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Selective incorporation of vRNP into influenza A virions determined by its specific interaction with M1 protein

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ABSTRACT

Influenza A viruses contain eight single-stranded, negative-sense RNA segments as viral genomes in the form of viral ribonucleoproteins (vRNPs). During genome replication in the nucleus, positive-sense complementary RNPs (cRNPs) are produced as replicative intermediates, which are not incorporated into progeny virions. To analyze the mechanism of selective vRNP incorporation into progeny virions, we quantified vRNPs and cRNPs in the nuclear and cytosolic fractions of infected cells, using a strand-specific qRT-PCR. Unexpectedly, we found that cRNPs were also exported to the cytoplasm. This export was chromosome region maintenance 1 (CRM1)-independent unlike that of vRNPs. Although both vRNPs and cRNPs were present in the cytosol, viral matrix (M1) protein, a key regulator for viral assembly, preferentially bound vRNPs over cRNPs. These results indicate that influenza A viruses selectively uptake cytosolic vRNPs through a specific interaction with M1 during viral assembly.

1. Introduction

Influenza A virus, a major respiratory pathogen that belongs to the family of *Orthomyxoviridae*, frequently causes seasonal epidemics and periodic pandemic outbreaks. Genetic reassortment between human and animal viruses caused emergence of many pandemic viruses (de Silva et al., 2012; Neumann et al., 2009; Watanabe et al., 2012). Therefore, a thorough understanding of the molecular mechanisms underlying influenza A virus replication, genome packaging and viral assembly is needed to clarify the reassortment process, which will also allow identifying potential drug targets.

Influenza A virus contains eight segments of single-stranded, negative-sense viral RNA (vRNA) (Knipe and Howley, 2013). The viral genomes are encapsidated with nucleoproteins (NP) and associated with the trimeric polymerase complexes to form helical-shaped viral ribonucleoproteins (vRNPs) (Arranz et al., 2012; Moeller et al., 2012; Noda and Kawaoka, 2010; Zheng and Tao, 2013). Unlike other RNA viruses, influenza A virus transcribes and replicates its genome in the nucleus of the host cell (de Silva et al., 2012; Knipe and Howley, 2013; Neumann et al., 2009; Watanabe et al., 2012). Within the nucleus of infected cells, positive-sense full-length complementary RNAs (cRNAs) are synthesized. The cRNPs are replicative intermediates used as templates to generate nascent vRNPs. Progeny vRNPs are exported to the cytoplasm via chromosome region maintenance 1 (CRM1) nuclear exporter. Substantial evidence indicates an important role of viral nuclear export protein (NEP) (Huang et al., 2013; Iwatsuki-Horimoto et al., 2004; Knipe and Howley, 2013; Ma et al., 2001; Neumann et al., 2000; O'Neill et al., 1998) and matrix (M1) protein (Arranz et al., 2012; Bui et al., 2000; Cao et al., 2012; Ma et al., 2001; Martin and Helenius, 1991; Moeller et al., 2012; Noda and Kawaoka, 2010; Zheng and Tao, 2013) in vRNP nuclear export via a CRM1-dependent pathway. The current model proposes that vRNP forms a daisy chain complex with M1 and NEP. This vRNP-M1-NEP complex interacts with CRM1 in association with RanGTP, a small GTPase, resulting in the translocation of vRNP into the cytoplasm (Akarsu et al., 2003; Baudin et al., 2001; Ma et al., 2001; Neumann et al., 2000; Shimizu et al., 2011). However, other studies suggested that NP can directly interact with CRM1, which also regulates vRNP nuclear export (Chutiwitoonchai and Aida, 2016; Elton et al., 2001). After nuclear export, vRNPs are transported to the site of assembly at the apical plasma membrane through Rab11a-regulated recycling endosome (Amorim et al., 2011; Chou et al., 2013; Eisfeld et al., 2011). At the plasma membrane, M1 protein together with viral envelope proteins are considered to play a major role in incorporation of viral genome

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into budding virions during viral assembly (Ali et al., 2000; Nayak et al., 2009; Noton et al., 2007; Rossman and Lamb, 2011; Wang et al., 2010; Wu et al., 2011).

