A Comparison of the Inhibition of Nucleocytoplasmic Trafficking by Viral Effectors from Cardioviruses and Rhinoviruses

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Abstract
Cardioviruses and Enteroviruses of the Picornaviridae family exhibit similar infections. Viruses from the two genera inhibit nuclear import/export through the nuclear pore complex (NPC), a channel between the cytoplasm and nucleus. However, the diseases caused by viruses within these two genera vary in severity. Encephalomyocarditis virus (EMCV), a cardiovirus, uses a small potent protein called Leader (L) to inhibit trafficking through the NPC, thereby causing encephalitis in pigs and other animals. Human rhinovirus (HRV), an enterovirus, uses a protease, 2A, to inhibit trafficking through the nuclear pore and infection is associated with the common cold. It is unknown why cardioviruses and enteroviruses cause diseases of varying severity. In addition, little information is known about whether the mechanism of these viruses’ toxic proteins in inhibiting nuclear transport through the NPC, may correlate with the severity variation of the disease phenotypes of these viruses. To test this, we would compare the rates of nuclear efflux by three different Cardioviruses and three serotypes of HRV. Molecular techniques would be used to clone and express recombinant L proteins of the cardioviruses. Both nuclear efflux and nuclear import assays would be performed using recombinant green fluorescent protein (GFP) to track infection in HeLa cells for the effects of either L protein or 2A protease. This is done to determine the extent of inhibition of nuclear trafficking at the NPC. Knowledge of the kinetics between cardioviruses and HRV could hint at the different pathogenicities of these viruses. Also, it could add to our understanding of whether the genotype of a virus can infer the phenotype of the disease it causes.

Introduction
Picomaviruses are small, non-enveloped, positive-sensed single-stranded RNA viruses (Gustin & Samow, 2002). The genome is approximately 7500 nucleotides long and is packaged in an icosahedral capsid (Lin, et al., 2009). The RNA viruses, within the Picornviridae family, cause different diseases of varying pathogenicity. Of the enteroviruses, poliovirus causes flaccid paralysis while the human rhinovirus causes the common cold and can exacerbate asthma. On the other hand, viruses of the Cardiovirus genus such as Encephalomyocarditis virus (EMCV) have been shown to be associated with outbreaks of myocarditis and encephalitis in pigs, non-human primates and other mammals (Oberste, et al., 2009). Also in the Cardiovirus genus, Theiler's murine encephalomyelitis virus (TMEV), has a neurovirulent strain and a persistent strain (thought to cause multiple sclerosis in mice) while saffold virus (SAFV) is asymptomatic in humans (Sato, Tanaka, Hasanovic, & Tsunoda) (Zoll, et al., 2009). Upon infection of cells, viral proteins of picomaviruses are recruited to aid and facilitate in viral replication, transcription and translation, as well as, evading the host cell immune response. One way by which picomaviruses shut-off host cellular processes is by inhibiting trafficking through the nuclear pore complex (NPC) (Porter & Palmenberg, 2009). The NPC is a large multi-subunit complex of approximately ~60,000 kDa that is comprised of 30 different proteins called nucleoporins (Nups) (Fontoura, Faria, & Nussenzveig, 2005). It is an aqueous pore within the nuclear envelope that allows the transport of molecules between the nucleus and cytoplasm (Lin, et al., 2009) (Sorokin, Kim, & Ovchinnikov, 2007). Small molecules, such as water and ions are transported by diffusion through the NPC, but it acts as a barrier to macromolecules such as DNA, RNA and proteins (Lin, et al., 2009).

In transporting macromolecules, each cargo needs to bear a recognition signal, either a nuclear localization sequence (NLS) for importing to the nucleus or a nuclear export sequence (NES) for exporting to the cytoplasm (Terry & Wente, 2009). Transport receptors called karyopherins associate with cargos containing either an NLS or NES (Lin, et al., 2009). The karyopherins dock the cargos at different Nups as they travel through the NPC (Fontoura, et al., 2005). Active trafficking through the NPC is mediated by a GTPase, Ran, which aids in the
dissociation and association of karyopherins and their cargos (Lin, et al., 2009). During infection by some viruses, the activities of the Nups are disrupted by viral proteins (Porter & Palmenberg, 2009).

