followed by the initiation of replication and transcription. Immune-electron microscopy (EM) studies provided evidence that LCMV uses a clathrin-independent pathway for cell entry (Borrow and Oldstone, 1994). On permissive cells, LCMV particles attached primarily to regions of the plasma membrane outside of coated pits and virus particles became incorporated into smooth-walled vesicles with a diameter of approximately 150–300 nm, which lacked a clathrin coat (Borrow and Oldstone, 1994). More recent studies reported that cellular entry of LCMV and LASV is sensitive to cholesterol depletion (Shah et al., 2006; Rojek et al., 2007c; Vela et al., 2007). While attachment of LCMV to its cellular receptor α-DG was not dependent on membrane cholesterol, cholesterol depletion blocked subsequent virus internalization (Rojek et al., 2007c), suggesting a cholesterol-dependent endocytotic pathway. Initial characterization of LCMV entry provided evidence for a pathway that is independent of clathrin and caveolin, and does not require the GTPase dynamin (Fig. 2) (Rojek et al., 2007c). Neither the structural integrity nor the dynamics of the actin cytoskeleton are required for infection, excluding mechanisms of internalization by macropinocytosis (Borrow and Oldstone, 1994; Rojek et al., 2007b). Interestingly, the characteristics of the endocytotic pathway used by LCMV resemble in some aspects those of a recently discovered cholesterol-dependent, clathrin- caveolin- and dynamin-independent pathway used by SV40 in cells devoid of caveolae (Damm et al., 2005; Kirkham and Parton, 2005). However, in contrast to SV40, which is targeted to pH-neutral compartments like caveosomes and the endoplasmatic reticulum (Pelkmans et al., 2001), the pH profile of LCMV membrane fusion suggests delivery of the virus to late endosomes. As the cellular factors involved in LCMV entry are so far largely unknown, a possible relationship between the two pathways remains speculative at this point.

Human pathogenic New World arenaviruses use transferrin receptor 1 and enter cells via clathrin-mediated endocytosis

Transferrin receptor 1 (TfR1) has recently been identified as the first cellular receptor for the South American HF viruses JUNV, MACV, GTOV and SABV (Radoshitzky et al., 2007). In a proteomic pull-down approach using a recombinant receptor-binding GP1 moiety of MACV as bait, a specific high-affinity virus-binding protein was isolated from permissive VeroE6 cells and identified as transferrin receptor 1 (TfR1). Expression of human TfR1, but not TfR2, greatly enhanced the susceptibility of otherwise rather resistant hamster cell lines with pseudotypes of JUNV, MACV, GTOV and SABV, but not LASV or LCMV.
Likewise, infection of human cells with JUNV, MACV, GTOV and SABV, but not LASV, was efficiently blocked with anti-TfR1 antibodies. Together, this provides strong evidence for a role of TfR1 as a major cellular receptor for the South American HF viruses.

TfR1 is a ubiquitously expressed class II membrane protein abundant in activated immune cells and vascular endothelial cells, cell types critically involved in arenavirus pathogenesis (Geisbert and Jahrling, 2004). In the host cell, TfR1 is a classical example of a cargo receptor and plays a central role in cellular iron metabolism and associates with clathrin-coated pits of the plasma membrane (Mellman, 1996). Binding of iron-loaded transferrin to TfR1 induces clathrin-mediated endocytosis delivering the transferrin–receptor complex to early endosomes. Under the mildly acidic pH of the early endosome (pH c. 6.0), iron is released from transferrin. The resulting apotransferrin–receptor complex is recycled to the cell surface, with each TfR1 molecule cycling up to 100 times (Wang et al., 2000; McCaffrey et al., 2001). Addition of neither holo-transferrin nor apo-transferrin interfered with or enhanced virus infection of cells, indicating the use of non-overlapping binding sites (Radoshitzky et al., 2007). TfR1 serves also as a receptor for at least two other groups of viruses, the canine and feline parvoviruses (Parker and Parrish, 2000; Parker et al., 2001) and mouse mammary tumour virus (Ross et al., 2002).

