Influenza infection modulates vesicular trafficking and induces Golgi complex disruption

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Abstract Influenza A virus (IFV) replicates its genome in the nucleus of infected cells and uses the cellular protein transport system for genome trafficking from the nucleus to the plasma membrane. However, many details of the mechanism of this process, and its relationship to subsequent cytoplasmic virus trafficking, have not been elucidated. We examined the effect of nuclear transport inhibitors Leptomycin B (LB), 5,6 dichloro-1- β -D-ribofuranosyl-benzimidazole (DRB), the vesicular transport inhibitor Brefeldin A (BFA), the caspase inhibitor ZWEHD, and microtubule inhibitor Nocodazole (NOC) on virus replication and intracellular trafficking of viral nucleoprotein (NP) from the nucleus to the ER and Golgi. Also, we carried out complementary studies to determine the effect of IFV on intracellular membranes. Inhibition of the CRM1 and TAP-P15 nuclear transport pathways by DRB and LB blocked completely the export of virus. Inhibition of vesicular trafficking by BFA, NOC, and ZWEHD also affected influenza infection. Interestingly, IFV infection induced fragmentation of the Golgi complex resulting in diffuse distribution of large and small vesicles throughout the cytoplasm. Live-cell microscopy revealed expansion of Golgi localization signals indicating progressive dispersion of Golgi positive structures, resulting in the disassembly of the Golgi ribbon structure. Other vesicular components (Rab1b, ARF1 and GBF1) were also

found to be required for IFV infection. Furthermore, the exact step at which IFV infection disrupts vesicle trafficking was identified as the ER-Golgi intermediate compartment. These findings suggest that IFV NP is trafficked from the nucleus via the CRM1 and TAP pathways. IFV modulates vesicular trafficking inducing disruption of the Golgi complex. These studies provide insight on the ways in which IFV affects intracellular trafficking of different host proteins and will facilitate identification of useful pharmaceutical targets to abrogate virus replication.

Keywords Influenza virus · Golgi fragmentation · Vesicular components

Introduction

Influenza A virus (IFV) is a major human disease that causes seasonal influenza, which has the capacity to elicit pandemic infection, and can be the major cause of morbidity and mortality around the world. Seasonal strains of IFV cause the death of over 50,000 people per year [28]. In addition to seasonal strains pandemic strains of IFV, epitomized by the 1918 strain that caused 50 million deaths, can cause widespread severe disease. New virus strains arise through reassortment of the genome segments during co-infection by two different IFV strains. This is exemplified by the 2009 H1N1 strain that has infected over 22 million people [8]. A comprehensive understanding of the mechanisms involved in IFV replication and interaction with host cells is imperative. The molecular and functional details of interaction of IFV with its host cell would provide a more complete picture of the influenza virus replication cycle and facilitate our ability to address questions concerning species-specificity and pathogenicity.

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Moreover, specific essential processes that take place during host-IFV interaction which are required for virus replication could be exploited for use in the development of novel prevention or intervention strategies including the formulation of new anti-viral drugs.

Influenza viruses are enveloped viruses that possess eight negative-strand RNA genome segments. In an early phase of infection, the vRNA segments are delivered to the cell nucleus where they serve as template for transcription and later, for genome replication. Newly synthesized viral proteins (such as neuraminidase and hemagglutinin) can be transported to the cell surface through the Golgi, while other proteins including NP and components of the viral polymerase complex (PB1, PB2, and PA) are trafficked to the nucleus to bind vRNA and form new viral genome particles [35]. In the nucleus, viral RNA (vRNA) segments are encapsidated into viral ribonucleoprotein (RNP) particles, in which the vRNA is associated with multiple nucleoprotein (NP) subunits and the viral polymerase complex [25]. vRNPs undergo nuclear export by binding with viral M1 and NEP (NS2) proteins, which are recognized by PHAX (phosphorylated adaptor for RNA export). This multiprotein-RNA complex then binds to CRM1 and Ran GTPases, and upon hydrolysis of GTP the vRNPs are exported to the cytoplasm.