Inhibition of active trafficking at the NPC can prevent nuclear import of antiviral signals, cellular signals that are necessary for cellular gene expression and activation of innate immunity pathways (Porter & Palmenberg, 2009).

The genera of the Picornaviridae family appear to use different mechanisms to achieve nuclear transport blockage at the NPC. The enteroviruses (e.g. poliovirus and human rhinovirus, HRV) use an encoded 2A protease to cleave nucleoporins. On the other hand, the cardioviruses (e.g. Encephalomyocarditis virus (EMCV), and Theiler’s Murine Encephalitis Virus (TMEV)) lack a 2A-like protease. Instead they encode a leader (L) protein which is used to regulate nuclear trafficking (Porter & Palmenberg, 2009). In EMCV, it has been shown that the presence of L causes phosphorylation of the nucleoporins. L, in EMCV, is thought to bind to Ran thereby inhibiting import of signal molecules and export of cellular mRNA through the NPC (Porter, Bochkov, Albee, Wiese, & Palmenberg, 2006).

In this research, we proposed to clone and express EMCV, TMEV, saffold (SaN) L protein and determine if TMEV and SaN, like EMCV disrupt the NPC by phosphorylation of the Nups. Alongside this, we proposed to compare the rates at which the cardioviruses (TMEV, EMCV and SaN) and rhinovirus inhibit nuclear import and enable nuclear efflux at the NPC. We propose that the cardioviruses (EMCV, TMEV and SaN) and the rhinoviruses will target and inhibit nuclear import into the NPC with different kinetics. Our hypothesis was that the difference in kinetics of these viruses in the disruption of nuclear import, contributes to the different disease phenotypes and the severity of the diseases caused by these picomaviruses. Knowledge of the kinetics of these viruses is significant as it could help to shed light on the varying disease severities of these picomaviruses.

**Results**

GST was amplified and digested, as well as viral L in pTriEx 1.1 vectors. Undigested pTriEx 1.1 was used as a control in the digestion of viral vectors in pTriEx 1.1. Colonies were gotten for both transformed recombinant EMCV L as well as recombinant TMEV L, however a diagnostic digest using an enzyme called Dra 1 showed that the viral L vectors did not contain the GST insert. GST-GFPNLs was purified with high purity. Most of the recombinant GFPNLs appeared to be present in the eluted protein fraction. Both the SDS-PAGE gel and the Western blot (incubated with GST antibody) showed the presence of purified GST-GFPNLs in the eluted portion of the purification process. GST-EMCV L was also purified however with ~40% purity and some GST truncations. Both the SDS-PAGE gel and the Western blot (incubated with GST antibody) showed the presence of GST-EMCV L in the eluted fraction of the purification process.

GST-EMCV L is shown to be active as an upward mobility shift (as indicated by the red box) is observed. Phosphorylation of the Nups is indicated in a Western blot by an upward mobility shift. Similar upward mobility shifts were not seen in reactions with either GST or cytosol only. The control (reaction containing GST-EMCV L provided by L. Ciomperlik) appeared not to show an upward mobility shift.

**Discussion**

For this project, we postulated that cardioviruses and rhinoviruses would inhibit nuclear trafficking through the NPC at different rates, and that this difference in inhibition could elucidate on the varying disease severities of these viruses. Previous work in the lab had shown that recombinant EMCV L phosphorylates nucleoporins, thereby preventing interaction with karyopherins that bind cargo NLSs and inhibiting import into the NPC (Porter & Palmenberg, 2009). It is unknown whether TMEV Land SaV L block import in a similar manner. To determine if TMEV Land SaV L phosphorylates nucleoporins like EMCV L, we needed to clone and express C-terminal GST-tagged viral L proteins for TMEV Land SaV L. The position of a GST tag can affect the activity of a protein. It has been shown previously that a GST tag at the N-terminus region of TMEV L and SaV L renders the L proteins inactive (Basta et. al, unpublished data).

