How Viruses Use the Endoplasmic Reticulum for Entry, Replication, and Assembly

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To cause infection, a virus enters a host cell, replicates, and assembles, with the resulting new viral progeny typically released into the extracellular environment to initiate a new infection round. Virus entry, replication, and assembly are dynamic and coordinated processes that require precise interactions with host components, often within and surrounding a defined subcellular compartment. Accumulating evidence pinpoints the endoplasmic reticulum (ER) as a crucial organelle supporting viral entry, replication, and assembly. This review focuses on the molecular mechanism by which different viruses co-opt the ER to accomplish these crucial infection steps. Certain bacterial toxins also hijack the ER for entry. An interdisciplinary approach, using rigorous biochemical and cell biological assays coupled with advanced microscopy strategies, will push to the next level our understanding of the virus-ER interaction during infection.

To trigger infection, a virus binds to receptors on a host cell's plasma membrane. This interaction induces virus internalization, and initiates a complex journey of the viral particle into the host's interior that leads to either nonproductive or productive infection (Mercer et al. 2010). In nonproductive infection, the virus may be targeted to and trapped in organelles unsupportive of viral membrane fusion or penetration, events which normally enable the viral nucleic acid access to the host cytosol or nucleus. Alternatively, the virus could be transported to a degradative intracellular compartment in which it is destroyed. In contrast, for productive infection, a viral particle must avoid these non-productive routes and traffic along a pathway that allows it to reach the appropriate replica-

tion and assembly site. Successful infection is usually completed when the newly assembled particle is released into the extracellular milieu, in which it can promote another infection round. Thus, the ability to co-opt a host cell entry pathway leading to efficient replication and assembly ultimately dictates the fate of an incoming virus.

For proper entry, replication, and assembly, viruses often rely on the complex membranous network surrounding and residing within the host cell, such as the plasma, endolysosomal, and endoplasmic reticulum (ER) membranes. Selecting the suitable membrane system requires several considerations. To support entry, the membranous system must possess triggers capable of inducing the necessary conformational

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changes that facilitate viral membrane fusion or penetration (Inoue et al. 2011). Examples of cellular triggers include receptors, low pH, proteases, chaperones, and reductases. Additionally, because viral replication and assembly often occur in the context of virus-induced membranous structures derived from host membranes, the membranous network of choice should accommodate these remodeling reactions (Miller and Krijnse-Locker 2008). Moreover, as a virus commonly manipulates the host immune system to sustain infection, a membrane's ability to provide the virus with such an opportunity would offer tremendous advantages during the infection course (Takeuchi and Akira 2009).

A wealth of data implicates the endoplasmic reticulum (ER), one of the most elaborate membranous networks in a cell (Shibata et al. 2009), as the organelle many viruses exploit during infection. This review focuses on how viruses co-opt the ER to enter, replicate, and assemble in the target cell. We will also draw parallels from the mechanisms by which bacterial toxins use the ER for entry. Together, these insights should unveil clues regarding why many viruses select the ER during infection.

THE ER

Structurally, the ER is a continuous membranous system consisting of the nuclear envelope, and peripheral sheets and tubules emanating from it (Voeltz et al. 2002). Recent studies suggest the membrane sheets correspond to the rough ER whereas the tubules represent the smooth ER (Voeltz et al. 2006; Shibata et al. 2010). Functionally, the rough ER is responsible for translating secretory and transmembrane proteins, whereas the smooth ER possesses specialized roles including lipid and glycogen metabolism (reviewed in Hopkins 1978). Once a newly synthesized protein is translated and folded properly in the ER lumen, it exits the ER to reach the Golgi apparatus via membrane budding mediated by the COPII complex. By contrast, should a protein misfold in the ER, an endogenous ER quality control system called ER-associated degradation (ERAD) alleviates the build-up of misfolded ER proteins (Brodsky

and Skach 2011; Smith et al. 2011). To do so, a network of ER factors recognizes and retro-translocates the misfolded protein to the cytosol. On reaching the cytosol, another cohort of cytosolic factors engage and ubiquitinate the substrate, targeting it to the proteasome for degradation.

Strikingly, some of ER's general properties are beneficial to viruses. For example, as the ER-to-cytosol retro-translocation machinery is an inherent apparatus in the ER, it represents an ideal conduit for certain viruses and bacterial toxins to enter the cytosol.

Additionally, ER membrane's ability to undergo constant budding reactions plays a crucial role during viral replication and assembly when viruses deform and rearrange the ER membrane to generate ER-derived structures used to support these processes.