Following nuclear export, vRNPs initially accumulate near the microtubule-organizing center (MTOC) and subsequently may associate with microtubules during transit to the plasma membrane [2]. Avalos et al. [4] showed the association of vRNPs with actin microfilaments. However, another study [3] demonstrated that actin is not essential for vRNP transport as IFV replication was not affected by disruption of actin polymerization. In addition, there is evidence that vRNPs may be transported to the plasma membrane through association with the cytoplasmic tails of HA and NA [24]. This latter “piggy-backing” transport mechanism is not mutually exclusive with transport in association with the cytoskeleton.

Vesicular trafficking of cellular components appears to be inhibited during IFV infection [17]. While the mechanism of this inhibition by IFV is unclear, studies with other viruses indicate that the Golgi can fragment or disperse during infection [7, 13], and this may result in inhibition of host protein processing and concomitant promotion of viral replication [5, 20, 40]. The Golgi normally undergoes fragmentation during mitosis as well as in apoptotic cells. Since the Golgi plays a key role in the processing of viral envelope glycoproteins, and successful infection depends on the transport of newly synthesized viral proteins from the endoplasmic reticulum (ER) to the Golgi [7], abrogation of Golgi function during infection would be incompatible with virus replication. During the process of viral protein transport from the ER to Golgi and other vesicular

transport, several Golgi and vesicular proteins, including COPs and Rabs, associate with viral proteins to facilitate their trafficking [26, 38].

Here, we use fluorescence microscopy to investigate the cellular trafficking of NP during IFV infection. Our results show that IFV likely uses two major nuclear pathways (CRM1 and TAP-P15) for export of NP from the nucleus. Moreover, IFV infection has two major effects on the cytoplasmic secretory complex. First, IFV blocks ER-to-Golgi traffic as evidenced by modulation the major vesicular components-Rab1b, GBF1, ARF1 and COPI. Second, IFV induces dramatic disruption of Golgi complex. Inhibitors that affect ER-Golgi traffic or maintenance of Golgi structure maintenance inhibited IFV replication.

Materials and methods

Viruses, cells, plasmids, antibodies and inhibitors

Virus NS1-GFP(A/Puerto Rico/8/34-GFP), PR8 (A/Puerto Rico/8/34) and IFV strains *A. memphis* (A/Memphis/4/1973) were used for infections. NS1-GFP virus was provided by Adolfo Garcia-Sastre. For multi-cycle growth experiments after inhibitor treatment, cells grown in 12 well plates were infected at a MOI 1.0. Supernatants were collected from IFV infected cells at 24 h and virus titrations were performed in MDCK cells.

A549 and MDCK cells were grown in MEM and DMEM respectively, with 5 % FBS and 100 units/ml of both penicillin and streptomycin at 37 °C. Id1F cell lines were provided by Monty Krieger, Cambridge, US. Plasmid expressing vesicular stomatitis virus G-GFP (VSVG-ts045-GFP) was purchased from Addgene. Plasmids ARF1-RFP and GBF1-GFP were from Nihal Altan Bonnet, Newark, NJ and Rab1-GFP plasmid was from Matthias P Machner, NIH. GM130 antibody was purchased from BD Transduction laboratories. β -COP and IFV Nucleoprotein (NP) antibody were purchased from Santa Cruz.

Transfections and infections

A549 cells were transfected with Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. In most experiments, A549 cells were infected with IFV A PR8 at the appropriate multiplicity of infection (MOI) for 1 h at 34 °C, medium was changed after 1 h, and the infection was continued for 24 h at 34 °C.