Therefore to compare the L activities of EMCV, TMEV and SaV, GST needed to be positioned at the C-terminus end of the L proteins of the three cardioviruses.

A few colonies of *E. coli* transformed with the ligation reactions that grew on ampicillin plates but diagnostic digests of the bacterial DNA plasmid showed that there were no GST insert in the plasmid. A number of possible explanations could be derived for the inefficient ligation of GST to the viral L vectors. One possible explanation is that the enzymes (Bsu361 and Xhol) used to digest the insert and vector did not digest the insert properly. Also, the
insert to vector ratio during ligation reaction may be too low and would need to be increased for more effective ligation reactions. Nevertheless, new primers have been designed to amplify GST from another plasmid for cloning into the viral L vectors. In addition, recombinant GFP<sub>NLS</sub> was purified with high purity. To test its activity, a nuclear import assay was performed. However, during a test nuclear import assay, there was little to no green fluorescence in the nuclei of HeLa cells incubated with just GFP<sub>NLS</sub>. However, under ultraviolet (UV) light, green fluorescence was observed indicating that the protein was active in emitting green light. Previous nuclear import assay with recombinant GFP<sub>NLS</sub> and EMCV L using digitonized HeLa cell and Xenopus laevis oocytes cytosol had been successful. Similar experiments had not been done using HeLa cell cytosol. Our preliminary test nuclear import assay suggest that Hela cell cytosol was not sufficient in supplying cellular factors needed for import of GFP<sub>NLS</sub> into the nuclei to show nuclear import inhibition. Porter et al proposed that possibility of EMCV L interacting with a cellular factor in the cytosol to induce phosphorylation of the Nups and that active transport through the NPC required factors in the cytosol (2009). A nuclear efflux assay was considered for an alternative method to test GFPNLs import to the nuclei and be further used for the comparison of the nuclear trafficking inhibition by recombinant EMCV L and HRV-16. Similar to the problem associated with the nuclear import assay, nuclear efflux assay using HeLa cell cytosol was difficult. Optimization the nuclear efflux assay or using Xenopus cytosol would need to be looked at for future directions.

In continuation of the project, recombinant EMCV L with the GST tag on the N-terminus region was made to perform the nuclear import and export assay. Protein purification of the recombinant EMCV L showed that it was ~40% pure with some GST truncations. The activity of the purified recombinant EMCV L was tested using a phosphorylation assay showing a slight upward mobility shift of Nup 62 from HeLa cells. GST and cytosol incubated HeLa cell extract did not show the same mobility shift. This result is concurrent with previous studies that show that EMCV L phosphorylates Nup 62 (Porter & Palmenberg, 2009). Although, EMCV-L was shown to be active signified by the upward mobility shift, the control did not show similar result. One possible reason could be that the EMCV – L used as the control might not have be active. However, the electrophoresis of the SDS-PAGE gel shown needed to run longer in order clearly show mobility shift of the phosphorylation of Nup 62 by recombinant EMCV L.

In conclusion, recombinant EMCV L and GFP<sub>NLS</sub> was successfully purified and optimization has begun for the nuclear import and nuclear efflux assays. Further work with this project would be effectively clone and express recombinant EMCV L, TMEV L and SaN L with the GST tag on the C-terminus region. The next step would be to perform a nuclear import assay using digitonized HeLa and Xenopus cytosol to assess the inhibition of nuclear transport through the NPC by the three cardiovirus L and HRV-16 2A. Conclusions drawn from the experiment would help enlighten our understanding of the varying disease severities of these picornaviruses.

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