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Rerouting the traffic from a virus perspective

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1. ABSTRACT

Viruses are important human and animal pathogens causing disease that affect global health and the economy. One outcome of many virus infections is the regulation of cellular trafficking machinery. Viral proteins recruit and interact with cellular trafficking proteins to divert the normal trafficking of key proteins or to induce the formation of novel membrane structures in the host cell. These alterations often increase replication efficiency by mislocalizing immune regulators or restriction factors ot by creating platforms for replication and assembly of new virus particles. Our knowledge of how viruses interact with the cellular trafficking machinery is still limited and furthering this understanding will be important for the future development of prophylactic and therapeutic treatments. This review provides a glimpse of the types of interplay between viral and cellular factors that result in a disruption of cellular trafficking or modifications to cellular membranes.

2. INTRODUCTION

The membrane system of eukaryotic cells is a complex, highly developed network of distinct compartments consisting of unique repertoires of lipid and protein composition. The diverse membrane composition and shape of organelles provide distinct identities and allow for execution of specific functions. The sophisticated network of transport machinery in eukaryotic cells ensures a balanced flow of membrane and membrane cargo between these compartments. The regulated and organized flux among compartments is essential for maintaining organelle identity and membrane homeostasis. Viruses, many of which require interactions with membranes at multiple steps during their replication cycle, often alter the membrane profiles of cells. These alterations have several benefits for the virus and may be essential for immune evasion strategies or to create a novel milieu optimal for replication. These changes may be a direct result of viral proteins targeting cellular machinery, or an indirect effect associated with viral replication. Whether direct or indirect, these changes are remarkable. In this review, we address both viral-mediated regulation of trafficking events and the morphological alterations to the membranes of cellular organelles. Recognizing the large amount of quality research in this area, we focus on post-entry events, namely alterations associated with genome replication and virion assembly and consider only a subset of the mechanisms of viral-mediated regulation of membrane transport and organelle morphology.

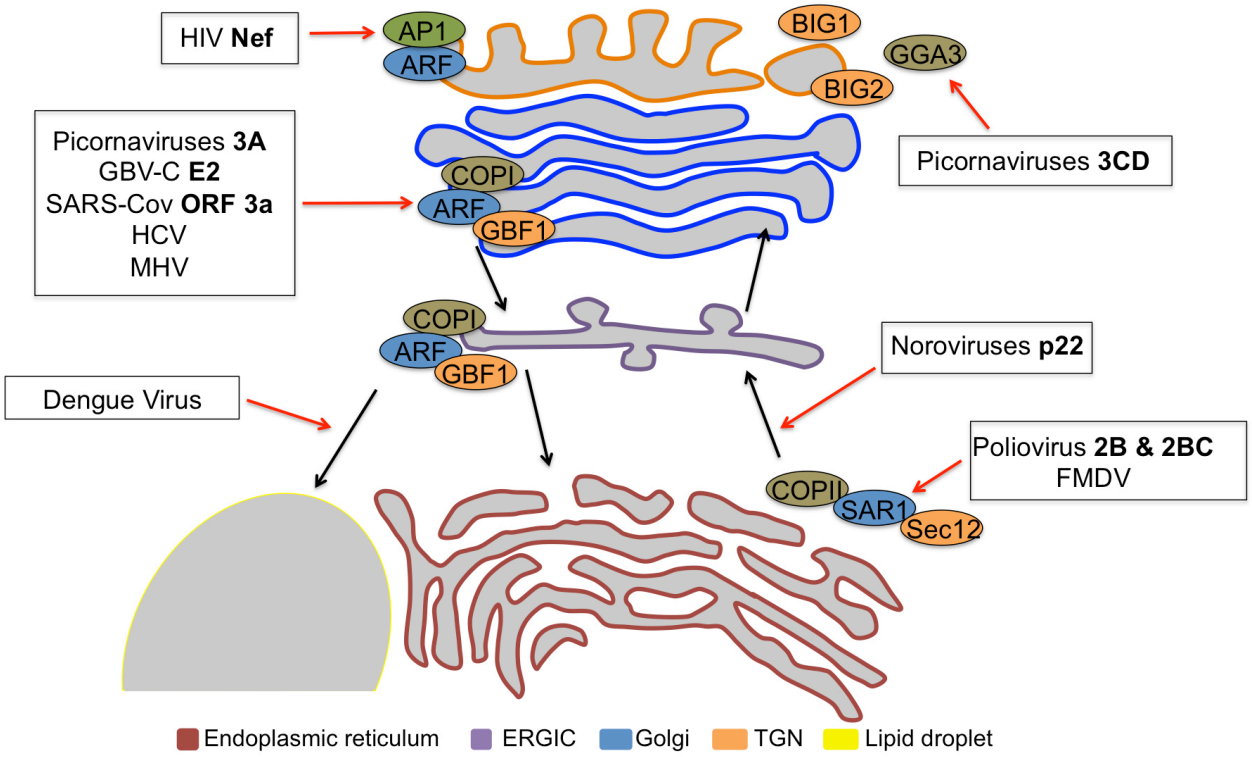

Figure 1. Schematic representing select points of regulation of cellular trafficking by viruses. The picornavirus 3A and 3CD proteins regulate trafficking by binding Arf GEFS, GBF1 or BIG1/2, respectively. Other viral proteins that target Arf directly or components of the Arf pathway include GBV-C E2 and SARS-CoV ORF 3a. Other viruses such as MHV or HCV regulate the Arf pathway, but the viral effectors involved remain uncharacterized. Arf1 is also involved in the lipid droplet delivery of DENV protein C and the Nef-mediated rerouting of MHC-I during HIV infection. COPII vesicles are also targeted by viruses. The poliovirus proteins 2B & 2BC promote formation of COPII vesicles and FMDV utilizes COPII vesicles to support infection. The p22 protein of Noroviruses blocks protein secretion and may do so by rerouting COPII vesicles, preventing them from reaching their Golgi destination.