Inhibitor treatment

A549 cells were either treated with drugs such as Leptomycin B (LB) at 10 ng/ml, 5,6 dichloro-1- β -D-

ribofuranosyl-benzimidazole (DRB) at 50 μ M, the combination of LB and DRB drugs at 10 ng and 50 μ M respectively, caspase inhibitor 1 (Z-Trp-Glu(OMe)-His-Asp(OMe)-FMK)(ZWEHD) at 20 μ M, Brefeldin A (BFA) at 10 μ g/ml, Nocodazole (NOC) at 5 μ M concentrations for 30 min before infection and for 1 h during initial IFV infection. The medium was then changed and infection was allowed to proceed for an additional 24 h.

VSV-GFP-045 assay

VSV-G protein from the ts045 mutant strain of vesicular stomatitis virus has been widely used to study membrane transport because of its misfolding and retention in the ER at high temperature (40 °C) and its ability to correctly fold and move out of the ER and into the Golgi complex upon temperature reduction to 32 °C. A549 cells were grown to confluence in 8-chamber slides (Nunc). Cells were transfected with VSVG-GFP plasmid and kept at 40 °C overnight. Cells were infected with IFV strain *A. memphis* and again kept the cells at 40 °C for another 4 h to maintain VSVG protein in the Endoplasmic Reticulum. Cells were then either fixed or subsequently incubated at 32 °C for various time points prior to fixation.

Immunofluorescence and microscopy

Cells grown on 8-chamber slides (Nunc) were fixed for 10 min with 4 % paraformaldehyde at room temperature, washed three times with PBS, and incubated with 0.05 % Triton X 100 in PBS for 20 min. Cells were then incubated with 5 % Goat serum for 1 h at RT and further incubated with primary antibody containing goat serum for 1 h at RT. After washing three times with 0.05 % Tween 20 in PBS (PBST), cells were incubated with fluorescently labeled secondary antibody. Cells were washed with PBST three times and incubated with mouse serum for 1 h. Cells were further incubated with IFV NP antibody overnight at 4 °C. Cells were again washed with PBST and incubated with fluorescent-labeled secondary antibody for 1 h followed by washing with PBST. The slides were mounted with ProLong Gold antifade containing DAPI (Invitrogen). Images were acquired with a Zeiss Axioplan II microscope (Carl Zeiss, Thornwood, NY). Series of horizontal optical sections (0.3 μ m each) were collected and subsequently deconvolved using Slidebook 5.0 software (Intelligent Imaging Innovations, Denver, CO). Post-collection processing of the images was performed using Volocity software.

Live cell microscopy

Cells were seeded onto cover slips and BODIPY-Ceramide was added after 24 h, using manufacturer's guidelines.

Cells were then imaged at 34 °C on a Zeiss Axioplan II microscope with a LCI Plan-Neofluar 63 $\times$ objective (NA = 1.3) (Zeiss, Jena, Germany) over a period of 20 h. After 2 h, cells were infected with NS1-GFP virus. Every 10 min, a set of three images (DIC, GFP, RFP) was taken with a CCD SensiCam (PCO, Kelheim, Germany). The system was controlled using Slidebook software (Intelligent Imaging Innovations, Denver, CO), and individual frame overlays and videos were prepared using Slidebook software.

Results

Influenza infection disrupts the Golgi complex

IFV traffics through the Golgi to the plasma membrane during virus egress. To determine whether IFV affects Golgi integrity we infected A549 cells with IFV strain PR8. After 24 h we examined using immunofluorescence the intracellular distribution of two Golgi markers, GM130 and β -COP. GM130 is a resident Golgi protein and β -COP, a component of COP-I vesicles, is present at high steady state concentrations in cis-Golgi membranes. Figure 1a (i) (GM130) and c (i) (β -COP) shows the resident Golgi markers in uninfected cells. The cytoplasmic display of these two proteins is indicative of an intact Golgi. Following IFV infection, both GM130 [Fig. 1b (v)] and β -COP [Fig. 1d (v)] were markedly dispersed, with GM130 fluorescence showing a discrete punctate pattern and β -COP presenting in a more generally diffuse pattern. Figure 1b (i) and d (i) display intracellular NP distribution.

