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In vivo processing and isolation of furin protease-sensitive alphavirus glycoproteins: a new technique for producing mutations in virus assembly

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Abstract

Sindbis virus particles are composed of three structural proteins (Capsid/E2/E1). In the mature virion the E1 glycoprotein is organized in a highly constrained, energy-rich conformation. It is hypothesized that this energy is utilized to drive events that deliver the viral genome to the cytoplasm of a host cell. The extraction of the E1 glycoprotein from virus membranes with detergent results in disulfide-bridge rearrangement and the collapse of the protein to a number of low-energy, non-native configurations. In a new approach to the production of membrane-free membrane glycoproteins, furin protease recognition motifs were installed at various positions in the E1 glycoprotein ectodomain. Proteins containing the furin-sensitive sites undergo normal folding and assembly in the endoplasmic reticulum and only experience the consequence of the mutation during transport to the cell surface. Processing by furin in the Golgi results in the release of the protein from the membrane. Processing of the proteins also impacts the envelopment of the nucleocapsid in the modified plasma membrane. This technique provides a unique method for studying the mechanism of virus assembly and protein structure without altering crucial early events in protein assembly, folding, and maturation.

Keywords: Alphavirus; Sindbis; Membrane glycoproteins; Furin protease; Virus assembly

Introduction

As the prototype of the *Alphavirus* genus, Sindbis virus (SV) has long served as a model system for the investigation of virus structure and the processes of assembly, host recognition, and infection (Strauss and Strauss, 1994). SV is an arthropod-borne virus with a positive polarity single stranded RNA genome that is 11,703 nt in length. The viral genome encodes four nonstructural proteins (nsP1 to nsP4) and three structural proteins (C/E2/E1). The structural proteins are translated from a polycistronic subgenomic 26S RNA which has the potential to produce the polyprotein NH_2-Capsid-PE2-6K-E1-COOH. The intrinsic autoproteolytic activity of the capsid protein (C) releases it from the developing polypeptide. This processing event exposes a signal sequence which first arrests translation and then directs the translational complex to the endoplasmic reticulum where translation reinitiates producing NH_2-PE2-6K-E1-COOH. Signal peptidase activity results in the excision of 6K from the polyprotein producing PE2 and E1 (Lilijestrom and Garoff, 1991). In the Trans Golgi Network (TGN), the PE2 precursor is processed to E2 by the endoprotease furin and E1 is converted from a stable to a metastable high-energy protein. The endoprotease furin is a member of the PACE enzyme superfamily (Paired basic Amino acid Cleaving Enzyme) (Moehring et al., 1993). Furin is a calcium dependent subtilisin-like Kex-2 analog endoprotease that carries out post-translational modifications within vesicular membranes of the secretory pathways of mammalians cells and is also found in insect cells. Furin cleaves PE2 to E2 and E3 at the conserved amino acid sequence Arg-X-Arg/Lys-Arg. The E3 protein is not found in mature SV particles.

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A mature SV particle contains 240 copies of the three structural proteins in a 1:1:1 stoichiometric arrangement with the glycoproteins E1 and E2 making up the icosahedral surface protein lattice. The outer protein shell adopts a $T = 4$ icosahedral conformation and is composed of 80 E1/E2 heterotrimeric spikes (Paredes et al., 1993, 1998). The $T = 4$ icosahedral conformation is also adopted by the internal core of the virus which is composed of 240 capsid proteins (Paredes et al., 1993). Receptor recognition by the E2 protein is postulated to be the first step in infection. Sindbis has the ability to infect a diverse range of hosts suggesting a receptor common to many species. While candidates have been found, the specific receptor(s) has yet to be identified (Klimstra et al., 2003). Once host cell recognition is established, genome introduction is the second step of infection and the E1 glycoprotein is implicated in this step. The ectodomain of the E1 glycoprotein has been demonstrated to be composed of two disulfide-bridge constrained domains; a functional domain (amino acids 1–129) and structural domain (amino acids 130–436) separated at amino acid 129 (Phinney and Brown, 2000) based on sensitivity to reducing agent (Anthony et al., 1992). These two domains contain intramolecular disulfide-bridges involving 12 cysteine residues, which stabilize the protein in a compact, energy-rich conformation. Short-term treatment with reducing agent eliminates virus infectivity (functional domain) but does not affect the integrity of the virion. Extended treatment with reducing agent dissolves the outer protein lattice (structural domain) (Anthony et al., 1992). The energy stored in the E1 protein may be used to breach the cell membrane of the host to initiate genome delivery. The mechanism by which cell penetration takes place is unclear. There are data supporting the model that penetration takes place by membrane fusion in acidic endosomes (Kielian, 1995). Other data however suggest acid-independent penetration at the cell surface by formation of a proteinaceous pore in the absence of membrane fusion (Paredes et al., 2004).