3. VIRUSES, THE GOLGI APPARATUS AND ARFS

ADP ribosylation factors (Arfs) constitute a family of GTP binding proteins that regulate membrane trafficking pathways. There are three classes of mammalian Arf proteins, distinguished by size and homology (1). As a generalization, class I and II Arfs mainly localize to the trans-Golgi network (TGN), endoplasmic reticulum-Golgi intermediate complex (ERGIC) and Golgi apparatus to mediate membrane trafficking between these compartments, whereas the class III Arf6 localizes to the plasma membrane and is involved in endocytosis (2–4). Like other GTPases, Arf proteins cycle between the active GTP-bound and inactive GDP-bound states. Upon activation, a myristoylated N-terminus is exposed to promote membrane binding and activity. Upon inactivation, Arf dissociates from membranes and returns to the cytosol. This cycle is mediated by guanine nucleotide exchange factors (GEFs) and guanine nucleotide activating proteins (GAPs) known as Arf GEFs and Arf GAPs. A key feature of Arf GEFs is the presence of a 200 amino acid Sec7 domain which catalyzes release of GDP (5). GBF1 is an Arf GEF that acts on both class I and class II Arfs, whereas BIG1 and BIG2 preferentially activate class I Arfs (6–8). GBF1, BIG1 and BIG2 are mostly at the Golgi and are sensitive to the trafficking inhibitor brefeldin A. Many of the initial observations linking the Arfs to virus replication are based on the observation that replication is sensitive to brefeldin A. It should be noted that some Arf GEFs, like ARNO and EFA6, are resistant to brefeldin A treatment.

Activated Arfs have several effector proteins. The outcome of Arf activation often depends on the Arf GEF and where activation occurs. For example, GBF1 is localized to both the ERGIC and cis-Golgi and is associated with recruitment of the COPI coat. COPI, consisting of seven subunits, mediates trafficking from the cis-Golgi to the endoplasmic reticulum. Arf recruitment of COPI was the first to be described and is well studied. In contrast, BIG1 and BIG2 localize to the TGN and recycling endosomes, and these Arf GEFs are associated with recruitment of adaptor protein complex 1 (AP1) and Golgi-localized gamma-ear-containing Arf-binding (GGA) protein complexes. Both complexes are involved in clathrin-dependent trafficking. In addition to these coats, Arf proteins also stimulate effectors that modulate lipid composition. These include phospholipase D and phosphatidylinositol 4-phosphate 5-kinase (9–11).

Thus Arf proteins modulate a number of different cellular processes. Many of the processes intersect with important stages of viral replication and as such, many viruses modulate Arf proteins to optimize replication conditions (summarized in Figure 1).

3.1. Altering trafficking by modulating the Arf pathway

One outcome of infection with poliovirus, a member of the picornavirus family, is the rapid inhibition of protein secretion (12). This block in secretion promotes immune evasion by decreasing MHC class I and TNF receptors at the cell surface and blocking secretion of interleukins 6, 8 and beta-interferon (13–15). The block occurs in trafficking from the ER or ERGIC to the Golgi apparatus, and is replicated by expression of protein 3A from either poliovirus or the related coxsackievirus B (CVB) (12, 16–18). This inhibition is a result of the direct binding of 3A to GBF1, a guanine nucleotide exchange factor for Arf1 (19, 20). Overexpression of either GBF1 or Arf1 rescues the 3A-mediated block in protein secretion (19). Furthermore while virus replication is normally inhibited by brefeldin A, a resistant form of GBF1 containing a single amino acid substitution is sufficient to support viral replication in the presence of the drug (21). Thus, the 3A-mediated regulation of GBF1 is an important step for blocking protein secretion during coxsackievirus and poliovirus infection.

Other picornaviruses utilize different strategies to modulate protein secretion. Expression of 3A from human rhinovirus 14 (HRV14), foot-and-mouth disease virus (FMDV), enterovirus 71 (EV71), hepatitis A, Theiler's virus and encephalomyocarditis virus (EMCV), does not inhibit protein secretion (20, 22). Analysis of the 3A protein from HRV, which is closely related to that of CVB and poliovirus, reveals a reduced ability of this 3A protein to bind GBF1, which likely accounts for the inability of HRV 3A to block protein secretion. In another case, the EV71 3A interacts with GBF1, which together with Arf1 and Arf3, is essential for replication of the virus (23). This result suggests that EV71 3A regulation of GBF1 is essential for viral replication, even though it doesn't block protein secretion as it does for the related CVB and poliovirus. EV71, and the other viruses mentioned above, have 3A-independent strategies to block protein secretion. At least for FMDV, the activity is associated with the nonstructural protein 2BC, as the coexpression of both the 2B and 2C proteins together, but not expression of the 3A protein from FMDV, was responsible for perturbing protein secretion (24, 25). Thus, picornaviruses have multiple strategies for blocking protein secretion.

In addition to GBF1, other Arf GEFS are regulated during enterovirus infection. Whereas 3A regulates GBF1, the viral protein 3CD, through regulation of BIG1 and BIG2, promotes the membrane-association of Arfs (26). The GBF1 recruitment by 3A brings COPI to membranes. In contrast, 3CD helps recruit the GGA3 coat to membranes (26). Mutations in 3CD that abrogate Arf activation also impair virus production. Thus, 3CD activation of Arfs through BIG1 and BIG2 is not redundant with the 3A-mediated activation of GBF1, and each Arf GEF may mediate a different activity during virus replication. Thus, by regulating various Arf effectors different enterovirus proteins can direct alterations in the infected cell that are essential for replication.