POLYOMAVIRUS CO-OPTS THE ER DURING ENTRY

A decisive virus entry step necessitates the incoming viral particle to breach a host cell's membrane barrier (Fig. 1). This barrier could be the plasma (Fig. 1, pathway 1), endolysosome (Fig. 1, pathway 2), or ER (Fig. 1, pathway 3) membrane. Although many viruses such as influenza virus, HIV, human poliovirus (PV), and adenovirus cross the plasma or endolysosome membrane (Fig. 1, pathways 1 and 2), only polyomavirus (Py) family members penetrate the ER membrane during entry (Fig. 1, pathway 3).

As Py lacks a surrounding lipid bilayer that defines enveloped viruses, it is classified as a non-enveloped virus. Prominent Py family members include the murine Py (mPy), simian virus 40 (SV40), and the human BK (BKV), JC (JCV), and Merkel Cell (MCPy) polyomaviruses. Although mPy and SV40 can induce tumors in experimental animals (Gross 1953; Sweet and Hilleman 1960), human polyomavirus's role as human cancer-causing agents is less certain. Perhaps the strongest link is observed in MCPy, in which the virus is positively correlated with incidences of Merkel cell carcinoma, a rare but aggressive skin cancer of neuroendocrine origin (Feng et al. 2008). Regardless of their role in

Figure 1. Virus entry across different cellular membranes. To enter cells, viruses penetrate the plasma membrane (PM) (pathway 1), endolysosome membrane (pathway 2), or ER membrane (pathway 3) to reach the cytosol. Whereas many viruses including influenza virus, HIV, and PV breach the plasma or endolysosome membranes, only Py transports across the ER membrane during cell entry.

cancer, human polyomaviruses are well-established causative agents for other human diseases, including hemorrhagic cystitis and nephropathy (by BKV), and the fatal demyelinating disease progressive multifocal leukoencephalopathy (by JCV) (Jiang et al. 2009a).

Structurally, Py particles are composed of a layer of the outer coat protein VP1. This single protein, arranged as 72 pentamers, forms the shell surrounding the viral genome (Liddington et al. 1991; Stehle et al. 1994). Each VP1 pentamer engages the internal protein VP2 or VP3 via hydrophobic interactions (Chen et al. 1998). Additionally, VP1 binds directly to its DNA genome harbored within the viral particle (Carbone et al. 2004). To stabilize the virus structure,

each VP1 within a pentamer extends its carboxy-terminal arm to an adjacent pentamer. Calcium ions bound to the virus further strengthen this interpentamer interaction (Stehle et al. 1996). Finally, a network of VP1 disulfide bonds reinforces its overall architecture (Stehle et al. 1996).

To initiate infection, Py binds to glycolipid receptors called gangliosides on the plasma membrane (Smith et al. 2003; Tsai et al. 2003; Low et al. 2006). After internalization, the virus is transferred first to the endolysosome (Eash et al. 2004; Querbes et al. 2006; Qian et al. 2009; Engel et al. 2011) and then the ER (Tsai et al. 2003; Gilbert and Benjamin 2004; Qian et al. 2009). Py transport to the ER, a phenomenon documented over 20 years ago by Helenius

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and coworkers using electron microscopy (EM) (Kartenbeck et al. 1989), is unique as most extracellular ligands do not reach the ER after endocytosis. On reaching the ER, Py penetrates the ER membrane to gain access to the cytosol and then the nucleus, in which ensuing transcription and replication of the viral genome lead to lytic infection or cell transformation. Insights into how Py crosses the ER membrane to gain entry into the cytosol are emerging. Conceptually, Py's entry into the cytosol via penetration of the ER membrane can be divided into three steps. First, the virus undergoes host-triggered conformational changes in the ER, priming it for membrane penetration. Second, the structurally altered virus transports across the ER membrane. Third, the virus is released into the cytosol.

In the first membrane penetration step, ER factors perturb the major forces stabilizing Py's structure to generate a membrane transport-competent intermediate. One of the major forces is the VP1 disulfide bond network. ER-resident redox-active protein disulfide isomerase (PDI) family members, including the canonical PDI, ERp57, and ERp72, can isomerize or reduce the virus disulfide bonds (Fig. 2A, step 1a) (Gilbert et al. 2006; Schelhaas et al. 2007; Walczak and Tsai 2011; Nelson et al. 2012). However, the precise combination of PDI proteins engaging a specific Py family member may differ because of subtle differences in the viral disulfide bond arrangements. For some Pys, a dimeric redox-inactive PDI protein called ERp29 untangles the VP1 carboxy-terminal arms (Magnuson et al. 2005; Rainey-Barger et al. 2007a,b; Nelson et al. 2012) exposing hydrophobic VP2 and VP3 to generate a hydrophobic viral particle (Fig. 2A, step 1b) (Magnuson et al. 2005; Rainey-Barger et al. 2007a; Geiger et al. 2011). The hydrophobic virus is likely maintained in a soluble state by binding to the Hsp70 chaperone BiP (Fig. 2A, step 1c) (Geiger et al. 2011; Goodwin et al. 2011) in a reaction regulated by the J-domain containing cochaperone ERdj3 (Goodwin et al. 2011). The J domain typically stimulates Hsp70's ATPase activity to control Hsp70-substrate interaction (Kampinga and Craig 2010). No ER factors have been found