Live-cell uptake of the Golgi marker BODIPY TR ceramide in uninfected cells revealed compact juxtanuclear marker accumulation, typical of the normal ribbon-like structure of the Golgi apparatus in uninfected cells (Fig. 2). After IFV the marker was expanded and more diffuse indicating progressive dispersion of Golgi-positive structures, and consistent with disassembly of the Golgi ribbon structure. Over time, detectable Golgi elements were progressively smaller in size and larger in number, again consistent with the idea that IFV infection induces fragmentation of the Golgi.

The Golgi coatomer complex is required for transport of NP from the nucleus

The coatomer protein complex is required for Golgi membrane budding, and β -COP is an essential component of the coatomer complex. IdIF cells are derived from CHO cells and harbor a temperature-sensitive version of β -COP. In IdIF cells incubated at the nonpermissive temperature (40 °C), β -COP is degraded, COPI function is impaired,

Fig. 1 Golgi fragmentation after Influenza infection and redistribution of GM130 upon treatment with BFA, NOC, ZWEHD. A549 cells were pretreated with mock, NOC, ZWEHD and BFA for 1.5 h and then infected with Influenza at MOI 1.0. Slides were fixed with PFA and processed for indirect immunofluorescence. **a** (i–iv) Cells were either stained with GM130 antibody alone to detect Golgi (red) or

b (i–viii) costained with Influenza NP antibody (green) to detect intracellular Influenza infection. **c** (i–iv) Cells were either stained with Beta-Cop antibody alone to detect COPI (red) or **d** (i–viii) co-stained Influenza NP antibody (green) to detect intracellular Influenza infection

and the Golgi is disrupted [11]. We examined the effect of Golgi disruption on IFV NP transport by examining the intracellular distribution of NP in IdIF cells at the non-permissive temperature. CHO cells, which contain wild type β -COP, and IdIF cells were infected in parallel with IFV. Twenty four hours later Golgi markers and NP were

detected by immunofluorescence microscopy. The data indicate that NP accumulated in the nucleus in the absence of functional β -COP consistent with a defect in efficient Golgi function [Fig. 3a (i–v)]. Inhibition of Golgi function leads to aberrant accumulation of products at upstream locations including the nucleus and ER, for proteins that

Fig. 2 Live cell microscopy of Influenza infected cells. A549 cells were seeded on cover slips and stained for BODIPY-TR ceramide to detect Golgi and kept under microscope for focusing the cells and then NS1-GFP virus was provided to cells and made movie for 18 h

traffic from the nucleus and into the vesicular transport pathway.

We next wanted to verify general upstream accumulation of protein products that are transported via the Golgi. Thus, we used Rab1b and VSV-G fusion proteins containing GFP tags. Both of these proteins are translationally expressed into intracellular membranes. Rab1b regulates the vesicular transport between the ER and Golgi and is found in that locale. VSV-G protein is the envelope glycoprotein of Vesicular stomatitis virus and is transported from the ER to the Golgi and through vesicles to the plasma membrane. In IdIF cells at the nonpermissive temperature both Rab1b and VSV-G were restricted to the perinuclear space (Fig. 3b). When cells were infected with virus, consequently, viral NP was limited to the nucleus and perinuclear space. It is likely that Golgi disruption, which would impair both antero and retrograde trafficking, which would in turn affect ER activity. In the context of virus replication it is probable that inefficient transport of NP and other viral proteins would be manifested in reduction of sufficient amounts of these proteins returning to the nucleus for maintenance of replication.

