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Genetic analysis of heptad-repeat regions in the G2 fusion subunit of the Junín arenavirus envelope glycoprotein

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Abstract

The G2 fusion subunit of the Junín virus envelope glycoprotein GP-C contains two hydrophobic heptad-repeat regions that are postulated to form a six-helix bundle structure required for the membrane fusion activity of Class I viral fusion proteins. We have investigated the role of these heptad-repeat regions and, specifically, the importance of the putative interhelical ***a*** and ***d*** position sidechains by using alanine-scanning mutagenesis. All the mutant glycoproteins were expressed and transported to the cell surface. Proteolytic maturation at the subtilisin kexin isozyme-1/site-1-protease (SKI-1/S1P) cleavage site was observed in all but two of the mutants. Among the adequately cleaved mutant glycoproteins, four positions in the N-terminal region (I333, L336, L347 and L350) and two positions in the C-terminal region (R392 and W395) were shown to be important determinants of cell–cell fusion. Taken together, our results indicate that α-helical coiled-coil structures are likely critical in promoting arenavirus membrane fusion. These findings support the inclusion of the arenavirus GP-C among the Class I viral fusion proteins and suggest pharmacologic and immunologic strategies for targeting arenavirus infection and hemorrhagic fever.

Keywords: Arenavirus; Junín virus or Argentine hemorrhagic fever virus; Envelope glycoprotein; GP-C; Membrane fusion; Six-helix bundle; Mutagenesis

Introduction

Arenaviruses are endemic in rodent populations worldwide (Salazar-Bravo et al., 2002) and are transmitted to humans by exposure to infected animals. Infection by Old World arenaviruses such as Lassa virus, or New World species such as the South American group including Junín, Machupo and Guanarito viruses, are responsible for recurring and emerging outbreaks of viral hemorrhagic fevers with high mortality (McCormick and Fisher-Hoch, 2002; Peters, 2002). Prophylactic and therapeutic options to combat arenavirus infection are limited, and the development of effective immunogens and antiviral agents to interfere with arenavirus entry may have broad public health benefits.

The arenaviruses are enveloped bisegmented RNA viruses whose genome consists of two single-stranded RNA molecules (Buchmeier et al., 2001; Clegg et al., 2000). During biogenesis, arenaviral particles assemble and bud at the plasma membrane. The mature envelope glycoprotein complex of the arenavirus consists of three noncovalently associated subunits derived from the GP-C precursor by proteolytic cleavage events: a stable myristoylated 58 amino-acid signal peptide (SSP), the receptor-binding G1 subunit and the transmembrane G2 fusion protein (Buchmeier, 2002; Eichler et al., 2003; York et al., 2004). The initial cleavage to yield SSP is likely mediated by the cellular signal peptidase, and the mature G1 and G2 subunits are subsequently generated by the cellular SKI-1/S1P protease. Both cleavage events, as well as the presence of the myristoylated SSP subunit, are required for envelope glycoprotein-mediated membrane fusion (York et al., 2004). Entry of virion particles into host cells is initiated by G1 binding to cell surface receptors followed by endocytosis of the virion into smooth vesicles (Borrow and Oldstone, 1994). Although α-dystroglycan serves as a binding receptor for the Old World

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arenaviruses (Cao et al., 1998), the receptor utilized by the major New World group of arenaviruses is unknown (Spiropoulou et al., 2002). Membrane fusion is pH-dependent and is activated upon acidification of the maturing endosome (Borrow and Oldstone, 1994; Castilla et al., 1994; Di Simone and Buchmeier, 1995; Di Simone et al., 1994).

The molecular basis for envelope glycoprotein-mediated membrane fusion in the arenaviruses is largely unknown, however, sequence analysis of the G2 ectodomain of Lassa virus and lymphocytic choriomeningitis virus (LCMV) has revealed two heptad-repeat regions that can be represented to form amphipathic helices (Gallaher et al., 2001). This sequence motif is found among the large group of viral envelope glycoproteins that promote membrane fusion through the formation of a fusion-active core structure comprising a stable bundle of six α-helices. These Class I fusion proteins include those of the retroviruses, orthomyxoviruses, paramyxoviruses, filoviruses and coronaviruses. In these proteins, the six-helix bundle structure involves a central coiled coil, formed by N-terminal heptad-repeat regions of the trimeric fusion subunit ectodomain, surrounded by three anti-parallel helices of the C-terminal heptad repeats which bind to the conserved hydrophobic grooves on the coiled-coil surface. Formation of this thermodynamically favored six-helix bundle brings the viral and cell membranes into apposition and is thought to provide the driving energy to initiate membrane fusion (Earp et al., 2005; Eckert and Kim, 2001; Hughson, 1997; Weissenhorn et al., 1999 and references therein). In keeping with the proposal that the arenavirus G2 ectodomain might also form a fusion-active six-helix bundle, Gallaher and colleagues have shown that a synthetic peptide derived from the N-terminal heptad-repeat region of LCMV is able to assume α-helical content (Gallaher et al., 2001).

