In Search of Cathepsins: How Reovirus Enters Host Cells

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AS OBLIGATE INTRACELLULAR PARASITES, viruses must gain access to the intracellular environment by exploiting host mechanisms to transport macromolecular cargo from the cell surface to the cytoplasm. Although cell entry mechanisms used by viruses are diverse (Mercer *et al.*, 2010), general principles of virus entry are conserved: viruses attach to target cells, internalize, cross a lipid bilayer, and deliver their nucleic acid payload to an intracellular site for replication. Some viruses require host factors for the disassembly events required to penetrate membranes. For example, cathepsin proteases have emerged as a class of host factors required for productive cell entry by many viruses. Interestingly, some bacterial toxins (e.g., *Clostridium difficile* toxins A and B and *Vibrio cholera* toxin, among many others) use similar strategies to gain access to the cell interior. Thus, understanding mechanisms by which viruses enter cells can illuminate mechanisms shared by diverse pathogenic microbes.

The *Reoviridae* are nonenveloped, segmented, double-stranded RNA (dsRNA) viruses that include rotavirus, the most common cause of gastroenteritis in children (Parashar *et al.*, 1998), bluetongue virus, an economically important pathogen of cattle and sheep, and mammalian orthoreovirus (reovirus), which infects humans, although disease is restricted to the very young (Dermody *et al.*, In Press; Ouattara *et al.*, 2011). Reovirus contains ten dsRNA segments packaged in two concentric protein shells, an outer capsid and core. During reovirus cell entry (Fig. 1), the outer capsid undergoes acid-dependent proteolysis catalyzed by cathepsin proteases, resulting in formation of infectious subviral particles (ISVPs). ISVPs are characterized by the loss of viral capsid protein $\sigma 3$ and cleavage of capsid protein $\mu 1$ into particle-associated fragments ϕ and δ . Further conformational rearrangements of the viral particle result in loss of the $\sigma 1$ attachment protein and release of the transcriptionally active viral core into the cytoplasm via a penetration mechanism governed by the $\mu 1$ cleavage products (Chandran *et al.*, 2002; Chandran *et al.*, 2003; Odegard *et al.*, 2004; Nibert *et al.*, 2005; Danthi *et al.*, 2008).

Reovirus binding to host cells is mediated by interactions of $\sigma 1$ protein with cell-surface sialic acid and junctional adhesion molecule-A (JAM-A) (Barton *et al.*, 2001a, 2001b; Forrest *et al.*, 2003; Campbell *et al.*, 2005). These receptors

mediate reovirus attachment via an adhesion-strengthening mechanism in which low-affinity interactions with sialic acid tether virions to the cell surface before high-affinity binding to JAM-A (Barton *et al.*, 2001a). However, JAM-A does not appear to participate in internalization of viral particles. The cytoplasmic tail of JAM-A, including a PDZ-binding domain that promotes the binding of JAM-A to scaffolding proteins in tight junctions (Severson and Parkos, 2009), is dispensable for reovirus infection (Danthi *et al.*, 2006; Maginnis *et al.*, 2006).

After receptor engagement, virions are internalized by receptor-mediated endocytosis in a manner dependent on $\beta 1$ integrin (Maginnis *et al.*, 2006; Maginnis *et al.*, 2008). Uptake of virions is dependent on clathrin under certain conditions (Ehrlich *et al.*, 2004; Maginnis *et al.*, 2008), but there is mounting evidence that other mechanisms are involved in virion internalization (Maginnis *et al.*, 2008; Schulz *et al.*, 2012). It is not known whether $\beta 1$ integrin mediates internalization of viral particles through a direct or indirect mechanism. However, viral core protein $\lambda 2$ has solvent-exposed RGD and KGE integrin-binding motifs that could serve as $\beta 1$ integrin contact residues. NPXY motifs in the cytoplasmic tail of $\beta 1$ integrin are required for efficient internalization and infection by reovirus (Maginnis *et al.*, 2008). Substitution of tyrosine residues with phenylalanines in the NPXY motifs leads to distribution of viral particles to degradative lysosomes (Maginnis *et al.*, 2008). NPXY motifs are required for the association of $\beta 1$ integrin with the actin cytoskeleton and also regulate integrin binding and signaling (Moser *et al.*, 2009). Thus, it is possible that reovirus engagement of $\beta 1$ integrin elicits recruitment of cellular molecules that promote internalization and proper endocytic sorting of reovirus particles.

After internalization, reovirus is transported to endosomal organelles where cathepsin proteases mediate stepwise proteolytic disassembly of virions (Ebert *et al.*, 2002; Johnson *et al.*, 2009; Doyle *et al.*, 2012). Reovirus virions distribute to early, late, and recycling endosomal compartments during cell entry, but only those that traverse early and late endosomal compartments can establish infection (Mainou and Dermody, 2012). Particles that fail to induce proper signaling upon JAM-A and $\beta 1$ integrin engagement may mis-sort into

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FIG. 1. Model of reovirus cell entry. After attachment to cell-surface sialic acid and junctional adhesion molecule A (JAM-A), reovirus enters cells by receptor-mediated endocytosis (1) in a $\beta 1$ integrin-dependent manner. Activation of Src kinase (2) at the site of entry targets reovirus to early endosomes (3), inhibited by inactive cathepsin proteases (cathepsin zymogens). In a productive infection, viral particles remain associated with JAM-A, and perhaps $\beta 1$ integrin, and traverse to late endosomes where active cathepsin proteases mediate disassembly of viral particles, resulting in the release of the transcriptionally active viral core into the cytoplasm (4). In a nonproductive infection (5), viral particles traffic to fast and slow recycling compartments. The fate of viral particles within recycling endosomes is not known, but it is possible that these particles are redirected to the cell surface where they can reinitiate the entry process or disengage from receptors.

recycling endosomes and return to the cell surface, where they could reinternalize or float free. Within endosomes, JAM-A codistributes with reovirus virions during entry (Mainou and Dermody, 2012), suggesting that virus and receptor remain engaged after adsorption and are transported together to the site of viral disassembly. The fate of $\beta 1$ integrin during reovirus endocytic transport is not clear.