ER-to-Golgi inhibitors reduce IFV transport

We next studied the effect of cellular trafficking inhibitors on the Golgi in both IFV- and mock-infected cells. Cells were treated with Brefeldin A (BFA), Nocodazole (NOC) or the caspase inhibitor 1 (ZWEHD), and the intracellular location of IFV NP and host Golgi markers, GM130 and β -COP was determined using immunofluorescence. In uninfected cells the Golgi disassembles in the presence of BFA as evidenced by diffuse distribution of GM130 and β -COP compared with untreated cells [Fig. 1a (iv), c (iv)]. IFV-

infected cells treated with BFA displayed the same pattern of Golgi (GM130 and β -COP) staining as in uninfected cells [Fig. 1b (viii), d (viii)]. However, BFA treatment markedly altered the intracellular distribution of NP in infected cells [Fig. 1b (i, iv), d (i, iv)]. In presence of NOC, the Golgi forms “minstacks,” as originally reported by Thyberg and Moskalewski [29] [Fig. 1a (ii), c (ii)]. As with treatment with BFA, NP co-localized with anomalously distributed Golgi material (i.e. NP co-localized with GM130 and β -COP following BFA or NOC treatment. Examination of mini-stack distribution has been previously interpreted as association of Golgi material with ER exit sites [10]. Treatment with either BFA or NOC significantly reduced virus titer (Fig. 4). This is consistent with the idea that alteration of normal Golgi structure inhibits virus replication through perturbation of normal trafficking of viral components.

While treatment with BFA and NOC reduced titer, we wanted to test the idea that virus-induced fragmentation of the Golgi into a greater number of Golgi elements might be a viral mechanism to promote IFV assembly. Alternatively, Golgi fragmentation might reflect one or more antiviral host responses to infection such as apoptosis. We treated uninfected and infected cells with ZWEHD, which prevents Golgi fragmentation, and then examined the intracellular distribution of NP, GM130, and β -COP. Confocal imaging revealed that the Golgi was not fragmented in presence of inhibitor ZWEHD in either uninfected and infected cells [Fig. 1a (iii), b (vii), c (iii), d (vii)]. Thus, ZWEHD effectively blocks IFV-induced Golgi fragmentation. Interestingly, in ZWEHD-treated cells, the intracellular distribution of NP was consistent with segregation of NP from the Golgi [Fig. 1b (iii, vii), b (iii, vii)]. Whereas the cytoplasmic locale of the Golgi markers GM130 and β -COP appeared similar in both ZWEHD-treated and untreated cells, NP was distributed in non-Golgi associated puncta. As with treatment with BFA and NOC, ZWEHD treatment reduced virus titer and reduced in inhibitor treated cells, which was also seen in Florescent focusing assay (Fig. 4). Since ZWEHD unexpectedly affected association of NP with intracellular membranes, it was not possible to distinguish between the possibility that this altered cytoplasmic association of NP, or whether inhibition of IFV-induced Golgi fragmentation by ZWEHD, was responsible for reduction in titer.

Viral gene expression requires ARF1 and GBF1, the upstream effectors of COPI

Since the COPI complex was affected by IFV infection we wanted to determine whether factors upstream of the COPI pathway have a role in IFV transport. We transfected A549 cells with plasmids expressing Rab1b-GFP, ARF1-RFP

Fig. 3 Influenza infection is inhibited in Idl-F cells at the non-permissive temperature. **a** CHO wild type and IdlF cells rescued with epsilon-COP were incubated at 34 °C or the non permissive temperature (40 °C) for 3 h. Cells were then left uninfected or infected with Influenza at MOI 1.0 for 4 h. Cells were fixed and processed for indirect immunofluorescence and cells were analyzed

for Golgi makers, GM130 or β -COP (red) and NP Influenza protein (red). **b** IdlF cells were transfected with Rab1b-GFP and VSVG-GFP plasmid and kept the cells at the permissive temperature and infected the cells with Influenza at MOI 1.0 and fixed and processed for indirect immunofluorescence