The disulfide-bridges formed by the cysteine residues in the E1 ectodomain are important for proper structure and function of the virus (see above). In a series of papers (Carleton et al., 1997; Mulvey and Brown, 1994, 1995, 1996), we have demonstrated that the E1 glycoprotein of Alphaviruses is folded progressively into a compact highly constrained and energy-rich configuration as it is assembled in the ER. Initially, E1 is found in a relaxed, extended conformation referred to as E1α (Mulvey and Brown, 1994). A series of folding intermediates, stabilized by disulfide-bridges, are formed as the E1 protein matures to a compact, stable, energy-rich conformation referred to as E1 ε. E1ε is found in the heterotrimers with PE2 which are exported from the Endoplasmic Reticulum (ER) to the trans-Golgi network (TGN) (Carleton et al., 1997). As E1 is delivered into mature virions, it is converted from a relatively stable to a metastable configuration. We have shown that treatment of mature virus with detergent results in the conversion of the E1 protein into at least 5 protein species, which can be distinguished by PAGE under non-denaturing conditions. All of these protein species migrate slower than the compact E1 form, which is assembled in the ER. This reorganization occurs rapidly upon exposure to detergent and cannot be prevented by thiol-blocking agents (Mulvey and Brown, 1994).

Detergent extraction from mature virions followed by proteolytic removal of the transmembrane domain has allowed crystallization of the Semliki Forest Virus E1 glycoprotein ectodomain and has yielded a structure at atomic resolution (Lescar et al., 2001). Likewise, release of the TBE E glycoprotein from virions by protease treatment followed by purification in detergent produced a crystal structure for Flavivirus E (Heinz et al., 1991; Rey et al., 1995). In other experiments, the ectodomain of the Dengue virus E protein (also a Flavivirus) has been crystallized in detergent after expression as a truncated protein without the membrane-anchoring domain (Modis et al., 2004). These analyses produced remarkably similar structures. All of the studies which have examined the structure of isolated Alphavirus E1 or Flavivirus E have involved steps in which the protein is exposed to detergent prior to analysis (Bressanelli et al., 2004; Gibbons et al., 2004; Heinz et al., 1991; Lescar et al., 2001; Modis et al., 2004; Rey et al., 1995; Wengler and Rey, 1999). It is therefore unclear whether or not the structures determined are of the native protein or of a reorganized product of detergent treatment as described above (Mulvey and Brown, 1994). All of the crystal structures published of E1 and E are of an extended and relaxed configuration and it is therefore possible that the crystal structure produced is one of a non-native low energy form of the protein. We have shown, using protein modification and mass spectrometry of intact virions at neutral pH, that tyrosines 1 and 15 of E1 are exposed on the surface of the virion (Phinney et al., 2000). In the crystal structure, these amino acids are buried in the fold. The protocol for production of membrane glycoproteins we describe herein may generate material which can confirm the published structure E1 or provide an alternative configuration.

To avoid complications which may arise from detergent extraction of integral membrane proteins, we have made use of the endoprotease furin to produce truncated E1 glycoproteins that are secreted from BHK-21 cells. Three functional domain mutants (furin E1 aa 130, 133, 139) predicted to release the functional domain (NH terminal domain described above) and two ectodomain mutants (furin E1 aa 392, 393) predicted to release entire ectodomain of the E1 protein were constructed. For all of these mutants, proteolytic cleavage of the E1 protein occurs after critical folding and assembly events in the ER. The data presented show that the correctly folded high-energy conformation of the E1 is transported from the ER to the TGN in the non-permissive BHK-21 cell line where it is cleaved by furin at the installed furin protease-sensitive motif. This proteolytic