Other virus families also have developed mechanisms to block protein secretion. One effector is the non-structural protein precursor NS4A/B of Hepatatis C virus (HCV), a flavivirus (27). A link between the NS4A/B-mediated block in secretion and the Arf pathway has not yet been demonstrated, although GBF1, Arf1 and COPI components all are critical for HCV replication (28–30). HCV proteins also alter membrane morphology, producing membrane buds at the rough ER and forming a membranous web that is tightly associated with vesicles (31). GBF1 does not appear to be required for membranous web formation, suggesting that alternative trafficking pathways deliver membranes to the HCV replication compartment (29). In contrast, the Arf1 pathway is required for HCV proteins to localize to this compartment. In the absence of functional Arf1, NS3 an NS5A, two multifunctional viral proteins that both play a role in RNA replication, are redirected from replication compartments to the periphery of lipid droplets, resulting in reduced replication of viral RNA (28). In addition, Arfs are required for delivering cellular factors to sites of replication. During HCV replication, PI4KIIIbeta is delivered to replication membranes in an Arf-dependent manner to generate a PI4P enriched environment (32). In this case, HCV redirects transport to ensure important cargo is delivered to its replication membranes. Of note, HCV is also dependent on COPI and requires the secretory pathway for virion maturation and exit. Thus, HCV relies heavily on Arf-mediated events for its replication.

HCV is not the only flavivirus that regulates Arfs during infection. GB virus C (GBV-C) is a flavivirus that infects humans. Clinically, even though the virus has not been associated with its own disease, GBV-C appears to delay progression of AIDS in HIV-infected patients (34). Among the different mechanisms that have been proposed to explain this block in disease progression by GBV-C, one is that HIV gag is unable to be delivered to the plasma membrane due to the altered trafficking associated with GBV-C E2 regulation of Arf (33). The E2 protein of GBV-C decreases Arf1 levels by promoting its degradation, resulting in disrupted Golgi morphology and impaired vesicle trafficking to and from the Golgi (33). In this context,

the inhibition of protein trafficking by GBV-C may have unintended and beneficial clinical consequences that are unrelated to GBV-C replication. Thus, global inhibition of protein secretion is a feature shared by several virus families and not only may help viruses avoid an immune response but also may produce additional unappreciated outcomes.

Dengue virus infection relies on a unique form of Arf-mediated trafficking. In infected cells, the Dengue C protein accumulates around lipid droplets (35). Transport of C from the ER to lipid droplets uses the GBF-Arf1-COPI pathway (36). Delivery of cellular proteins to the surface of lipid droplets also is COPI-dependent (37). However, because lipid droplets contain a phosopholipid monolayer, as opposed to most transport vesicles that contain a lipid bilayer, transport to lipid droplets is likely to be different from canonical vesicle trafficking. More work is needed to elucidate this trafficking pathway in both uninfected and infected cells and Dengue virus infection could provide a useful model.

A number of other viruses require a functional Arf pathway for replication. Proper processing of the G protein of the vesicular stomatitis virus (VSV), a negative-strand RNA virus, requires an intact secretory system. Its topology and processing were first investigated several decades ago, and since then it has become a membrane protein among the most well-studied by both virologists and cell biologists (38). A recent human genome-wide siRNA screen revealed that COPI subunits are required for a productive infection by VSV, as are Arf1 and GBF1 (39). Unexpectedly though, COPI, Arf1 and GBF1 are required for an early step in replication, viral gene expression. Furthermore, the block in gene expression is independent of the entry and uncoating of the virus that requires endosomal transport, since gene expression is also blocked in the absence of COPI when the genome is delivered by transfection (39). Similar observations were made for two other negative-strand RNA viruses, the arenavirus lymphocytic choriomenigitis (LCMV) and the paramyxovirus parainfluenza virus type 3 (HPIV3). As reported for VSV, knockdown of Arf1 and COPI subunits inhibits LCMV gene expression. In contrast, for HPIV3, COPI subunit knockdown, but not Arf1 (at least to the level reported), prevents HPIV3 gene expression (39). How the Arf pathway contributes to the gene expression of these viruses and the differential requirements for pathway components remains an open question. These findings highlight the important contribution that cellular trafficking events have on multiple stages of infection.

The HIV multifunctional protein Nef is a known regulator of intracellular trafficking. Nef prevents the plasma membrane localization of a number of key immune regulatory proteins, including MHC-I. Nef binds MHC-I early in the secretory process and reroutes it from the TGN to lysosomal compartments for degradation (40). Nef accomplishes this rerouting by promoting a direct interaction between the mu subunit of the clathrin coat adaptor protein AP1 and MHC-I. Arf1 activates AP1 at the TGN and recent structural studies of the AP1:Arf1 multimer promoted by Nef reveal a previously unappreciated organization to the inner layer of the AP1-clathrin coat (41). This is an example of how studying a viral-mediated trafficking event can provide clues to the normal function of these factors. In addition to rerouting traffic early in the secretory pathway Nef1 also interacts with trafficking machinery at the plasma membrane to change its protein composition. A direct interaction between Nef1 and the plasma membrane localized AP2 complex is required for the Nef-mediated downregulation of CD4, which is important for HIV infection (42). Thus, Nef has evolved distinct strategies to modulate protein trafficking at different locations in the cell.

Infection with other clinically important viruses also involves Arf-mediated trafficking. Ebolavirus virion production requires Rab1a-dependent activation of GBF1 (43). Influenza virus requires COPI indirectly for entry and perhaps more directly for protein production and assembly (44). The Kaposi's sarcoma-associated herpesvirus (KSHV) regulates Arf1 during infection with clinically important implications. The KSHV protein kaposin A protein binds the Arf-GEF cytohesin-1, resulting in activation of Arf1 and regulation of integrin-mediated cell adhesion (45). This regulation is important for KSHV-mediated cellular transformation and disease, as a mutant cytohesin-1 that is unable to catalyze guanine nucleotide exchange does not transform cells.

