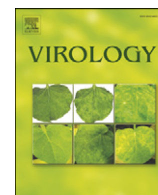
Virology 404 (2010) 139–147

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro



La Crosse virus (LACV) Gc fusion peptide mutants have impaired growth and fusion phenotypes, but remain neurotoxic

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ARTICLE INFO

Article history:
Received 21 December 2009
Returned to author for revision
5 February 2010
Accepted 13 April 2010
Available online 31 May 2010

Keywords:
La Crosse virus
Bunyaviridae
Glycoprotein
Fusion
Pathogenesis
Neuropathogenesis
Recombinant virus

ABSTRACT

La Crosse virus is a leading cause of pediatric encephalitis in the Midwestern United States and an emerging pathogen in the American South. The LACV glycoprotein Gc plays a critical role in entry as the virus attachment protein. A 22 amino acid hydrophobic region within Gc (1066–1087) was recently identified as the LACV fusion peptide. To further define the role of Gc (1066–1087) in virus entry, fusion, and neuropathogenesis, a panel of recombinant LACV (rLACV) fusion peptide mutant viruses was generated. Replication of mutant rLACVs was significantly reduced. In addition, the fusion peptide mutants demonstrated decreased fusion phenotypes relative to LACV-WT. Interestingly, these viruses maintained their ability to cause neuronal loss in culture, suggesting that the fusion peptide of LACV Gc is a determinant of properties associated with neuroinvasion (growth to high titer in muscle cells and a robust fusion phenotype), but not necessarily of neurovirulence.

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Introduction

La Crosse virus (LACV) is a member of the *Bunyaviridae* (genus *Orthobunyavirus*) and a common cause of pediatric encephalitis and aseptic meningitis in the Midwestern United States, the principal location of its major mosquito vector, *Ochlerotatus* (formerly *Aedes*) *triseriatus* (Thompson et al., 1965). About 300,000 systemic LACV infections are estimated to occur annually, though fewer than 1.5% of these are clinically apparent (Kalfayan, 1983). Approximately half of the children with LACV encephalitis have seizures during the acute illness, and about 10% will develop epilepsy. A subset of those with encephalitis (approximately 2%) will have persistent paresis, learning disabilities, or cognitive defects. It is possible that the incidence of LACV encephalitis may be under-reported because of clinical similarities with herpes simplex virus encephalitis (McJunkin et al., 1998). Importantly, because there is a well developed mouse model for LACV encephalitis that mimics many features of the human disease, LACV infection has been used to study the neuroinvasion and neurovirulence of arthropod-borne viruses (Janssen et al., 1986).

The LACV genome consists of three single-stranded RNA segments of negative polarity, designated by size as large (L), medium (M), and small (S). These segments encode the viral polymerase, two viral glycoproteins (Gc and Gn) and the nucleocapsid protein, respectively. In addition, the M and S segments each encode nonstructural proteins, NSm and NSs. The three negative-stranded RNA segments of the LACV genome (L, M, and S) have defined roles in virus pathogenesis. Previous studies have mapped the neuroinvasive phenotype of LACV to its M RNA segment (Gonzalez-Scarano et al., 1982; Janssen et al., 1984, 1986; Griot et al., 1993, 1994), whereas at least in one instance, neurovirulence mapped to the L segment (Endres et al., 1989).

The two M-segment glycoproteins, Gn and Gc, are cleaved co-translationally from a precursor polyprotein and associate as a heteromultimer (Bupp et al., 1996; Eshita et al., 1985; Fazakerley et al., 1988). This Gc/Gn heteromultimer is targeted to the Golgi apparatus, the site of viral assembly and budding, by a Golgi localization signal that has been mapped to the carboxy terminus of Gn (Lappin et al., 1994; Ruusala et al., 1992). Gc is the exclusive target of neutralizing antibodies against LACV and has been demonstrated to play a critical role in entry as the virus attachment protein (Pekosz

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0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.04.012

et al., 1995b); it is the larger of the two glycoproteins in the genus *Orthobunyavirus*. In addition, Gc and recombinant soluble forms that lack a transmembrane domain form homomultimers and undergo a pH dependent conformational change that is associated with virus-cell or cell-to-cell fusion (Pekosz et al., 1995b; Gonzalez-Scarano, 1985; Gonzalez-Scarano et al., 1984).

Previous studies from our group have demonstrated that the region corresponding to the membrane proximal two-thirds of Gc, amino acids 860–1442, is critical in LACV fusion and entry (Plassmeyer et al., 2005; Pekosz et al., 1995a,b); this is consistent with the previously defined role of Gc as the exclusive target of neutralizing antibodies generated against LACV. Moreover, computational analysis identified structural similarities between the LACV Gc amino acid region 970–1350 and the E1 fusion protein of two alphaviruses: Sindbis virus and Semliki Forrest virus (SFV). Collectively, these studies suggested that the LACV Gc functions as a class II fusion protein, much like the alphavirus E1 glycoprotein and the flavivirus E glycoprotein, and led to the identification of a 22 amino acid hydrophobic segment (1066–1087) within Gc that was predicted to correlate structurally with a hydrophobic domain of SFV and Sindbis virus (Garry and Garry, 2004; Plassmeyer et al., 2005). This hydrophobic domain is highly conserved among the Bunyaviridae and closely resembles the fusion domains of SFV E1 and avian sarcoma-leukosis virus (ASLV) E in the placement of its cysteine residues, which form an internal loop (Levy-Mintz and Kielian, 1991).