Fig. 4 Graph showing viral titer in supernatant of different inhibitors treated cells. A549 cells were treated with LB, DRB, combination of LB and DRB, NOC, ZWEHD, BFA for 30 min before infection and 1 h with or without Influenza infection and afterwards medium was changed and infection was proceed for next 24 h. Supernatants were collected and used for infecting MDCK cells. After 24 h, MDCK cells were fixed and processed for Fluorescent focusing assay

and GBF1-GFP fusion proteins and infected them with IFV. In uninfected cells, the vesicular components, Rab1b and ARF1 reside in the Golgi. In contrast, GBF1 localizes to the ER exit sites, VTC/intermediate compartment structures, and early Golgi compartments [23] (Fig. 5a). In IFV-infected cells these vesicular markers were dispersed from their juxta-nuclear location as expected, consistent with our previous data. Interestingly, in virus-infected cells ARF1 and GBF1 appeared to co-localize with NP, while Rab1b was more diffusely distributed than in uninfected cells. The co-localization of GBF1 with NP indicates that a subset of specific vesicular components may be involved in IFV transport, reflected by continued association of NP with these components. Alternatively, these data indicate that virus-induced Golgi fragmentation results in reconfiguration of membrane-associated components.

CRM1 and TAP-P15 inhibitors block nuclear export of influenza virus

To further examine the interplay between CRM1, TAP-P15, IFV replication and the Golgi complex, we investigated the effect of DRB (inhibitor of TAP-P15 pathway) and LB (inhibitor of CRM1 pathway) on NP and GM130 expression. A549 cells were infected with IFV, and at 90 min post-infection, treated with different concentrations of LB, DRB or a combination of both. It was found that the concentrations 90 μ M of DRB and 15 ng/ml of LB were required for complete blockage of nuclear export of IFV NP as measured 8 h post-infection (Fig. 6). We further tested the same combinations of LB and DRB for 24 h and observed that NP localization was restricted to the perinuclear space, which further affected the organization of Golgi complex as seen in Fig. 6.

Secreted protein cargo accumulates in the ER-to-Golgi intermediate compartment during IFV infection

To determine the exact step at which IFV disrupts vesicle trafficking and protein secretion, we investigated the integrity and function of early secretory compartments using a temperature-sensitive variant of vesicular stomatitis virus (VSV-G) containing a GFP fusion peptide (VSVG-ts045-GFP) [14]. Normally VSV-G is co-translationally translocated into the ER and, within few minutes exits the ER and transported to the Golgi through the ERGIC [15]. However, VSVG-ts045 is misfolded at 40 °C in ER-like structures. When the temperature is reduced to 32 °C, VSVG-ts045-GFP is moved out from the ER and localizes in the ERGIC and Golgi (Fig. 7a). We thus examined the fate of VSVG-ts045-GFP in IFV-infected cells upon release from retention in the ER. In contrast to uninfected cells (Fig. 7a), little Golgi-like VSV-G staining was observed in infected cells after 30 min of incubation at the permissive temperature. Instead, the VSVG-ts045-GFP was found in both diffuse, ER-type membranes and bright punctate structures (Fig. 7b). This indicates that IFV likely affects the general transport of membrane cargo.

Discussion

It is well known that the Golgi plays a role in the processing of IFV glycoproteins and the exocytosis of virions [36]. Further, the effects of changes in Golgi structure on transport of viral proteins have been the subject of extensive investigations. Using the Golgi markers, GM130 and β -COP we found that virus infection results in Golgi fragmentation and dispersion. The breakdown and dispersal of the Golgi stacks has been reported to lower the efficiency of protein export [29] and can facilitate the replication of some intracellular pathogens [13]. Herpes Simplex Virus-1-induced Golgi fragmentation has been found to increase the efficiency of virion release into the extracellular space [5]. It is possible that Golgi fragmentation is for facilitating the intracellular trafficking of virions during IFV infection.