In line with the use of TfR1 as a primary receptor, recent studies on the cellular entry of JUNV provided evidence for virus internalization by a clathrin-dependent pathway (Fig. 2). Infection of cells with JUNV was specifically blocked by chlorpromazine, a drug that interferes with the assembly of clathrin-coated pits at the plasma membrane (Martinez et al., 2007). In contrast, sequestration of membrane cholesterol by nystatin had only a mild impact in JUNV entry. In line with the pharmacological data, over-expression of dominant-negative mutants of the clathrin coat-associated protein Eps15 and dynamin, which is required for clathrin-mediated endocytosis, significantly reduced JUNV infection (Rojek et al., 2007c). Immune-EM visualized JUNV particles in coated vesicles (Martinez et al., 2007), further supporting clathrin-mediated endocytosis as a major route of entry of JUNV.

As mentioned above, upon binding to transferrin, TfR1 is internalized and recycles through the early endosome, which has a pH around 6.0. However, the GP of JUNV requires a significantly more acidic pH (< 5.5) for optimal fusion activity (York and Nunberg, 2006), which would require delivery of the virus to late endosomal compartments. This apparent discrepancy suggests that for productive infection, the virus must somehow re-direct TfR1 from the normal recycling pathway towards late endosomes. A possible answer to this puzzle may lie in the multivalent nature of the arenavirus particle. Indeed, while monomeric transferrin recycles through early endosomes in 4–10 min, an artificial 10-mer of transferrin was retained in the endosome for over 1 h, suggesting that the trafficking of TfR1 critically depends on the oligomeric state of the ligand (Marsh et al., 1995). A similar alteration in cellular trafficking of TfR1 may be induced by arenavirus binding, providing another example of how viruses manipulate the host cell’s endocytotic machinery for their purposes.

So far, no studies have been performed to address modulation of TfR1 expression and/or cellular trafficking by New World arenavirus infection. However, an interesting correlation between cellular iron concentrations and...
susceptibility to New World arenavirus infection has been discovered (Radoshitzky et al., 2007). On most cells, expression of TfR1 is subject to regulation by the cellular iron, with an inverse correlation between cytosolic iron concentration and the surface expression levels of TfR1 (Templeton and Liu, 2003). Iron depletion in the culture medium enhanced, and iron supplementation decreased infection of cells with retroviral pseudotypes of MACV and JUNV (Radoshitzky et al., 2007), raising the interesting possibility that iron deficiency may enhance susceptibility to these pathogens. This issue is of considerable concern in some of the endemic regions of the South American HF viruses where iron deficiency due to malnutrition is common.

While TfR1 has emerged as a major receptor for human pathogenic Clade B New World arenaviruses, studies on receptor use and cellular tropism revealed remarkable differences between the South American HF viruses and closely related non-pathogenic Clade B viruses like Amapari (AMPV) and Tacaribe virus (TACV) (Reignier et al., 2007a,b). The SSP of arenavirus GP has an unusual length of 58 amino acids, contains two distinct hydrophobic domains (Eichler et al., 2003a; Froeschke et al., 2004; York and Nunberg, 2004), and undergoes myristoylation at its N-terminus (York et al., 2004). In the viral membrane, mature arenavirus GPs form tripartite complexes consisting of GP1, GP2 and SSP. Both N- and C-termini of SSP are located in the cytosol (Agnihothram et al., 2007) and SSP associates non-covalently with the cytoplasmic domain (CTD) of GP2 (Agnihothram et al., 2006). Recent studies discovered a highly conserved series of six cysteine and histidine residues in the CTD of arenavirus GP2 that form zinc-binding domains and are essential for the GP2–SSP interaction (York and Nunberg, 2007b). Interestingly, amino acid substitutions that diminish the positive charge at the conserved lysine K33 in the ectodomain loop of SSP lower the pH optimum for fusion (York and Nunberg, 2006), indicating a molecular interaction of K33 in SSP with the fusion machinery in the ectodomain of GP2. The dual interaction of SSP with the CTD and the fusion-active ectodomain of GP2 provides a novel mechanism by which the fusion activity of a viral GP can be modulated by factors located at the inner face of the viral membrane and vice versa. This novel and unique mechanism of regulation of the fusogenic activity of arenavirus GPs likely plays an important role in viral entry and may provide promising targets for the development of novel antiarenaviral drugs.