3.2. Viruses and Golgi morphology

Virus infection can result in gross morphological changes in organelles. Alterations of picornavirus-infected cells were first observed over a half-century ago (46, 47). Disappearance of the Golgi apparatus is accompanied by the appearance of an extensive membrane network used as a platform for replication. Although the loss of the Golgi might be linked to the block in protein secretion, several lines of evidence indicate that the two processes are separate. First, synthesis of viral proteins has differing effects on the two processes. Protein 2C disrupts the Golgi but has no apparent effect on protein secretion (12, 48). Conversely, protein 2B inhibits protein secretion without noticeably altering Golgi morphology (12), unless high levels of 2B are produced (49). Second, after infection with a mutant virus with a single amino acid insertion in protein 3A that reduces the ability to inhibit protein secretion, dispersion of the Golgi resembles that produced by wildtype virus, once again demonstrating the uncoupling of the inhibition of protein secretion from Golgi dispersion (18). Thus, viral-induced membrane

alterations can result directly from viral regulation and are not merely a by-product of the block in trafficking. Tomographic analysis of infected cells can trace membrane rearrangements throughout infection. Initial formation of single membrane branching tubules early in infection gradually transform into double-membrane structures and ultimately into the double-membrane vesicles present during the late stages of infection (50). Elucidating the mechanism behind this extensive membrane rearrangement, including the source of the membrane, has been the subject of extensive study as well as some controversy. Mechanistically, production of the viral proteins 2C or 2BC leads to membrane rearrangements that include the formation of vesicles and the disappearance of Golgi stacks (48). The cellular factors involved in this 2C and 2BC-mediated mechanism remained to be elucidated.

A number of other viruses also induce morphological alterations to the Golgi. The ORF 3a protein of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is multifunctional. One of its functions is to induce Golgi fragmentation, which is restored by overexpression of Arf1, suggesting that 3a directly regulates Arf1 or an upstream factor such as GBF1 (51). Like other positive-strand viruses, SARS-CoV infection modifies intracellular membranes, forming double membrane vesicles, convoluted membranes and vesicle packets (52, 53). Consistent with the role of ORF 3a in regulating Arf1 and modulating Golgi morphology, double membrane vesicles do not form in its absence (51). However, replication of the viral genome is unaffected. Because the double membrane vesicles are not required for genome replication, they could be merely a consequence of the regulation of Arf1 and protein trafficking by ORF 3a rather than a requirement for productive infection.

In mouse hepatitis virus (MHV)-infected cells, inhibition of the Arf1 pathway reduces the number of double-membrane vesicle replication compartments. This reduction appears to be important for infection, because expression of a dominant-negative mutant of Arf1 reduces infection by about 75%, while expression of a constitutively active Arf1 mutant results in wildtype levels of virus (54). Arf1 activation during MHV infection is associated with GBF1, but not BIG1 or BIG2. Arf1 does not associate directly with the replication compartments (54). Thus, it does not act at replication sites and may instead facilitate the delivery of key components, such as phospholipids, necessary for generating the compartments.

In summary, viruses often alter cellular trafficking, in many cases by targeting the Arf pathway. In some cases this modulation may morphologically alter the membrane landscape, while in more subtle cases it may simply be to direct and concentrate cargo to a new location important for viral replication.

4. EXITING THE ENDOPLASMIC RETICULUM

The Arf-related GTPase Sar1 regulates coat protein complex II (COPII). COPII consists of Sec23, Sec24, Sec13 and Sec31, which together form a complex capable of forming vesicles from membranes (55, 56). COPII acts on the cytosolic face of the ER by inducing membrane curvature, concentrating cargo, and releasing budding vesicles. The complex is formed in a sequential manner, beginning with the activation and recruitment of the Arf-related GTPase Sar1 by the ER-resident GEF Sec12 (57). The N-terminal amphipathic helix of Sar1 is inserted in to the ER membrane and the ER-bound Sar1-GTP recruits the Sec23/24 heterodimer by binding to Sec23 (56, 58). Sec24 is the main adaptor protein of the COPII coat and interacts directly with cargos and cargo-bound receptors (59, 60). Sec23 recruits another heterodimer, Sec13/Sec31, by binding to Sec31 (61). Sec13/31 forms the outer coat of the forming vesicle and its cage-like formation drives bending and curvature formation of the membrane (62–64). Sec23 also is the GAP for Sar1 and with Sec31 promotes GTP hydrolysis and ultimately the release of vesicles from ER exit sites (65, 66). This GTPase activity is opposed by Sec16, preventing premature vesicle scission. Smaller vesicles are generated in the absence of this Sec16 regulation (67). Thus, the formation of COPII vesicles requires the concerted action of a number of factors regulated spatially and temporally, providing multiple points for viral intervention.

4.1. Viral hijacking of COPII vesicles

Poliovirus infection, as it does for the Arf pathway, also alters Sarl and COPII-mediated trafficking. Vesicles that form the poliovirus replication complex are associated with COPII. The nonstructural proteins 2B and 2BC are sufficient to generate these vesicles (68). Furthermore, Sec16, which interferes with COPII GTPase activity increases early in infection (67, 69, 70). The increase in Sec16 occurs simultaneously with an increase in COPII-derived vesicles, and this transient increase in vesicles may increase the pool of membranes that are available to form replication compartments (70). Thus, poliovirus exemplifies how a virus can target multiple aspects of the cellular trafficking system, the Arf pathway and formation of COPII vesicles, to generate an environment optimal for viral replication.