Incorporation of influenza genome is a sophisticated process, as eight segments of negative-sense vRNPs are required to make an infectious particle. Electron tomography and fluorescent *in situ* hybridization (FISH) studies demonstrate that the majority of progeny virions incorporate a single copy of all eight vRNPs (Chou et al., 2012; Lakdawala et al., 2014; Noda and Kawaoka, 2012; Noda et al., 2006). However, how influenza virus selectively uptakes vRNPs, while excluding the replicative intermediates, cRNPs, is not known. cRNPs are produced in the nucleus, but whether they remain in the nucleus throughout infection or are exported to the cytoplasm is also not clear. In fact, a very limited analysis has been done regarding localization of cRNPs in infected cells. In this study, we investigated two possible mechanisms that facilitate selective incorporation of vRNPs over cRNPs into influenza infectious virions: 1) cRNPs remain in the nucleus throughout the virus life cycle and therefore are not incorporated into progeny virions, or 2) both vRNPs and cRNPs are exported from the nucleus, but selective uptake of vRNPs occurs during trafficking to or assembly at the plasma membrane. We used a specific and sensitive qRT-PCR approach to study production and distribution of vRNAs and cRNAs at various time points after infection. Our data indicate that both vRNPs and cRNPs are exported from the nucleus to the cytoplasm, but their export mechanism seems to be different. We also showed that influenza M1 protein preferentially interacts with vRNPs over cRNPs, suggesting that influenza A viruses selectively uptake cytosolic vRNPs through a specific interaction with M1 during viral assembly.

2. Results

2.1. Influenza cRNPs are exported from the nucleus of an infected cell

To determine the translocation of cRNPs during virus replication, we first quantified the amount of cRNAs in the nuclear and cytoplasmic fractions at various times after infection and compared them with those of vRNAs and mRNAs. We prepared nuclear and cytoplasmic fractions from MDCK cells infected with A/WSN/1933 (H1N1) at 1, 4, 8, 12, and 24 h post infection (hpi). Extracted RNAs were applied for strand-specific qRT-PCR to quantify the amount of vRNAs, cRNAs and mRNAs of the neuraminidase (NA) RNA segment (Kawakami et al., 2011). First, we confirmed the quality of nuclear/cytosolic fractionations by Western blot analysis using lamin A/C as a nuclear marker and tubulin as a cytosolic marker. The presence of lamin A/C (nuclear marker) in the cytosolic fraction and tubulin (cytosolic marker) in the nuclear fraction was less than 2.8% and 0.9%, respectively, showing efficient separation of the nuclear and cytosolic samples (Fig. 1A). We also confirmed the specificity of strand-specific qRT-PCR primers using *in vitro*-transcribed RNA template (see Materials and Methods). The vRNA primer set minimally detected cRNA and mRNA (0.48% and 0.8%, respectively). Similarly, the cRNA primer set detected only 0.99% and 2.67% of vRNA and mRNA, respectively. The mRNA primer set also minimally detected vRNA and cRNA (0.08% and 0.93%, respectively) (Fig. 1B). These data indicate that the strand-specific qRT-PCR primers were highly specific to distinguish viral vRNA, cRNA, and mRNA as previously reported (Kawakami et al., 2011).

