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The protease-sensitive loop of the vesicular stomatitis virus matrix protein is involved in virus assembly and protein translation

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ABSTRACT

To study the contribution of the protease-sensitive loop of the VSV M protein in virus assembly we recovered recombinant VSV (rVSV) with mutations in this region and examined virus replication. Mutations in the highly conserved LXD motif (aa 123–125) resulted in reduced virion budding, reduced virus titers and enhanced M protein exchange with M-ribonucleocapsid complexes (M-RNPs), suggesting that the mutant M proteins were less tightly associated with RNP *skeletons*. In addition, viral protein synthesis began to decrease at 4 h post-infection (hpi) and was reduced by ~80% at 8 hpi for the mutant rVSV-D125A. The reduced protein synthesis was not due to decreased VSV replication or transcription; however, translation of a reporter gene with an EMCV IRES was not reduced, suggesting that cap-dependent, but not cap-independent translation initiation was affected in rVSV-D125A infected cells. These results indicate that the LXD motif is involved in both virus assembly and VSV protein translation.

Introduction

Vesicular stomatitis virus (VSV) is a simple, enveloped, non-segmented negative-strand RNA virus that has served as a good model to study virus replication and assembly. There are two major serotypes of VSV found in the United States, VSV-Indiana (VSV-Ind) and VSV-New Jersey (VSV-NJ), both of which are part of a larger group of related vesiculoviruses that belong to the family *Rhabdoviridae*. The genome of VSV encodes the nucleocapsid (N) protein, the phosphoprotein (P), the matrix (M) protein, the glycoprotein (G), and the large (L) polymerase protein. The viral genomic RNA is encapsidated by the N protein and is associated with the RNA-dependent RNA polymerase (RdRp), which consists of a complex of the L and P proteins. Together, the encapsidated genome and associated RdRp make up the ribonucleoprotein particle (RNP). During virus assembly at the plasma membrane, RNPs localize to membrane microdomains containing G protein to form budding sites (Brown and Lyles, 2003a, 2003b). The M protein, which is found in both the cytoplasm and associated with the inner leaflet of the plasma membrane, is recruited into budding sites (Swinteck and Lyles, 2008) where it condenses the RNP into a tightly packed helix called the *skeleton* (Newcomb and Brown, 1981; Newcomb et al., 1982). Recently, cryo-electron microscopy (cryo-EM) of VSV-Ind virions revealed that the *skeleton* is a left-handed helix with M on the outside of the RNP anchoring it to the viral membrane (Ge et al., 2010). Surrounding the *skeleton* is the viral envelope derived from the host cell plasma membrane with G protein trimers protruding from the surface. The M protein of VSV plays a key role in virus assembly by condensing the RNP and it also contributes to the release of virions by budding from the cell surface (Chong and Rose, 1994; Harty et al., 2001; Jayakar et al., 2000). The M protein is also responsible for inducing the cytopathic effects (CPE) characteristic of a VSV infection, which include disruption of cytoskeletal organization, inhibition of host mRNA expression, inhibition of host translation, impeding nucleocytoplasmic transport, and induction of apoptosis (Ahmed and Lyles, 1998; Ahmed et al., 2003; Connor and Lyles, 2005; Faria et al., 2005; Petersen et al., 2000).

Biochemical studies suggest that an exposed protease-sensitive loop (amino acids 120-PAVLADQGQP-129) in M may be important for assembly of virions. Cleavage of this region in the M protein from VSV-Ind with the protease thermolysin resulted in an M protein that no longer self-associates (Gaudier et al., 2001, 2002). M self-association has been implicated in condensation of RNPs (Gaudin et al., 1995; Newcomb et al., 1982), which is critical for the formation of the bullet-shaped particles characteristic of rhabdovirus virions (Lyles et al., 1996; Mebatsion et al., 1999). The recent cryo-EM analysis of VSV virions led to a model of virion assembly where the loop region was important for M-M interactions between consecutive helical turns of the RNP (Ge et al., 2010). This was supported by the determination of the crystal structure of the M protein from VSV-NJ, which included amino acids 122–127 of the loop, and which showed that the N-terminal region of an adjacent M monomer interacted with the loop region in self-assembled M oligomers (Graham et al., 2008).

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To understand the role of the protease-sensitive loop of VSV-Ind M in virus assembly, Connor and Lyles (Connor et al., 2006) mutated the hydrophobic core of the loop from AVLA to KDQQ. Interestingly, the effect on virus assembly was minor, but the mutations resulted in reduced viral protein synthesis. Here, we report on the effect of additional mutations within the loop region on viral protein synthesis and VSV-Ind assembly. Characterization of the mutants revealed no effect on the cellular distribution or the in vitro budding activity of the M mutants compared to wild-type M protein (Mwt); however, attempts to recover recombinant virus encoding some of the mutants were unsuccessful. For those that were recovered, the viruses grew to lower titers and produced less total virus, indicating that the mutations in the loop may have affected virus assembly. Metabolic labeling studies with rVSV-infected cells revealed that one of the mutants (D125A) had reduced levels of viral protein synthesis beginning at 4 hpi, similar to that reported previously by Connor et al. (2006). The reduced protein synthesis for our mutant was not due to reduced genome replication or mRNA transcription. These results suggest that the protease-sensitive loop in VSV-Ind M is important for VSV assembly and that it also positively regulates translation by an unknown mechanism. Sequence analysis of this region identified a conserved LXD motif (residues 123–125 for VSV-Ind M) that is found in the matrix proteins of other vesiculoviruses, suggesting that the LXD sequence may represent an interaction motif important for reversible assembly–disassembly reactions, such as is needed during virus assembly and uncoating.