Unlike poliovirus and other enterovirus family members, FMDV replication is resistant to brefeldin A. Further, dominant-negative versions of Arf1 or Rab1a that completely disrupt Golgi morphology actually enhance FMDV replication (71). Instead, replication is sensitive to inhibition of Sar1a function, indicating that FMDV requires COPII-mediated trafficking. A dominant-active form

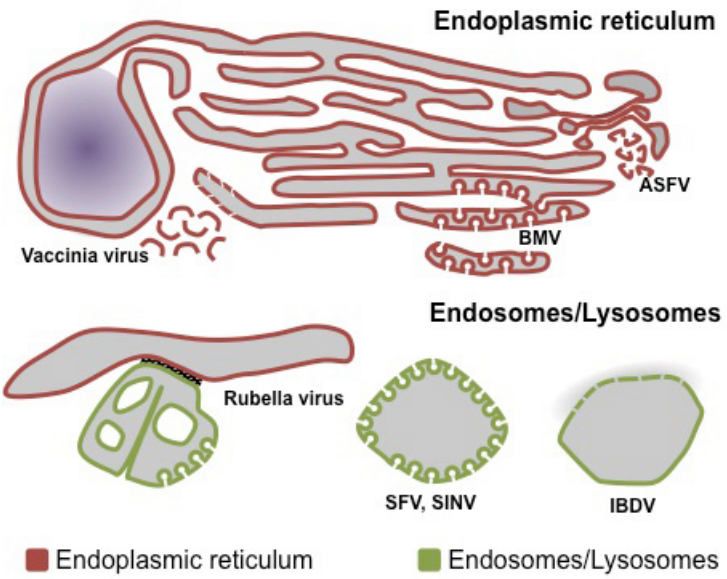

Figure 2. Cartoon representation of viral-induced alterations to the morphology of the endoplasmic reticulum, endosomes and lysosomes. Alteration of ER membrane during BMV infection involves the formation of spherules. During infection with Vaccinia virus and ASFV, ER membranes are ruptured to generate membranes used in virion assembly. Additionally, ER membranes encircle the virus factory or "mini-nucleus" formed during Vaccinia virus infection. Viruses also modify membranes of endosomes and lysosomes. SFV & SINV form spherules around the limiting membrane. Rubella also forms small vesicles or spherules in addition to large vesicles and straight elements. These modified compartments are often in close proximity to ER, mitochondria and Golgi membranes, even forming protein contacts with ER and Golgi, as depicted in the above picture with ER membrane. While endosomal membranes are not grossly altered during infection with IBDV, the pep46 protein induces small pores of less than 10 nm in endosome membranes.

of Sar1a that completely disrupts both the ERGIC and the secretory pathway by stabilizing COPII coats also supports infection (71). This result suggests that FMDV utilizes early secretory membranes for replication and also creates a replication compartment like that of other virus family members. However, unlike related viruses that target the Arf pathway, FMDV does so by targeting formation of COPII vesicles.

Norwalk virus, a single-stranded, positive-sense RNA virus, also disrupts protein secretion. The mechanism is unknown, however the viral protein p22 is sufficient to both block protein secretion and disrupt Golgi morphology (72). p22 contains an ER-export mimic sequence that allows it to incorporate into COPII vesicles (72). One hypothesis is that p22 reroutes the COPII vesicles from their normal Golgi destination. Disrupting the flow of incoming vesicles to the Golgi would inhibit protein secretion and disassemble the Golgi apparatus. Important, unanswered questions include whether the block in protein secretion is essential for a productive infection. Investigations of the p22 homologues of non-human noroviruses may be informative. Whereas p22 from human noroviruses both blocks protein secretion and disassembles the Golgi, the murine homologue, p18, disassembles the Golgi but reduces protein secretion only modestly (73). The feline calicivirus homologue, p30, does not block protein secretion or disrupt the Golgi. Thus, these two activities do not appear to be essential conserved features of norovirus p22 homologues, and at least in these species are not required for productive infection.

5. VIRUS ALTERATIONS TO ORGANELLES

In addition to regulating the transport between organelles, viruses often directly modify organelles to generate replication platforms. These modifications often result in gross alterations to the organelle morphology. For some viruses, cell death and lysis is the end game, and whether the organelles remain functional or not is inconsequential to the replication strategy. However, some viruses have protracted replication periods and must maintain at least some semblance of normal cell function. For organelles, maintenance of function may limit the kinds of changes in structure and morphology that can be tolerated. In the sections below, we will discuss some viral-induced morphological alterations to organelles (Figure 2), and describe the viral and cellular proteins responsible for these modifications.

5.1. Altering the ER

Many viruses utilize ER membranes as a platform for genome replication or as the site of envelopment. Two notable modifications to ER structure are the formation of replication spherules and the regulated rupture of collapsed ER membranes. Brome Mosaic Virus (BMV), a small, positive-strand

RNA plant virus, generates small spherules that bud into the ER. Expression of a single viral protein, 1a, is sufficient to form these 50–70 nm unscissioned vesicles, which protrude inward into the ER (74). Several cellular proteins also contribute to spherule formation. BMV is notable because it can infect yeast, and the ease in genetically manipulating yeast cells facilitates the identification and analysis of cellular factors that participate in BMV infection. Screening of yeast gene collections has led to the identification of at least 123 cellular genes that either enhance or inhibit BMV replication (75, 76). One outcome is the discovery of a role for both ESCRT and reticulon proteins in the proper formation of ER spherules (77, 78). Reticulons, which normally contribute to ER morphology, may help establish the spherules by reducing the curvature of vesicles lined with the viral protein 1a. The ESCRTs probably function at the neck of the bud, similar to their role in intraluminal vesicle formation, to maintain the spherule opening. Because ESCRT-mediated reactions usually lead to membrane scission, to stabilize the ER spherules BMV must somehow stall the progression of the ESCRT-mediated deformation event at the open bud. Thus BMV spherule formation is example of how a virus modulates host machinery to take advantage of some of its functions while preventing others.

African Swine Fever Virus (ASFV), a double-stranded DNA virus member of the nucleocytoplasmic large DNA viruses (NCLDV), also modifies ER membranes. ASFV virion membranes may be derived from open membrane precursors that originate from ruptured ER (79). Although the mechanism of this rupture is not known, the defined diameter of the membrane curls suggests that the process is carefully regulated and involves scission of the ER at regular intervals. The viral protein p54/J13Lp is necessary for the appearance of membrane precursors at virus factories (80). p54 is sufficient to induce collapse of ER cisternae, which occurs by two separate interactions mediated by distinct domains of p54. The cytoplasmic domains between p54 proteins located on neighboring cisternae form antiparallel interactions, while the luminal domains of proteins on opposite membranes of the same cisternae form disulphide bonds (81). The p54-mediated collapse of the ER cisternae may be a prerequisite for ER rupture and membrane curl formation.