Following subcellular fractionation of infected cells, we isolated RNAs, which were subjected to qRT-PCR using the specific primer sets. At 1 hpi, we detected a small amount of vRNAs (1.7×10^2 RNA copies/cell), which originated from inoculated viruses. The cRNAs were undetectable at this time-point. At 4 hpi, vRNAs remained constant, while the copy number of cRNAs began to rise, indicating initial genome replication from inoculated vRNA templates. Small quantities of viral mRNAs were detected at 1 hpi and began to increase at 4 hpi, indicating that primary transcription from vRNA templates occurs prior to genome replication. Viral mRNAs were present in both the nuclei and cytoplasm of infected cells, indicating nuclear export of viral mRNAs. Copy numbers of vRNAs and cRNAs increased over time from 8 hpi onwards. A significant quantity ($>10^3$ RNA copies/cell) of vRNAs were present in both the nuclei and cytoplasm of infected cells at late time points (8, 12, and 24 hpi), indicating nuclear export of newly synthesized vRNPs to the cytoplasm as anticipated (Fig. 1C). Intriguingly, we also detected cRNAs in the cytoplasm at a similar ratio with vRNAs. Although the difference between cytoplasmic and nuclear cRNA was not statistically significant, we did tend to observe more cRNA in the cytoplasm than in the nucleus (Fig. 1C). These data indicate that both influenza vRNPs and cRNPs are exported from the nucleus of infected cells.

2.2. cRNPs are exported from the nucleus via a CRM1-independent pathway

A current model of vRNP nuclear export proposes that vRNP forms a daisy chain complex with M1 and NEP, in which the N-terminal domain in NEP interacts with CRM1 to navigate vRNP nuclear export (Akarsu et al., 2003; Baudin et al., 2001; Iwatsuki-Horimoto et al., 2004; Neumann et al., 2000). In contrast, recent studies showed that vRNPs passively traverse across the caspase-induced enlarged nuclear pores caused by influenza infection (Mühlbauer et al., 2015; Wurzer et al., 2003). To test if cRNPs utilize CRM1 for their trafficking across nuclear pore complexes, we treated MDCK cells with leptomycin B (LMB), a potent CRM1 inhibitor immediately after infection and determined NP localization by immunofluorescent (IF) assay. Previous studies demonstrated nuclear retention of influenza NP as a surrogate for vRNP, when infected cells were treated with LMB (Elton et al., 2001; Ma et al., 2001; Watanabe et al., 2001). Consistent with previous reports, LMB treatment caused nuclear accumulation of NP at late time points (12 and 18 hpi) (Fig. 2A). Quantification of NP signals in the nucleus and cytoplasm of the 18 hpi-sample indicates a significant increase of nuclear/cytosol ratios of NP in the LMB-treated cells ($P<0.001$) (Fig. 2B). We also investigated the effect of LMB on production of infectious virions and found that LMB treatment caused over a hundred-fold reduction in infectious virion production ($P<0.05$) (Fig. 2C).

To determine if cRNP nuclear export is CRM1-dependent, we quantified the amount of both vRNAs and cRNAs in the nuclear and cytosolic fractions of cells treated with LMB for 18 h. LMB treatment increased the quantity of vRNAs in the nucleus 2.5-fold, while slightly decreasing their amount in the cytosol, compared to the untreated condition (Fig. 2D). LMB reduced the cytosol/nuclear ratio of vRNAs from 2.49 to 0.86 ($P<0.05$) (Fig. 2E), indicating an inhibition of vRNP nuclear export in the presence of a CRM1 inhibitor consistent with previous findings (Chase et al., 2011; Chou et al., 2013; Watanabe et al., 2001). In contrast, LMB treatment did not reduce nuclear export of cRNP. More cRNA copies were detected in the cytosol than nucleus as observed in untreated cells (Fig. 2D). There was no significant difference in the cRNA cytosol/nuclear ratio between treated and untreated cells (Fig. 2E). There results suggest that cRNP nuclear export is CRM1-independent.

To test if cRNPs are exported from the nucleus via a caspase-dependent passive transportation, we treated infected cells with a caspase 3/7 inhibitor (CI) at the concentration reported to block caspase activation (Mühlbauer et al., 2015) immediately after virus infection. CI treatment slightly increased nuclear accumulation of NP (Fig. 2A and B) and caused a 10-fold reduction in infectious virion production (Fig. 2C). However, we detected no significant difference in either vRNA or cRNA cytosol/nuclear ratio between CI-treated and untreated cells (Fig. 2D and E). Overall, these results suggest that cRNP nuclear export is CRM1- and caspase- independent.