to extract Py's bound calcium ions. Despite the actions of the ER factors on Py, biochemical analyses have revealed that the virus remains as a large and intact particle in the ER (Inoue and Tsai 2011).

In the second step, the hydrophobic virus integrates into the ER membrane to initiate membrane penetration (Fig. 2A, step 2) (Daniels et al. 2006; Rainey-Barger et al. 2007b; Geiger et al. 2011). At this juncture, ERAD membrane components including Derlin-1 (Schelhaas et al. 2007; Jiang et al. 2009b), Derlin-2 (Lilley et al. 2006), Sel1L (Schelhaas et al. 2007), RMA1 (Geiger et al. 2011), and BAP29/BAP31 (Geiger et al. 2011), have been proposed to facilitate Py's exit to the cytosol. However, as none of them have been shown to interact with Py physically, their actions on the virus may be transient or indirect. Additionally, given the relatively large viral particle size (40–50 nm in diameter) in the ER (Inoue and Tsai 2011) compared to cellular proteins, it is unlikely to cross a protein-conducting channel of the ERAD machinery that accommodates smaller cellular proteins. Instead, we hypothesize that Py penetrates the ER lipid bilayer directly. Trapping Py on the ER membrane to ascertain its interacting partners under this condition should distinguish these possibilities. Py penetration across a lipid bilayer and not a protein channel will be consistent with the general mechanism by which nonenveloped viruses breach their limiting membranes (Tsai 2007). If the large Py particle transports across the lipid bilayer, we envision this process to deform the ER membrane physically. The advent of electron tomography (ET) capable of visualizing cellular structures in three dimension (3D) at high resolutions may reveal such ER membrane remodeling during virus penetration. Clearly, deciphering Py's precise membrane transport mechanism will require more rigorous biochemical and imaging approaches.

In the final entry step, Py is released into the cytosol (Fig. 2A, step 3). How this step is accomplished is the least understood. The cytosolic ATPase p97, which normally extracts misfolded cellular proteins into the cytosol during ERAD (Ye et al. 2001), plays only a minor role in releasing Py into the cytosol (Geiger et al.

Figure 2. Model of Py and CT transport across the ER membrane. (A) On reaching the ER, PDI family members (i.e., PDI, ERp72, and ERp57) disrupt disulfide bonds on Py's surface (step 1a). ERp29 then extrudes the VP1 carboxy-terminal arm to expose VP2 and VP3 (step 1b), generating a hydrophobic virus. This particle is likely maintained in a soluble state by the BiP-ERdj3 chaperones (step 1c). The hydrophobic virus then integrates into the ER membrane (step 2). ERAD membrane components including Derlin-1, Derlin-2, Sel1L, BAP29/31, and RMA1 are postulated to mediate virus transport across the ER membrane. How Py is released into the cytosol is not well-understood (step 3), but may rely on the ER membrane J proteins (i.e., DNAJ B12, B14, and C18) stimulating binding between the virus and cytosolic Hsp70; reiterative cycles of this binding "pull" the virus into the cytosol. (B) CT arrives to the ER by binding to the host ganglioside GM1 receptor. In the ER, CT is transferred to the Derlin-1-Hrd1 membrane proteins (step 1) potentially mediated by BiP-J protein chaperones. An unidentified reductase reduces CTA to generate the CTA1 peptide (step 2). Next, PDI (bound to Derlin-1 and Hrd1), in its reduced state, unfolds CTA1 (step 3); ensuing PDI oxidation by Ero1 releases the unfolded toxin from PDI. The unfolded toxin presumably crosses the Hrd1 complex to reach the cytosol (step 4). Finally, an unidentified cytosolic factor extracts the toxin into the cytosol to complete the transport event (step 5).