The COPI complex is required for the replication of several pathogenic viruses including the flavivirus, hepatitis C virus (HCV) and the picornavirus, polio and vaccinia virus [38, 39]. The heptameric COPI complexes are connected with the Golgi membranes which leads to the budding of COPI-containing vesicles, and involved in the retrograde and intra-Golgi transport of cellular proteins and cargo [20]. In the present study, the pharmacologic inhibitor of COPI, BFA, inhibited viral growth. In addition, IFV NP export was blocked in IdLF cells, which had have

a Uninfected cells **b** Infected cells

Fig. 5 Colocalization of vesicular components during Influenza infection. **a** A549 cells were transfected with Rab1b-GFP, ARF1-RFP and GBF1-GFP plasmids. The cells were fixed and mounted and analyzed for GFP or RFP fluorescence. **b** Rab1b-GFP, ARF1-RFP and GBF1-GFP transfected cells were infected with influenza virus at an MOI of 1.0 for 24. Cells were fixed and analyzed for GFP or RFP fluorescence or the presence of NP Influenza protein

COPI function. Consequently, our results point toward a specific role of COPI in NP transport. Since COPI is also involved in endosomal transport [34], it is possible that the inhibition of correct NP localization could be indirectly due to disruption of endosomal transport required for IFV entry and uncoating. However, we favor the hypothesis that the blockage of NP transport observed in IdIF cells is due to an effect on intra-Golgi transport; if early events are inhibited this would in turn result in inhibition of subsequent steps in replication dependent on *de novo* synthesized NP. Vaccinia virus needs COPI for the viral protein transport albeit HIV does not require COPI [39]. The study presented here reveals a requirement for COPI in IFV infection, suggesting a critical role of this secretory pathway.

In the present study we observed a distinct staining patterns of modified intracellular distribution of Rab1, Arf1 and GBF1 in IFV infected cells, consistent with the

involvement of associated vesicular components during virus infection. Polio, HCV and coxsackie virus have been found to regulate the host vesicular components i.e. ARF1, GBF1, and COPI during their successful replication [6, 16]. The overexpression of Arf1 or GBF1 reversed the effects of coxsackie virus 3A protein by not inhibiting the trafficking in between ER-Golgi [33]. Yamayoshi et al. [37] also showed that dominant-negative mutants of the GTPase Rab1b inhibited the Ebola virus matrix protein VP40-mediated particle formation, consistent the use of this vesicular pathway in the exit of this filovirus. Recently it has been reported that cdc42, which shuttles between Golgi and cytoskeleton, regulates IFV NA transport [32]. Cdc42 appears to function downstream of ARF1, which controls COPI binding and acts as a master regulator of Golgi structure and function [32]. The involvement of ARF1 and GBF1 in affecting the NP expression indicates the function of the host cell secretory pathway is important during IFV replication. It will be of further interest to determine whether COPI complex directly modulates the IFV replication or it requires another factors. Expression of dominantly interfering mutants of these vesicular components might be helpful in illuminating the intracellular trafficking during IFV infection.

Infected

Fig. 6 Effect of DRB, LB and combinatorial treatment on Golgi and NP distribution in Influenza infected cells. A549 cells were pretreated with LB, DRB and in combination for 30 min and cells were infected with Influenza MOI 1.0. Cells were fixed after 24 h p.i. with PFA and

processed for indirect immunofluorescence. Golgi structure was stained with a murine monoclonal antibody against GM130 (red) and mouse monoclonal NP antibody was used to detect intracellular Influenza NP distribution (green)

Fig. 7 A549 cells were transfected with VSVG-ts045-GFP-expressing plasmid and incubated at 40 °C overnight to accumulate VSVG-ts045-GFP protein in the ER. **a** Uninfected cells were either maintained at 40 °C or shifted to 32 °C for 15 or 30 min as indicated. The cells were subsequently fixed and mounted for fluorescence

microscopy. **b** VSVG-ts045-GFP transfected cells were infected with influenza virus at an MOI 1.0 at 40 °C for 4 h and then shifted to 32 °C for 15 and 30 min. Cells were fixed and analyzed for GFP fluorescence or the presence of NP Influenza protein by indirect immunofluorescence