5.2. Displacing the TGN

In addition to rupturing the endoplasmic reticulum to form membrane curls, ASFV also disperses the TGN. Resident proteins, TGN46, p230, sialyltransferase and AP1 relocalize to vesicles at the periphery of the ASFV assembly compartment, also referred to as the virus factory (82, 83). The fates of the different TGN proteins are not uniform, as TGN46 and p230 appear to redistribute to distinct vesicles (82). Mechanistically, dispersion of the TGN markers requires an intact microtubule network and may involve an interaction between the viral protein CD2v and the adaptor complex AP1 (82, 84). Functionally, TGN dispersion slows down trafficking to the plasma membrane and lysosomes, an outcome that likely contributes to immune evasion. There are many remaining questions such as how the virus directs different TGN proteins to different compartments, and whether this is functionally important for virus production.

Kunjin virus, the Australian strain of West Nile virus, also morphologically alters the TGN. Among the several distinct membrane alterations is the appearance of vesicle packets that co-localize with TGN markers (85). These vesicle packets may arise from repurposed TGN membranes to serve as the site of viral RNA synthesis (86). Two other membrane structures, paracrystalline arrays and convoluted membranes, arise during Kunjin virus infection from ERGIC membranes in close association with or perhaps continuous with the rough ER (85, 87). Virions assemble at these rough ER membranes, enter the ER lumen and then transit through the secretory pathway for release (88). Which viral proteins direct formation of these distinct membrane structures, each commissioned for its unique function? An NS4A-NS4B cassette containing the viral protease (NS2B-3pro) is sufficient to produce the membrane rearrangements characteristic of viral infection (89). This result suggests that cleavage of the NS4A-4B poly-protein is a key event. How the cleavage products cause the dramatic alteration of cellular membranes and which cellular proteins are required remain to be elucidated.

Other flaviviruses replicate in specialized viral-induced membrane structures that vary in composition and originate from different organelles than the related Kunjin virus. Rather than TGN, the New York 99 strain of West Nile virus uses ER-derived membranes for replication (90). The NS4B protein of this strain associates with these compartments and is involved in initiating the formation of the viral-induced membrane structures, unlike the Kunjin homologue that does not alone induce the membrane rearrangements (89, 90). Another flavivirus, DENV, also induces the formation of vesicle packets and convoluted membranes that appear to originate from the ER (91). In this case, the NS4A protein produces membrane alterations resembling those during infection (92). Thus, among viruses of the same family, strategies that utilize different membrane origins and require distinct viral proteins lead to similar outcomes.

5.3. Endosomes and lysosomes

Endosomal and lysosomal membranes are also sites of viral modifications. Rubella virus, a togavirus, replicates in a “cytopathic vacuole” derived

from modified endosomes and lysosomes (93, 94). These vacuoles consist of vesicles of varying sizes at the periphery and an internal rigid membrane sheet that is packed with replicase proteins (95). These vacuoles are in contact with rough endoplasmic reticulum, the Golgi and mitochondria (94). Electron tomography shows that these factories are not only in close proximity to these organelles, but at least in the case of rough ER and Golgi also appear to form contacts with the cytopathic vacuole. These contacts include protein bridges, closely apposed membranes, and what is described as "fuzzy material" (95). Interestingly, the formation of these vacuoles in close association with other organelles does not affect endo-lysosomal trafficking and the ability to receive incoming material from the plasma membrane (95). Thus, despite drastic morphological alterations to accommodate viral replication, these organelles maintain their normal functionality.

Two alphaviruses, Semliki Forest virus and Sindbus virus, also utilize modified lysosomes and endosomes for replication. First observed nearly 50 years ago and historically referred to as type 1 cytopathic vacuoles, these compartments resemble those formed by rubella virus in that they have membrane invaginations or spherules of approximately 50 nanometers spaced around the limiting membrane of the vacuole (96). Their formation and endosomal association of replication proteins requires an intact polyprotein containing the viral non-structural proteins 1 and 3. The individual non-structural proteins are not sufficient (97). Although associated with lysosomes, in vertebrate cells these replication spherules appear to originate at the plasma membrane and are subsequently internalized and delivered to endosomal and lysosomal membranes (98, 99). Migration of the replication spherules depends on endocytosis that requires phosphatidylinositol-3-kinase, actin and myosin, followed by long-range transport on the microtubule network (100). With the exception of dynamin and to a lesser extent nocadazole (which may have other effects on replication), endocytosis inhibitors that prevent the migration of vesicles do not profoundly reduce viral replication, suggesting that the virus can replicate in spherules that remain located at the plasma membrane (99). Notably, in mosquito cells the distribution of alphavirus replication spherules between the plasma membrane and endosomal/lysosomal membranes is different from distribution in vertebrate (99). This may simply reflect a difference in endocytosis dynamics, or may be a more direct consequence of the actions of the particular viral proteins that direct compartment formation in each kind of cell.

Replication of infectious bursal disease virus (IBDV) of the *Birnaviridae* family also requires the endocytic compartment for replication. The virus causes immunosuppression in chickens and thus its control is economically important to the poultry industry. IBDV replicates on modified membranes of endocytic compartments that label with EEA1, Rab5, LAMP-1 and LAMP-2 (101). Unlike the membranes of the replication compartments of the viruses discussed above, those of IBDV compartments are not grossly altered. Rather, IBDV encodes a 46 amino acid peptide, pep46, which induces small pores of less than 10 nm in endosomal membranes. These pores allow the exchange of molecules that initiate replication (102). One model is that replication factors exit the endosome through the pores, allowing the viral protein VP3, which localizes to endosomes, to direct the association of these proteins with the limiting membrane. These replication factors would then remain associated with endosomal membranes as the endosomes traverse the microtubule network to the Golgi complex, where viral assembly is completed (101). In this model, viral modification of the organelle membrane allows entry, replication and assembly to be coordinated in a well-integrated, dedicated subcellular space.