2011). Furthermore, although there is evidence implicating the proteasome at this stage (Schelhaas et al. 2007; Jiang et al. 2009b; Inoue and Tsai 2011), it is unlikely to play a direct role. If cytosol release and virus disassembly are coupled, the cytosolic chaperone Hsc70 may be the

ideal candidate for catalyzing the release step as it disassembles Py in vitro (Chromy et al. 2006). Because all of these proposed cytosolic factors play many other cellular functions, whether they execute a direct role in catalyzing Py release into the cytosol remains inconclusive. What is

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needed is the establishment of an in vitro translocation assay in which defined cytosolic factors can be added to an ER membrane fraction preloaded with Py; these putative factors' ability to stimulate virus release from the ER membrane would offer compelling evidence of their direct role in promoting cytosol release.

Interestingly, a recent report identified an ER membrane-bound DNA J protein family (i.e., DNAJ B12, B14, and C18) in facilitating Py ER-to-cytosol transport (Goodwin et al. 2011). As these Hsp70 co-chaperones' J domains are expected to face the ER membrane's cytosolic surface, they may stimulate Hsp70's ATPase activity to promote Hsp70-Py interaction; reiterative cycles of this binding eventually "pull" the virus into the cytosol. This scenario is reminiscent of the manner by which BiP "pulls" substrates into the ER lumen in a reaction controlled by the J-domain containing Sec63 protein during posttranslational translocation (Matlack et al. 1999). Why so many members of this DNA J protein family promote Py's release into the cytosol from the ER is unclear.

ER AS THE ENTRY SITE FOR CHOLERA TOXIN

Similar to Py, certain bacterial toxins including cholera toxin (CT) and shiga toxin (ST) hijack the ERAD machinery to reach the cytosol to induce cytotoxicity (Teter and Holmes 2002; Lencer and Tsai 2003; Lord et al. 2005). As CT is this toxin group's prototype, we briefly describe how it co-opts the ERAD machinery during entry. On reaching the ER from the cell surface, CT is transferred to a membrane complex composed of the Derlin-1 (Bernardi et al. 2008; Dixit et al. 2008) and Hrd1 E3 ubiquitin ligase (Bernardi et al. 2010) membrane proteins (Fig. 2B, step 1) postulated to form the ERAD translocation channel (Carvalho et al. 2010; Smith et al. 2011). How this complex captures CT is unknown. After reaching the Derlin-1-Hrd1 complex, the toxin's CTA subunit is reduced to generate the toxic CTA1 fragment (Fig. 2B, step 2). PDI (bound to the Derlin-1-Hrd1 complex) then acts as a redox-dependent chaperone to unfold CTA1 (Fig. 2B, step 3) (Forster et al.

2009; Tsai et al. 2001). In its reduced state, PDI binds to and unfolds CTA1; PDI oxidation by Ero1 alters PDI's conformation, enabling it to release the unfolded toxin (Tsai and Rapoport 2002; Moore et al. 2010). A recent high-resolution PDI X-ray structure confirms that it undergoes a redox-driven structural change (Wang et al. 2012). That CTA1 hijacks ER redox proteins for cytosol entry parallels Py's use of these factors during its ER membrane transport.

Once CTA1 is released from PDI, it presumably transports across the Hrd1 complex (Fig. 2B, step 4) (Carvalho et al. 2010). The driving force propelling the toxin to the cytosol is unknown. One possibility is CTA1's propensity to refold (Rodighiero et al. 2002). In this scenario, the toxin's ability to refold rapidly on emerging the ER membrane's cytosolic surface prevents it from backsliding. A cytosolic factor then extracts the folded toxin from the ER membrane, releasing it into the cytosol (Fig. 2B, step 5). Similar to Py, p97 plays a modest role in this step (Abujarour et al. 2005; Kothe et al. 2005). Thus, we postulate another cytosolic factor ejects the toxin into the cytosol. An obvious distinction between CTA1 and a typical ERAD substrate's fate in the cytosol is that the toxin is not degraded by the proteasome. How the toxin evades this degradative machinery is unclear.