Results

Recovery and characterization of rVSVs with M loop mutations

Previous studies by Gaudier et al. (2001, 2002) identified a protease-sensitive loop in the M protein of VSV-Ind that, when digested with thermolysin, prevented the self-association of M and allowed M protein to be crystallized. Using coordinates from the M crystal structures of VSV-Ind and VSV-NJ (Graham et al., 2008), this region is clearly seen as a loop (Fig. 1A) extending from the main framework of the M structure. Alignment of the putative loop regions from several different vesiculovirus M proteins revealed that the leucine and aspartate residues within this region are conserved (Fig. 1A). Amino acids that were not present in the crystal structure of M-Ind are shown in lower case as are the residues released after thermolysin cleavage. Based on the vesiculovirus M alignments (Fig. 1A), we constructed a series of mutations (Fig. 1B) to determine whether the loop itself (Δ Loop), the size of the loop (2X Loop), or specific amino acids within the loop were involved in the assembly and budding of VSV. All of the constructs included a C-terminal tetracysteine (Lumio) tag, which we have shown does not affect M protein localization, budding activity, or uncoating when assembled into virus particles (Mire et al., 2009, 2010).

To examine the effect of the mutations on the cellular distribution and function of M, we transiently expressed the proteins in BHK cells and compared them to wild-type M protein (Mwt) by immunofluorescence microscopy (IF) and by an in vitro budding assay. There were no discernable differences in the intracellular distributions of the M loop mutants after staining with a monoclonal anti-M antibody (23H12) conjugated to Alexa-546 (Fig. 1C). The loop mutants (only D125A is shown) also had wild-type budding activity (Fig. 1D).

To determine if the M loop mutants could support virus assembly we replaced the Mwt gene in the viral genome with those encoding the loop mutants (Fig. 1B). In contrast to the results of the in vitro budding assay where all of the mutants showed wild-type budding activity, only viruses with single amino acid substitutions (D125A, L123A, and L123S) were recovered. Multiple attempts ($n=6$) to recover the other viruses were unsuccessful. One-step growth curves revealed that the L123A, L123S, and D125A mutant viruses grew more slowly, produced small plaques, and generated final titers that were approximately one-log less than rVSV-wt (Fig. 2A). Because all three

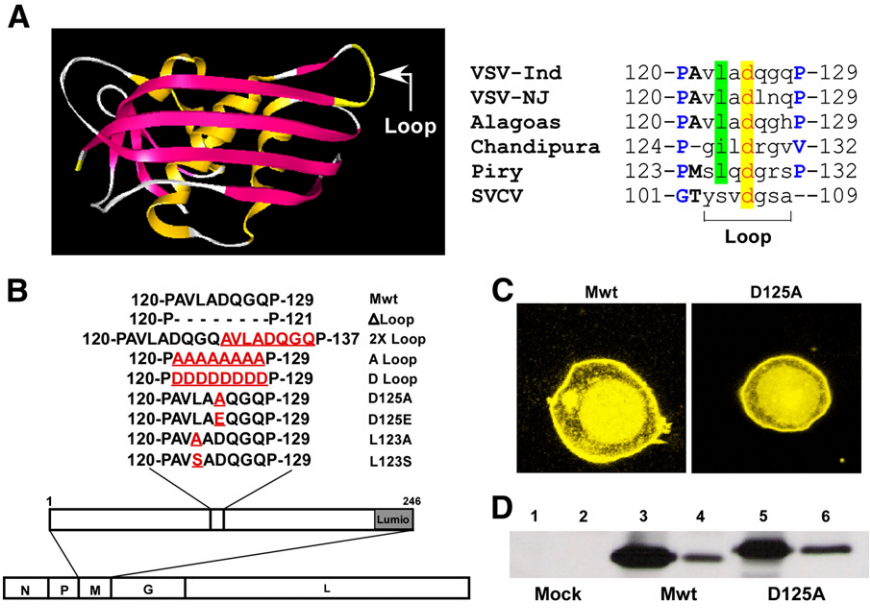

Fig. 1. Identification of a conserved LXD motif and characterization of M protein loop mutants. (A) Location of the protease-sensitive loop in VSV M protein is shown. The image was generated using coordinates from the VSV-Ind M crystal structure (MMDB ID: 50117; PDB ID: 1LG7 (Gaudier et al., 2002)) and viewed in 3D Molecule Viewer. The loop regions from 6 different vesiculoviruses were aligned using Clustal W and the location of the conserved aspartate residue (red font with yellow highlight) and semiconserved leucine/isoleucine (green highlight) are shown. (B) Schematic of mutations made in the protease-sensitive loop region of M and their location within rVSV genomes. The residues in red and underlined are either mutated to the amino acids shown, or are added (2X Loop). The Δ Loop mutant has all amino acids between the two proline residues at positions 121 and 129 deleted. All constructs have a tetracysteine Lumio tag at the C-terminus. (C) Transient expression of Mwt and the D125A loop mutant in BHK-21 cells stained using an anti-M monoclonal antibody conjugated to Alexa-546 and examined by LSCM. The images shown are 1 μm optical sections through the middle of the cell. (D) Budding assay showing intracellular protein expression (odd numbered lanes) and M protein budded into the supernatant in mock transfected (lane 2), Mwt (lane 4), and D125A (lane 6) transfected cells.