In this report, we have utilized alanine-scanning mutagenesis of the heptad-repeat regions of the New World Junín arenavirus envelope glycoprotein to obtain genetic evidence in support of this model for membrane fusion in the arenaviruses. We show that mutations at predicted interhelical positions affect the ability of the envelope glycoprotein complex to mediate pH-dependent cell–cell fusion. These findings suggest that membrane fusion by the arenavirus envelope glycoprotein complex is promoted via a refolding into a highly stable six-helix bundle structure characteristic of Class 1 viral fusion glycoproteins.

Results and discussion

Sequence analysis of the G2 ectodomain of the Junín arenavirus

The amino acid sequences of the heptad repeats of New World and Old World arenaviruses were aligned to demonstrate the high degree of conservation within the family (Fig. 1A). One key consideration in assigning a register to the proposed helical coiled coil of the heptad repeats (i.e., positions ***a*** through ***g***) was to maximize the degree of hydrophobicity at the interhelical ***a*** and ***d*** positions. Upon inspection of the aligned sequences, a unique register of ***a*** and ***d*** positions was apparent (Figs. 1A and B). The ***a*** and ***d*** positions of the proposed N-terminal helix consist of hydrophobic residues common to the central interface of a trimeric coiled coil (e.g., leucine, isoleucine, methionine and valine). Positions assumed to lie on the exterior face of the coiled coil (***b***, ***c*** and ***f*** positions) contain polar or charged residues. This pattern is repeated through the C-terminal heptad-repeat region and appears to end at the C-terminal-most ***d*** and ***a*** positions (K409 and D413, respectively). Hydrophilic residues at two ***a*** positions in the New World viruses (R392 and S399) are replaced by less polar amino acids in the Old World viruses (serine and alanine, respectively). The hydrophilic sidechains, if buried, may impart specificity to the process of coiled-coil folding at the expense of thermal stability (Ji et al., 2000; Lumb and Kim, 1995).

*Expression of Junín virus envelope glycoproteins bearing alanine mutations at **a** and **d** positions*

To investigate the proposed α-helical structures and their role in promoting membrane fusion in the arenaviruses, we subjected the heptad-repeat regions of the Junín virus envelope glycoprotein to scanning mutagenesis. Each ***a*** and ***d*** position amino acid was individually changed to alanine, a small residue that is a good helix inducer yet contributes little to the hydrophobic forces that predominantly stabilize coiled-coil structures. Alanine substitutions at these positions are likely to alter the stability of the proposed six-helix bundle, but not to disrupt its overall folding. We anticipated that alanine mutations at interhelical positions within the coiled-coil bundle may selectively affect the ability of the envelope glycoprotein to mediate membrane fusion.

For these studies, we utilized the GP-C gene of the pathogenic Junín virus isolate MC2 (York et al., 2004). The wild-type and mutant plasmids were introduced in Vero 76 cells, and the envelope glycoproteins were expressed using the T7 promoter of the plasmid vector and T7 RNA polymerase provided by infection with the recombinant vaccinia virus vTF7-3 (Fuerst et al., 1986). Cultures were metabolically labeled and subsequently solubilized. The envelope glycoproteins were immunoprecipitated using the mouse MAb BF11 (Sanchez et al., 1989) directed to the G1 subunit (York et al., 2004).

We have previously shown that the Junín virus envelope glycoprotein complex isolated by immunoprecipitation retains the three noncovalently associated subunits: the mature G1 and G2 glycoproteins as well as the stable signal peptide, SSP (York et al., 2004 and see Fig. 2). Cleavage by signal peptidase and the SKI-1/S1P protease are incomplete upon recombinant expression such that two precursor glycoproteins (full-length GP-C and the G1–G2 precursor lacking SSP) are observed. The glycoproteins isolated by immunoprecipitation of the wild-type envelope glycoprotein, and an SKI-1/S1P cleavage-site mutant (cd-JGPC; York et al., 2004), are illustrated in Fig. 2A (far left). The two precursor glycoproteins migrate with molecular weights of 65 kDa and 60 kDa,