ER'S ROLE IN VIRUS REPLICATION AND ASSEMBLY

After entry into the cytosol, an incoming bacterial toxin exerts its cytotoxic effect. In contrast, an incoming viral particle after entry replicates and assembles to generate new progenies to complete its infection cycle. Viral replication and assembly are often supported by a host cell's membranous network. These membranes function as scaffolds to recruit viral and host components necessary for replication and assembly. As examples, influenza virus (Compans and Dimmock 1969) and HIV (Ono and Freed 2001) assembly is supported by the plasma membrane (Fig. 3, site 1), Semliki Forest virus (Kujala et al. 2001) and Rubella virus (Magliano et al. 1998), RNA replication by the endolysosome (Fig. 3, site 2), Flock House virus

Figure 3. Viral replication and assembly in different membranous networks. Distinct host membranes support replication and assembly of different viruses. As examples, influenza virus and HIV assembly is supported by the plasma membrane (site 1), Semliki Forest virus and Rubella virus RNA replication by the endolysosome (site 2), Flock House virus genome replication by the mitochondria (site 3), Herpes simplex virus assembly by the inner nuclear membrane (site 4), and Hepatitis C virus, Dengue virus, vaccinia virus, and rotavirus replication and assembly by the ER membrane (site 5).

(Miller et al. 2001), genome replication by the mitochondria (Fig. 3, site 3), and Herpes simplex virus (Mettenleiter et al. 2006) assembly by the inner nuclear membrane (Fig. 3, site 4).

Remarkably, diverse viruses including RNA and DNA viruses belonging to both the enveloped and nonenveloped virus families rearrange the ER membrane to generate a vast array of ER-derived structures, with each structure postulated to facilitate viral replication and assembly (Fig. 3, site 5) (Stephens and Compans 1988; Miller and Krijnse-Locker 2008; den Boon and Ahlquist 2010). For instance, the enveloped

hepatitis C virus (HCV) promotes formation of an ER-derived membranous matrix referred to as “membranous webs” important for viral replication and assembly (Egger et al. 2002), and the enveloped Dengue virus (DENV) generates so-called vesicle packets and convoluted membranes also presumed to facilitate genome replication and virus assembly (Welsch et al. 2009). For the enveloped vaccinia virus (VV), the only known DNA virus to replicate in the cytosol and not the nucleus, the ER membrane encloses discrete cytoplasmic foci in which active viral DNA replication is thought to take place

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(Tolonen et al. 2001). In the case of the non-enveloped rotavirus, the virus buds into the ER lumen to generate a transient enveloped intermediate, with removal of the viral envelope in the ER lumen required to complete virus assembly. We will not describe each case in detail as they are already covered in many excellent reviews (Miller and Krijnse-Locker 2008; den Boon and Ahlquist 2010). Instead, we focus here on how HCV induces ER-derived membranous webs to support its replication and assembly, and how rotavirus uses the ER for assembly. These examples illustrate ER's remarkable versatility in facilitating different stages of the virus life cycle.

HCV, which infects approximately 170 million people worldwide, causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. This virus is a member of the Flavivirus family. Its positive-strand RNA genome encodes ten structural and nonstructural proteins (i.e., core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Reed and Rice 2000). The core proteins and the RNA genome constitute the nucleocapsid, and are surrounded by a host-derived envelope. Embedded in this envelope are the E1 and E2 glycoproteins that mediate viral attachment and entry. To infect cells, HCV binds to host receptors, becomes endocytosed, and undergoes pH-dependent membrane fusion in an endosomal compartment that enables the viral core proteins and genome access to the cytosol where genome replication ensues (Ploss and Evans 2012).

HCV replicates its genome specifically on the cytosolic surface of a virus-induced, ER-derived membranous system called the membranous web (Fig. 4A). Functionally, this web harbors the HCV replication complex that promotes replication with assistance from viral nonstructural proteins along with an increasing list of cellular factors (Wolk et al. 2008). Although the HCV membranous web is found juxtaposed to the rough ER (Egger et al. 2002), whether it is physically connected to or detached from the ER membrane network remains unclear. Web formation is thought to initiate when the ER membrane-bound viral nonstructural protein NS5A recruits and activates phosphatidylinositol 4-ki-

nase- α (PI4KIII α) (Fig. 4A) (Berger et al. 2009, 2011; Reiss et al. 2011), thereby increasing the local concentration of phosphatidylinositol-4-phosphate (PI4P). PI4P might itself be an important membrane constituent that supports membranous web structural integrity. Alternatively, PI4P may recruit downstream effector proteins such as oxysterol-binding protein 1 (OSBP1) to form a sterol-rich environment surrounding the membranous web essential for HCV replication (Amako et al. 2009). Recruitment of the host protein proline-serine-threonine phosphatase-interacting protein 2 (PSTPIP2) by the nonstructural proteins NS4B and NS5A to the membranous web also appears to mediate web formation (Chao et al. 2012). Interestingly, PSTPIP2 contains a F-BAR domain that has membrane-deforming activity (Frost et al. 2008). This property enables PSTPIP2 to induce membrane curvature on the ER membrane to promote web formation (Chao et al. 2012). In this context, NS4B itself can also cause web formation (Egger et al. 2002), suggesting that both viral and host components play key roles in sculpting the ER-derived membranous web.