In a subsequent study, we used site-directed mutagenesis of conserved residues within LACV Gc 1066–1087 to demonstrate that LACV Gc 1066–1087 functions as the LACV fusion peptide (Plassmeyer et al., 2007). The effects of mutations within LACV Gc 1066–1087 were assessed using conformational and non-conformational antibodies specific to Gc, a luciferase based cell-to-cell fusion assay, and pseudotype transduction assays (Ma et al., 1999; Plassmeyer et al., 2005, 2007). Several mutations within this hydrophobic domain affected glycoprotein expression to some extent, but all mutations either shifted the pH threshold of fusion below that of the wild-type protein, reduced fusion efficiency, or abrogated cell-to-cell fusion and pseudotype entry altogether. Notably, a mutation at position 1066 (W1066A) was particularly informative, because it did not affect glycoprotein expression, yet abrogated fusion and entry. Collectively, these results support a role for the region Gc 1066–1087, as the LACV Gc fusion peptide.

Blakqori and Weber developed a three-cDNA plasmid reverse genetics system for LACV based on a protocol previously established for Bunyamwera virus (Blakqori and Weber, 2005). Using this technology and the existing panel of LACV Gc 1066–1087 fusion peptide mutant constructs, we have generated several recombinant viruses; we used these recombinant viruses to examine the effect of mutations in the fusion peptide region on *in vitro* growth characteristics, fusion phenotype, and neurotoxicity in the context of a virus infection in physiologically relevant cells and cell lines. We found that recombinant viruses with mutations in LACV Gc (1066–1087) were growth-impaired in muscle cells, insect cells, and primary rat neuronal cultures and had a diminished ability to mediate fusion from within (FFWI). As in wild-type virus, FFWI was pH and temperature dependent in these fusion peptide mutants. Nevertheless, all fusion peptide mutant rLACVs tested were as neurotoxic as wild-type virus in primary neuronal cultures in spite of their decreased growth kinetics and fusion phenotypes; these data are similar to results obtained with a monoclonal selected variant virus (V22) with reduced fusion function but unaltered neurovirulence (Gonzalez-Scarano et al., 1985). Overall, this study suggests that the fusion peptide of LACV Gc is a determinant of properties associated with neuroinvasion (growth to high titer in muscle cells, robust fusion phenotype), but not of neurovirulence.

Results

Generation of recombinant La Crosse virus (rLACV) with specific mutations in Gc

We previously generated several mutant LACV Gc constructs (Plassmeyer et al., 2007). These constructs had either (a) a single amino acid substitution within LACV Gc (1066–1087), (b) a deletion of the entire fusion peptide region, LACV (Δ1066–1087), (c) a substitution containing the entire fusion peptide of SFV instead of the LACV fusion peptide, LACV (SFV-FP), or (d) a mutation outside the fusion peptide region, in the tryptic site located at Gc 761 (Table 1). Although there are many potential proteolytic sites in the Gc glycoprotein (Gentsch and Bishop, 1979), the tryptic site at position 761 is uniquely accessible in the whole virion prior to acidification, and its accessibility is affected by the conformational changes that are induced by low pH (Gonzalez-Scarano, 1985). All of the mutations were engineered into construct pBluescript II KS(+)-LAC(M), then subcloned into the expression vector pCAGGS (Niwa et al., 1991) and sequenced for verification, as previously described (Plassmeyer et al., 2005).

Subsequently, these mutant glycoprotein constructs were subcloned into pT7Ribo-LACV-cM and recombinant LACVs (rLACVs) with mutations in the fusion peptide region were generated. Four recombinant viruses with targeted mutations in Gc were rescued from BSR-T7/5 cells using a three-cDNA plasmid system consisting of the L, S, and mutant M segments in antigenomic orientation, transcribed by the T7 RNA polymerase (Table 1) (Blakqori and Weber, 2005). Based on our previous data using constructs containing these respective mutations in a luciferase based cell–cell fusion assay (Plassmeyer et al., 2007), these four rLACV Gc mutants represent a wide range of predicted fusion phenotypes. Namely, one mutant construct has a normal fusion phenotype (rLACV-R761H), one has a moderately impaired fusion phenotype (rLACV-G1067A), and two have severely impaired fusion phenotypes (rLACV-V1076A, and rLACV-D1078A) (Plassmeyer et al., 2007). We also generated a control recombinant virus with a wild-type glycoprotein sequence (rLACV). After repeated attempts, we were not able to rescue viruses with other specific mutations in LACV Gc (Δ1066–1087, W1066A, G1083L, L1074A, S1077N, and R761A), even though some of these mutant Gc proteins (L1074A, S1077N, and R761A) were capable of mediating cell-to-cell fusion in a plasmid-based fusion assay

Table 1
Rescue, growth, and fusion phenotypes LACV Gc (1066–1087) amino acid deletion and substitution mutant constructs and LACV Gc 761 tryptic site mutant constructs (Plassmeyer et al., 2007).

Construct	Transfer-capable VLPs generated	rLACV rescue	Growth in BHK-21	FFWI in BHK-21
rLACV-WT	Yes	Yes	++++	++++
W1066A	No	No		
G1067A	Yes	Yes	+++	+
L1074A	Yes	No		
V1076A	Yes	Yes	+++	+
S1077N	Yes	No		
D1078A	Yes	Yes	+++	++
G1083L	No	No		
Δ1066–1087	No	No		
SFV FP	No	No		
R761A	Yes	No		
R761H	Yes	Yes	++	++++

WT (wild-type LACV M-segment construct used to generate rLACV); VLP (virus-like particles); FFWI (fusion from within).
++++ WT growth or fusion phenotype where ≥80% of the monolayer is in a heterokaryon; +++ slight decrease in growth or fusion phenotype (70–80% of monolayer in a heterokaryon), decrease is not significant; ++ impaired growth or fusion phenotype (50–70% of monolayer is in a heterokaryon, decrease is significant (p<0.001); + severely impaired growth or fusion phenotype, significant (<50% of monolayer is in a heterokaryon).

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