Beyond the utilization of $\beta 1$ integrin in reovirus internalization, the cellular molecules that promote uptake of viral particles and sorting within the endocytic pathway are beginning to be defined. Src kinase, the prototype member of the Src family of tyrosine kinases, colocalizes with reovirus particles during cell entry (Mainou and Dermody, 2011). Reovirus infection results in increased levels of Src kinase phosphorylation at tyrosine 416 (Mainou and Dermody, 2011), which is associated with increased kinase activity (Cooper *et al.*, 1986; Parsons and Weber, 1989; Brown and Cooper, 1996). Furthermore, impairment of Src kinase activity with small-molecule inhibitors or decreased Src kinase

expression using RNA interference leads to aberrant targeting of viral particles to lysosomes with a concomitant reduction in the efficiency of infection (Mainou and Dermody, 2011). It is possible that Src kinase mediates endosomal sorting of reovirus particles by recruiting and modifying molecules necessary for endocytic transport to a site of viral disassembly. Interestingly, other viruses use Src kinases for efficient cell entry.

Coxsackievirus, a member of the *Picornaviridae*, also utilizes Src family kinases during cell entry (Coyne and Bergelson, 2006). Coxsackievirus activates Abl and Fyn kinases after cell-surface engagement of decay accelerating factor (DAF). Abl kinase activation triggers Rac-dependent actin rearrangements that promote transport of viral particles and DAF to tight junctions. Fyn activation promotes caveolin phosphorylation and viral particle internalization using a caveolin-dependent pathway (Coyne and Bergelson, 2006). While reovirus likely accesses tight junctions to engage JAM-A, reovirus does not appear to induce actin rearrangement or use caveolin-

dependent endocytic pathways during cell entry. As such, coxsackievirus and reovirus appear to use Src family kinases in distinct mechanisms to internalize into host cells.

The goal of reovirus endocytic transport is to gain access to an endocytic organelle replete with the enzymes capable of catalyzing virion disassembly. Cathepsins B, L, and S can mediate virion-to-ISVP disassembly *in vitro* and in cells (Ebert *et al.*, 2002; Golden *et al.*, 2004; Johnson *et al.*, 2009; Doyle *et al.*, 2012). Some cathepsin proteases (e.g., cathepsins B and L) are loaded into early endosomes in an inactive form, and after processing and acidification within endosomal organelles become active enzymes (Mohamed and Sloane, 2006; Guha and Padh, 2008). It is possible that multiple cathepsins catalyze disassembly during a single entry event. Cathepsins are active in a pH range of 4.5–8.0, depending on the specific cathepsin (e.g., the pH optimum is 5.0–6.0 for cathepsin B and 5.0–8.0 for cathepsin S) (Mohamed and Sloane, 2006; Guha and Padh, 2008). A gradient of cathepsin activity could provide a means for reovirus to regulate cathepsin-mediated disassembly by allowing specific cathepsins to act on the viral particle at distinct pHs as the virus traverses the endocytic pathway. Employment of multiple cathepsins for uncoating also may contribute to the broad tissue tropism and wide host range displayed by reovirus.

Several viruses, in addition to reovirus, exploit cathepsin proteases to achieve productive cell entry. After attachment and entry (Nanbo *et al.*, 2010), Ebola virus is transported to late endosomes where cathepsins B and L cleave and activate the viral glycoprotein (Chandran *et al.*, 2005; Misasi *et al.*, 2012). The cleaved glycoprotein binds to Niemann-Pick C1, promoting fusion and entry into the cytoplasm (Carette *et al.*, 2011; Cote *et al.*, 2011). Henipaviruses also utilize cathepsins during cell entry. Hendra virus uses cathepsin L (Pager and Dutch, 2005), while Nipah virus uses cathepsins B and L (Pager *et al.*, 2006; Diederich *et al.*, 2012) to proteolytically process the F protein, a step required for fusion with target cells. However, in the case of these viruses, cathepsin-mediated cleavage occurs prior to viral release. Severe acute respiratory syndrome coronavirus (SARS-CoV) uses cathepsins to proteolytically process the viral spike glycoprotein before fusion with the cell membrane during entry (Simmons *et al.*, 2005; Huang *et al.*, 2006). Since cathepsins are required by several viruses for cell entry, it is possible that cathepsin inhibitors might serve as broadly active antiviral agents. Indeed, treatment of mice with a cathepsin L-specific small-molecule inhibitor decreases reovirus disease severity and mortality rate (Johnson *et al.*, 2009).

Viruses use a variety of receptors and host molecules to target cells for replication and dissemination to new hosts. Viruses also exploit conserved cellular mechanisms, including kinase recruitment and activation, diminished pH, and cathepsin proteases to define specific locales within the cell to initiate endosomal egress. Further studies of pathogen uptake mechanisms will enhance an understanding of microbial pathogenesis and potentially illuminate new targets for anti-infective drug development.

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