In addition to supporting replication, the membranous web is also postulated to be the virus assembly site. Within this web, lipid droplets (LDs), a storage organelle for neutral lipids in cells (Guo et al. 2009), execute a key viral assembly role (Miyanari et al. 2007). Acting as a scaffold, LD captures HCV core proteins on its surface (Fig. 4B). The LD-core protein interaction is crucial for production of infectious virus (Bouillant et al. 2007; Shavinskaya et al. 2007). As LD is located proximal to the replication complex positioned on the surface of the membranous web, core proteins released from LD encapsulate the newly synthesized RNA genome, forming the nucleocapsid (Fig. 4B, step 1). This process likely occurs within an invagination on the membranous web distinct from where replication takes place. The viral P7 and nonstructural proteins NS2, NS3, and NS4A have been implicated in the release of core proteins from the LD (Boson et al. 2011; Counihan et al. 2011). Once the nucleocapsid is formed, it buds into the lumen of the membranous web, generating an enveloped

Figure 4. ER-derived membranous web as the HCV replication and assembly site (A) HCV replication and assembly occur in the context of a virus-induced, ER-derived membrane matrix called the membranous web. It remains unclear whether the membranous web is connected to or detached from the ER membrane network. To initiate web formation, the viral nonstructural protein NS5A recruits and activates PI4KIII α , generating PI4P. PI4P may be crucial to maintain web structural integrity, or it could recruit OSBP1 to create a sterol-rich environment that supports HCV RNA replication. Membrane remodeling proteins such as the viral nonstructural protein NS4B and host protein PSTPIP2 sculpt the ER membrane to promote web formation. (B) The membranous web is also thought to be the HCV assembly site. Lipid droplet (LD), situated proximal to the replication complex on the web surface, captures core proteins. Core proteins are released from LD, delivered to the assembly site on the membranous web, and encapsulate the RNA genome, generating the nucleocapsid (step 1). This reaction is mediated by P7 and the nonstructural proteins NS2, NS3, and NS4A. Nucleocapsid formation is thought to take place within an invagination on the membranous web distinct from where replication occurs. Once the nucleocapsid is formed, it buds into the lumen of the membranous web to form the enveloped virus with concomitant incorporation of the E1 and E2 glycoproteins (step 2). The HCV particle is then secreted to the extracellular milieu.

viral particle with E1 and E2 glycoproteins displayed on the surface (Fig. 4B, step 2). If the membranous web remains connected to the ER membrane network, the newly assembled viral particle can be secreted from the cell via the classic secretory pathway. Should the web be dis-

connected from the ER, the virus-containing vesicle can reconnect with the secretory pathway by fusing with elements of the secretory pathway such as the Golgi complex.

Replication and assembly of DENV, a flavivirus that causes dengue fever, show interesting

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parallels to and differences from HCV. For instance, akin to HCV's NS4B, DENV's nonstructural protein NS4A rearranges the ER membrane (Miller et al. 2007), leading to formation of vesicle packets and convoluted membranes (Welsch et al. 2009). Additional viral and host components (Khadka et al. 2011) are likely recruited to the ER membrane to promote this reaction. DENV also employs LD for particle formation (Samsa et al. 2009), similar to HCV assembly. However, in contrast to the less obvious physical connection between the HCV-induced membranous web and ER, high-resolution ET analyses show that the DENV-induced ER-derived structures are clearly interconnected in one continuous ER membrane network (Welsch et al. 2009). Perhaps a similar approach using ET will better clarify the physical relationship between HCV's membranous web and the ER membrane network.

The ER also aids in rotavirus assembly, particularly during the late stage of this process. This virus belongs to the reovirus family and is an important causative agent for childhood diarrhea. Structurally, the capsid is an icosahedral particle with a diameter of approximately 100 nm; its double-stranded RNA genome is enclosed within the particle (Trask et al. 2012). The capsid consists of three layers: the inner most layer (called core shell), the middle layer, and the outer layer. The core shell is formed by 120 copies of VP2 dimers arranged as an icosahedron, with the replication complex and the genome encapsulated (McClain et al. 2010). The middle layer is formed by up to 260 VP6 trimers that interact with VP2 dimers to stabilize the core shell (Mathieu et al. 2001). The outer layer is composed of VP7 and VP4: VP7 constitutes a large part of the outer layer and makes extensive contact with the middle layer, whereas VP4 protrudes radially from the particle as spike-like structures (Settembre et al. 2011). A viral intermediate containing the core shell and middle layer is called a double-layer particle (DLP) and a mature infectious particle harboring all three layers is referred to as a triple-layer particle (TLP). DLP-to-TLP morphogenesis is intimately associated with the ER.