It has been reported that vesicular trafficking is affected during IFV infection [17]. In the present study we wanted to determine whether inhibitors that affect vesicular components and the Golgi also affect correct NP transport. NOC inhibits microtubule polymerization which leads to trans-Golgi network fragmentation, whereas BFA disrupt the anterograde transport, and forms the trans-Golgi tubular network [9]. ZWEHD is an inhibitor of inflammatory caspases and inhibits Golgi fragmentation [13]. These inhibitors of vesicle function and integrity reduce virus replication as evidenced by a reduction in virus titer. Moreover, immunofluorescent microscopy revealed that these inhibitors affect correct intracellular trafficking of IFV NP. Our data indicate both an effect of IFV infection on normal Golgi and vesicle structure, and requirement for correct Golgi and vesicle function for efficient replication.

The replication cycle of IFV has a complex series of nuclear import and export events as IFV replicates in the nucleus and buds from the plasma membrane. Viral RNP components, including structural and nonstructural proteins, as well as vRNA and mRNA, move across the nuclear envelope in different phases of the infectious cycle. Whittaker et al. [36] demonstrated that NP shuttles between the nucleus and cytoplasm during IFV infection. The dependence of IFV virus export on the CRM1 pathway is well established and the TAP pathway has also been shown to be involved in IFV export [31]. In addition, several studies indicate specific inhibition of late viral gene expression after UV irradiation, LB, actinomycin D or

DRB treatment [1, 12, 21, 22, 27]. These studies reported that the drugs DRB and LB, which were added at the beginning of the infection, did not inhibit viral mRNA synthesis completely and that viral transcripts were retained in the nucleus, consistent with our microscopy results. In addition, the Protein Kinase C Inhibitor [also known as 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride, kinase inhibitor H7] was found to block viral late gene expression but not the viral RNA [18, 19]. Vogel et al. [30] showed that the H7 resulted in retention of HA in the nuclei of infected chick embryo fibroblasts. Amorim et al. [1] also showed that NP expression was resistant to DRB. In the present study we wanted to see if we inhibit the nuclear export of NP by inhibiting both CRM1 and TAP pathways and found that export of IFV NP is inhibited when in combination of both drugs were used. Our data indicate that both the CRM1 and TAP pathways are required for export of IFV NP as evidenced by the inhibition of nuclear export of NP by combined use of DRB and LB.

During infection with IFV export of host secretory and surface proteins is inhibited by 4 h post infection [30]. To study the specific step at which host protein secretion is blocked, we used a temperature-sensitive VSV-G-GFP fusion protein as a surrogate for general membrane protein processing. Monitoring the intracellular trafficking of tsVSV-G indicated that virus infection caused the peptide to remain in ER-like membranes and to co-localize in punctate structures with markers of the ER-to-Golgi

intermediate compartment (ERGIC). Furthermore, numerous vesicles filled with secretory cargo were observed in IFV virus-infected, as well as in uninfected, cells. This is consistent with the idea that IFV inhibits secretion by blocking traffic between the intermediate compartment and the Golgi. If ERGIC transport is inhibited this might be expected to be accompanied by dispersion of the Golgi, which occurs during IFV infection. Further investigation will be required to elucidate the mechanism leading to disruption of ERGIC to Golgi transport. Identification of individual viral proteins that mediate this effect would be useful in understanding this effect of IFV. It is possible that disruption of ERGIC-to-Golgi traffic during infection might be caused by Golgi dispersion. This effect could be mediated by the inhibition of Rab1b or GBF1 activation of Arf1 at ERGIC membranes by IFV.

We hypothesize that during IFV infection three sequential and separable effects on the host cell occur. ERGIC-Golgi trafficking is inhibited to facilitate the IFV intracellular trafficking. This in turn appears to lead to disruption in correct intracellular localization of vesicular trafficking components. This then leads to disassembly or fragmentation of the Golgi complex.

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