6. VIRUS-INDUCED COMPARTMENTS

In addition to modifying existing organelles, viruses often create their own environment or "organelle" that accumulates the viral and cellular proteins, lipids, and other factors required for optimal replication. This process often involves gross rearrangements and/or mixing of existing organelles. Replication of the aforementioned NCLDVs occurs in this type of viral "factory." The distinctive cytoplasmic virus factory of the NCLDV member vaccinia virus, a model poxvirus, transitions through several different states during infection. After the onset of viral DNA synthesis, the virus factory becomes completely enwrapped by ER membrane, a process that requires the viral E8R protein (103). This form of the viral DNA-containing membrane-bound compartment resembles a mini-nucleus. As virus assembly begins, DNA replication declines rapidly and the ER membrane dissociates from the factory (103). Subsequently, membrane crescents that will ultimately form the viral envelope associate with the compartment. These crescents are derived in the cytoplasm from small patches of pre-existing intracellular membrane (104). Unlike most viruses that acquire an envelope by budding through the plasma membrane or the limiting membrane of an organelle, poxviruses derive their primary envelope from coalescence of these crescents.

The viral proteins required for the proper formation, delivery and assembly of poxvirus membranes have been investigated by genetic analysis. Because many of these proteins are essential, conditional expression systems must be used to grow mutant viruses. Some of these systems are not without limitations, as inducible gene schemes and

temperature-sensitive mutants can be “leaky” under supposed null-production conditions. An alternative is to grow null viral mutants in complementing cell lines. A potential advantage of using both approaches is exemplified by genetic analysis of the vaccine virus H7 gene. Instead of the crescents formed in the presence of wild type virus, cells infected under non-inducing conditions by a virus with an inducible form of H7 accumulate small membrane arcs coated with spicules in association with dense inclusions that likely represent the viroplasm that is observed in wild type virus infection (105). In contrast, these membrane arcs are not observed in cells infected with viruses completely lacking H7, produced in a complementing cell line (106). These results show a concentration-dependent effect for H7 on membrane alterations and reveal a membrane intermediate under conditions where H7 is limiting. In addition to H7, similar genetic approaches have implicated vaccinia proteins D13, A14, A17, A6, A11, L2 and A30.5. in membrane precursor formation and envelopment (105, 107–119). Together with H7, the latter four proteins make up a group referred to as viral membrane assembly proteins, or VMAPs (109). The viral kinase F10 has been implicated in orchestrating the formation of membrane crescents, likely by phosphorylting A14, A17 and/or other candidate membrane-associated proteins (114, 120, 121). The properties of these proteins and how they contribute to formation of crescents and the different classes of vaccinia virions has been recently reviewed in detail (122).

The origin of the patches that give rise to the membrane crescents has been investigated for over half a century. It was originally proposed that the crescents were synthesized “de novo” because they preferentially incorporated newly synthesized phospholipid and had a different composition than host cell membranes (104). It was then proposed that the membranes originate from the ERGIC complex because some vaccinia proteins were found to associate with ERGIC membranes (123, 124). However, formation of the immature virions does not require transport between the ER and ERGIC or Golgi, suggesting that the virus directly trafficks from the ER to the sites of immature virion formation (125). A number of recent studies provide evidence that the membrane crescents form directly from ruptured ER, capturing spicule-coated structures trapped in the lumens of partially ruptured ER structures (109, 126, 127). EM tomography supports the hypothesis that the crescents consist of a single membrane and are formed by rupturing a pre-existing membrane (111). The mechanistic events of this ER rupture are emerging, however it is not currently known how or even whether viral proteins induce breaks in the ER. The uniformity of the membrane crescents suggests a highly regulated process. A17 and D13 are important in regulating the size and shape of the growing crescents. A17 is a reticulon-like protein with membrane remodeling capability that promotes extensive tubulation of the ER upon expression (128). The reticulon-like property of A17 helps shape the growing membrane crescents in combination with the D13 scaffold, which forms a lattice that supports the growing membrane crescent (111, 112). In summary, the unique events of vaccinia virus membrane acquisition provide opportunities for investigating novel protein-membrane interactions and their effects on membrane integrity, shape and size.

The virus factory of another NCLDV, ASFV, contains partially and fully assembled virions and is the site of virus assembly (Figure 3). This compartment excludes obvious cellular organelle markers. These factories, which form at the microtubule organizing center (MTOC), have several characteristics of aggresomes and may utilize similar features for their formation. For example, like aggresomes, ASFV viral factories are dependent on microtubules and dynein and are susceptible to disruption of the dynynein/dynactin complex by overexpression of p50 dynamitin (129, 130). Additionally, viral factories are surrounded by a collapsed vimentin cage, another trait shared with aggresomes (129, 131). Early in infection, vimentin forms an aster at the future site of the virus factory next to the MTOC, which eventually converts into a cage around the factory in a process dependent on calmodulin-dependent protein kinase II (132). Similarly, in cells infected by an unrelated virus, a positive stranded RNA picornavirus, enterovirus 71, vimentin is phosphorylated by CaMK-II and rearranged around replication centers (133). This rearrangement, characteristic of aggresome formation, may simply be part of the cellular response to the accumulation of viral proteins at the factory. Alternatively, viruses may actually coordinate the vimentin rearrangement to generate both a structural component in virus factory formation and later a cage to maintain a high local concentration of viral replication components. Along this line, vimentin not only surrounds the replication compartment of vaccinia virus, but also associates with the assembling immature virions (124). Thus at least in this case, vimentin is likely to play a role in the virus life cycle that extends beyond the cellular aggresome response.