To form TLP, DLP must gain access into the ER lumen to acquire the outer layer (Fig. 5). Once formed, TLP exits the host cell via lysis (Musalem and Espejo 1985) or secretion (Jourd'han et al. 1997). The unusual DLP-to-TLP conversion is composed of two steps. The first step requires DLP budding into the ER lumen, generating a transient enveloped intermediate (Fig. 5, step 1). The viral nonstructural membrane protein NSP4 executes a key role in this step. As a tetramer on the ER membrane (Bowman et al. 2000), NSP4 recruits DLP and VP4 to the ER membrane's cytosolic surface (Petrie et al. 1984). The energy source required to deform the ER membrane to bud DLP into the ER lumen is not clear, although multivalent interactions between NSP4 and DLP may trigger this reaction (Taylor et al. 1993). VP4 is dispensable for budding as its knockdown generates TLP (Dector et al. 2002), albeit spike-less as the resulting TLP lacks VP4. That the ER membrane supports DLP budding into the ER lumen reflects this membrane's propensity for promoting budding reactions, even though this reaction proceeds in the opposite direction to conventional ER budding.

How does VP7 assemble on DLP's surface after budding? Using its signal sequence, VP7 translocates into the ER lumen where the signal sequence is cleaved. However, this cleaved unassembled VP7 remains tethered to the ER membrane via noncovalent interactions with its signal peptide (Stirzaker and Both 1989). VP7 also interacts with NSP4's amino terminus on the ER membrane (Maass and Atkinson 1990). Thus the emerging picture of the transient enveloped intermediate is an ER-derived lipid bilayer encasing a VP4-bound DLP with unassembled VP7 decorated on the outer membrane surface (Fig. 5, transient enveloped intermediate).

In the second maturation step, the transient membrane layer is removed, which enables VP7 to dock and assemble on the VP4-bound DLP, forming infectious TLP; NSP4 release from DLP is coordinated with these events (Fig. 5, step 2). Although the mechanics of this process remains unclear, VP7 is thought to play a crucial role as VP7 knockdown fails to block viral

Figure 5. ER as the final rotavirus assembly site. Infectious rotavirus particle formation requires DLP-to-TLP conversion. This process requires two steps that occur in or surrounding the ER. The first step is DLP budding into the ER lumen, generating a viral enveloped intermediate (step 1). This step is initiated when NSP4 recruits DLP and VP4 to the ER membrane. ER membrane deformation by the DLP-NSP4-VP4 complex generates a transient enveloped intermediate. Because unassembled VP7 is tethered on the ER membrane's luminal surface prior to budding, formation of the transient enveloped intermediate “captures” VP7 that remains topologically facing the ER lumen. The second step necessitates removal of the transient membrane layer (step 2), a process likely mediated by VP7 and NSP4. This step is accompanied by VP7 conformational change that enables it to dock and assemble on DLP. Additionally, NSP4 is released from DLP during this process. Mature TLP finally exits the host cell via lysis or secretion.

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budding but causes enveloped intermediate accumulation in the ER lumen (Lopez et al. 2005; Cuadras et al. 2006). How might VP7 promote envelope shedding? In addition to serving as a structural protein, VP7 possesses membrane lytic activity (Charpilienne et al. 1997). There is evidence that unassembled VP7 tethered on ER membrane's luminal side exists in a different conformation when compared to its native assembled structure on TLP's surface (Kabcenell et al. 1988). Thus, VP7 likely undergoes conformational rearrangements after budding, exposing its membrane-lytic portion to disrupt envelope integrity. Although NSP4 also has membrane lytic activity (Browne et al. 2000), its function in removing the transient envelope is difficult to assess as NSP4 knockdown attenuates DLP assembly severely (Lopez et al. 2005). Topologically, VP7 but not NSP4 of the transient enveloped intermediate is exposed to the ER lumen. Thus, should ER factors trigger membrane disruption, VP7 would be the likely candidate to engage the ER proteins. Importantly, knockdown of ER-resident chaperones including BiP and PDI perturb proper rotavirus ER morphogenesis (Maruri-Avidal et al. 2008), further supporting ER's role during rotavirus DLP-to-TLP assembly.