Membrane fusion mediated by arenavirus envelope GPs: evidence for a novel mechanism of GP-mediated pH-dependent membrane fusion

Initial evidence for pH-dependent membrane fusion for arenavirus entry was provided by experiments showing that infection of cells with LCMV and JUNV could be inhibited at the entry stage by lysosomotropic weak bases (Borrow and Oldstone, 1994; Castilla et al., 1994). Subsequent analysis of the pH-dependence and kinetics of fusion of LCMV indicated that the GP spike is activated to a fusion active state by low pH followed by conformational changes of the GP that result in membrane fusion (Di Simone et al., 1994; Di Simone and Buchmeier, 1995). This model has been largely supported by more recent molecular modelling and biochemical studies that revealed similarities between arenavirus GP2 and class I viral fusion proteins of other enveloped viruses including retroviruses, paramyxoviruses and filoviruses (Gallaher et al., 2001; York et al., 2005; Eschli et al., 2006).

The principal features of the current model for arenavirus GP2 are a hydrophobic N-terminus and two anti-parallel helices separated by a glycosylated loop region. In the pre-fusion form, the N-terminal α-helices form a coiled-coil core structure. Low pH sets off a series of conformational rearrangements leading to the formation of the post-fusion six-helix bundle conformation. This state is characterized by the C-terminal α-helices packing into the hydrophobic grooves of the trimeric coiled-coil core formed by N-terminal α-helices. Thereby, the viral envelope and the target membrane are pulled together to give rise to the fusion pore.

While the overall architecture of arenavirus GP2 is similar to other class I viral fusion proteins, recent studies revealed a crucial role of the SSP of arenavirus GP in pH-dependent membrane fusion (York and Nunberg, 2006; 2007a,b). The SSP of arenavirus GP has an unusual length of 58 amino acids, contains two distinct hydrophobic domains (Eichler et al., 2003a; Froeschke et al., 2004; York and Nunberg, 2004), and undergoes myristoylation at its N-terminus (York et al., 2004). In the viral membrane, mature arenavirus GPs form tripartite complexes consisting of GP1, GP2 and SSP. Both N- and C-termini of SSP are located in the cytosol (Agnihothram et al., 2007) and SSP associates non-covalently with the cytoplasmic domain (CTD) of GP2 (Agnihothram et al., 2006). Recent studies discovered a highly conserved series of six cysteine and histidine residues in the CTD of arenavirus GP2 that form zinc-binding domains and are essential for the GP2–SSP interaction (York and Nunberg, 2007b). Interestingly, amino acid substitutions that diminish the positive charge at the conserved lysine K33 in the ectodomain loop of SSP lower the pH optimum for fusion (York and Nunberg, 2006), indicating a molecular interaction of K33 in SSP with the fusion machinery in the ectodomain of GP2. The dual interaction of SSP with the CTD and the fusion-active ectodomain of GP2 provides a novel mechanism by which the fusion activity of a viral GP can be modulated by factors located at the inner face of the viral membrane and vice versa. This novel and unique mechanism of regulation of the fusogenic activity of arenavirus GPs likely plays an important role in viral entry and may provide promising targets for the development of novel antiarenaviral drugs.

Perspectives

Research during the past decade has identified α-DG and TfR1 as major cellular receptors for pathogenic Old World and New World arenaviruses, respectively, and provided insights into the molecular mechanisms of receptor recognition by these pathogens. While several lines of evidence indicate that pathogenic New World arenaviruses take advantage of the clathrin-dependent endocytic

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pathway normally used by their cellular receptor TfR1, the low fusion pH of these viruses suggests that they are able to re-direct receptor trafficking to late endosomes. It will be of great interest to see how pathogenic New World arenaviruses alter the normal trafficking pattern of TfR1 in the host cell, in particular the role of multivalent attachment.

In contrast to the situation with New World arenaviruses and their receptor TfR1, no particular endocytotic pathway has been associated with the Old World arenavirus receptor α-DG and data on the prototypic LCMV suggest entry via a cholesterol-dependent, clathrin- and caveolin-independent pathway that does not require dynamin. The cellular factors associated with this endocytotic pathway are currently unknown. However, the advent of a powerful reverse genetic system for Old World arenaviruses (Flatz et al., 2006; Sanchez and de la Torre, 2006), combined with novel techniques and tools for the molecular dissection of cellular endocytotic pathways, including RNA interference and dominant negative mutants of critical cellular factors, will greatly advance this field in the near future. Furthermore, the recent discovery of novel and highly unusual mechanistic features of the pH-dependent membrane fusion mediated by arenavirus GPs will illuminate novel aspects of the fusion process. As viral entry is a promising step to attack pathogenic viruses before they can utilize the host cell’s machinery for replication, studies on arenavirus entry will likely suggest novel molecular targets for antiviral strategies to combat human arenavirus infection.

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