Human cytomegalovirus (HCMV) infection also extensively alters the cellular membrane landscape (Figure 3). The cytoplasmic viral assembly compartment (cVAC) of HCMV is in many ways different from the factories discussed above. One major functional difference is that because viral DNA is replicated and packaged into capsids in the nucleus, DNA replication and packaging do not occur in the cVAC. Rather, it is the site of tegument acquisition and envelopment. Morphologically, the compartment consists of nested cylinders of organelle specific vesicles derived from the Golgi, TGN and early and

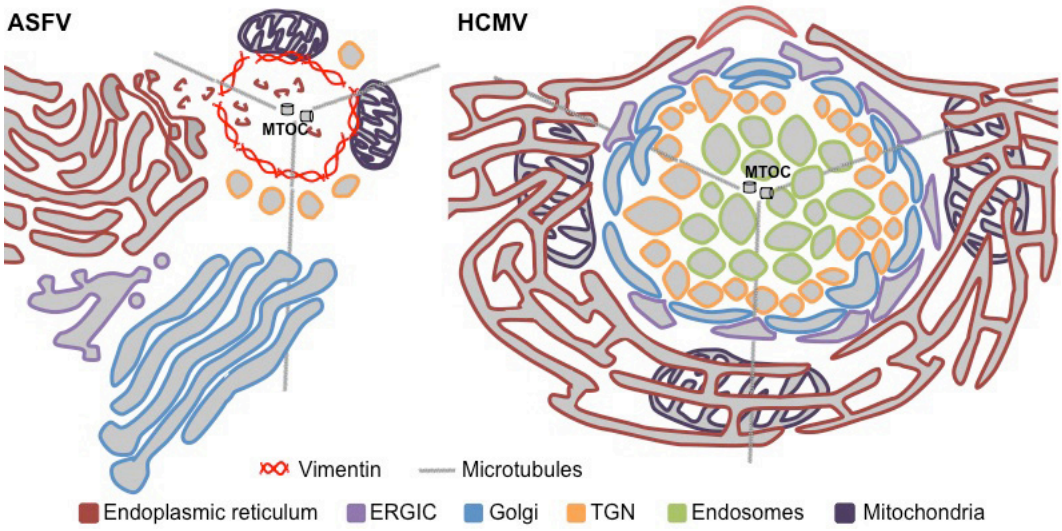

Figure 3. Depictions of the cytoplasmic compartments formed by ASFV and HCMV during infection. The assembly compartment of ASFV forms at the MTOC and is surrounded by a collapsed vimentin cage. The TGN is dispersed to vesicles at the periphery of the compartment. The assembly compartment of HCMV also forms around the MTOC. The Golgi apparatus and TGN form rings around an endosomal core. The ER fills the space around the compartment with mitochondria in close proximity.

recycling endosomes (134, 135). Like other virus factories, the cVAC forms at the perinuclear MTOC and requires an intact microtubule network and the molecular motor dynein, but unlike the other factories, the HCMV cVAC is not surrounded by a vimentin cage (136, 137). siRNA knockdown of candidate viral genes reveals three essential genes for cVAC formation: UL48, UL94 and UL103 (138). Their roles in cVAC formation have not yet been elucidated. A number of cellular proteins, many of which are involved in trafficking, are associated with the formation and/or maintenance of the cVAC,. The proteins include Rab11, Bicaudal D1, FIP4, BiP and, as mentioned previously, dynein (137, 139–141). Other cellular candidates include VAMP3, RAB5C, RAB11A, SNAP23 and CDC42, which are targeted by virus-encoded microRNAs (142). Ongoing studies of HCMV assembly should identify any additional cellular proteins and elucidate how each component participates in the redirection and reshaping of cellular organelles to form the cVAC.

What are the functional consequences of this drastic reorganization of the cellular membrane system? The HCMV life cycle can extend from four days to more than a week, depending on the cell type, and must maintain cell viability for most of this period. Viral glycoproteins, of which HCMV encodes no less than 65, must traverse the cellular secretory system for proper processing and localization, thus ruling out global inhibition of protein secretion as described above for other viruses. On the other hand, HCMV encodes a number of proteins dedicated to immune evasion by preventing the trafficking of specific cellular proteins that are required for recognition of the infected cell by the immune system. Recent reviews provide a comprehensive summary of these viral proteins and their immune evasion strategies (143, 144). Briefly, the HCMV proteins US3, US10, UL16, UL82, UL141 and UL142 block the progression of certain cellular proteins through the secretory pathway enroute to the plasma membrane (145–154). US18 and US20 direct MICA, a ligand that binds to immune cells expressing the NKG2D receptor, to the lysosomes for degradation (155). US2 and US11 target MHC class I molecules in the ER and promote their ER dislocation and subsequent proteasomal degradation (156–161). US10 can act in a similar manner to target HLA-G for degradation (162). UL20 contains an immunoglobulin-like ectodomain and is rapidly transported to the lysosome for degradation after synthesis, so that it never reaches the plasma membrane (163). Why would HCMV encode such as short-lived protein? Perhaps UL20 binds to and chaperones particular cellular proteins to the lysosome for degradation, which would add yet another layer of viral regulation of trafficking. Thus, although HCMV may not induce the global block in protein trafficking observed in other viruses, it has evolved a more targeted approach for deterring the trafficking of a number of proteins, primarily those involved in immune recognition that would compromise survival of the infected cell.

7. CONCLUSION

Viruses exhibit remarkable diversity in structure, genomes, replication and assembly strategies. Yet they face similar challenges by having to interact with the host cell environment to produce

infectious progeny. One aspect of the host system often targeted by viral proteins is the machinery involved in the transport of proteins and membrane. Viral products recruit cellular trafficking components to generate specialized compartments for optimal viral replication and assembly. They may inhibit trafficking components to prevent the proper localization of cellular proteins, a process particularly important for immune evasion. In many instances, virus infection alters the structure of organelles or disperses them altogether. In a few cases, the mechanism of action is known. In far more instances, very little is known. Increasing our understanding of how viruses interact with the cellular trafficking machinery will not only expand our knowledge of these fascinating entities, but also contribute to development of better therapeutics and vaccines.

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