Clarifying how the transient enveloped intermediate's membrane is removed to facilitate TLP formation is unquestionably the most crucial step in illuminating rotavirus assembly. What ER factors impart conformational changes on VP7 to render it membrane penetration-competent? What is the nature of these VP7 structural changes? Does the envelope simply "dissolve" to release DLP or does DLP cross the envelope leaving an intact membrane behind, potentially containing NSP4? And finally, how does VP7 dock and assemble on DLP as NSP4 is coordinately removed from DLP? Perhaps a reconstitution system in which ER proteins are added to various rotavirus ER intermediates to drive the next assembly step will provide a better understanding of this dynamic process. Moreover, high-resolution cryo-EM and ET approaches could be useful in revealing a 3D view of the ER-localized enveloped intermediate.

ER'S FUNCTION DURING VIRAL IMMUNE EVASION

In addition to supporting entry, replication, and assembly, the ER also affords viruses an opportunity to manipulate the host immune system to sustain the infection course. Prominent examples include proteasomal destruction of MHC class I molecule triggered by the human cytomegalovirus (HCMV)-encoded transmembrane proteins US2 and US11 (Wiertz et al. 1996; Machold et al. 1997) in a pathway that requires the ERAD machinery. By degrading MHC class I molecules, HCMV effectively prevents infected cells from properly presenting viral antigens on their surface. Similarly, the murine γ herpesvirus encodes a membrane-bound E3 ubiquitin ligase called mK3 that promotes MHC class I degradation via the ERAD pathway (Wang et al. 2006). Another salient example is observed in HIV, where its Vpu protein also coopts the ERAD machinery to down-regulate the host entry receptor CD4 (Willey et al. 1992; Magadan et al. 2010). CD4 down-regulation leads to a series of events including disruption of T cell activation (Lanzavecchia et al. 1988), ultimately contributing to robust HIV infection. As there are many reviews on how viruses use the ER to thwart the host immune system (Loureiro and Ploegh 2006; Lindwasser et al. 2007; Hansen and Bouvier 2009; Jackson et al. 2011), this topic will not be covered extensively in this article. Nonetheless, it unveils another aspect of the ER function that viruses hijack to maintain infection.

CONCLUDING REMARKS

A virus navigates through the host cell's environment to chart the most effective infection strategy. This strategy not only requires it to evade pathways that trap or degrade itself, but more importantly, delivers it to an appropriate intracellular destination where it can replicate and assemble. Increasing evidence shows host membranes play crucial roles in supporting virus infection. There is no better example than the ER organelle, where many viruses have evolved to co-opt ER's luminal and membrane

contents to achieve proper entry, replication, or assembly.

Although only Py family members use the ER for entry, many viruses including HCV, DENV, VV, and rotavirus, use the ER membrane to support their replication and assembly. In the case of Py entry, elegant studies have identified ER factors that prime the virus for ER membrane penetration. However, elucidating the specific ER membrane machinery and cytosolic factors that complete the transport reaction remains a challenge. This viral entry field should look to the field of ER membrane translocation where transport of a defined cellular substrate across the ER membrane can be reconstituted using purified translocation components (Brundage et al. 1990; Akimaru et al. 1991; Gorlich and Rapoport 1993). Such a feat can be accomplished for Py when more rigorous biochemical strategies are applied.

Powerful imaging approaches, in particular cryo-ET, have revealed formation of virus-induced ER-derived structures considered critical for viral replication and assembly. In many instances, co-localization of these structures with the replicated genome and assembling intermediates implicate these structures as virus replication and assembly sites. Although plausible, convincingly showing these structures support replication and assembly requires the imaging findings to be reinforced by in vitro and cell-based studies. For example, can the virus-induced ER-derived structures be isolated and shown to be active in promoting a specific replication or assembly step? In cells, will blocking formation of the ER-derived structures prevent virus replication or assembly? A more comprehensive approach combining classical biochemical and cell biological methods coupled with state-of-the-art imaging techniques is needed to establish firmly a causal relationship between these ER-derived structures and their roles in viral infection.

Historically, clarifying the nature of virus–host cell interactions has illuminated fundamental cellular processes. In this review, we observe how ER's potential functions can be unveiled through its interaction with viruses. Specifically, ER-resident factors not only interact with en-

dogenous substrates but also viral pathogens to trigger their entry and assembly. Additionally, the ER membrane that normally accommodates budding reactions can undergo virus-induced structural rearrangements to generate ER-derived structures postulated to be important for replication and assembly. Although not discussed, the abundant ER membrane surface can also serve to recruit different viral components to promote efficient genome replication. Such revelations regarding ER's functional capacity will only expand as we continue to explore its role in facilitating pathogen infection.

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