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An alphavirus temperature-sensitive capsid mutant reveals stages of nucleocapsid assembly

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ABSTRACT

Alphaviruses have a nucleocapsid core composed of the RNA genome surrounded by an icosahedral lattice of capsid protein. An insertion after position 186 in the capsid protein produced a strongly temperature-sensitive growth phenotype. Even when the structural proteins were synthesized at the permissive temperature (28 °C), subsequent incubation of the cells at the non-permissive temperature (37 °C) dramatically decreased mutant capsid protein stability and particle assembly. Electron microscopy confirmed the presence of cytoplasmic nucleocapsids in mutant-infected cells cultured at the permissive temperature, but these nucleocapsids were not stable to sucrose gradient separation. In contrast, nucleocapsids isolated from mutant virus particles had similar stability to that of wildtype virus. Our data support a model in which cytoplasmic nucleocapsids go through a maturation step during packaging into virus particles. The insertion site lies in the interface between capsid proteins in the assembled nucleocapsid, suggesting the region where such a stabilizing transition occurs.

Introduction

Alphaviruses comprise a genus of small, enveloped plus-sense RNA viruses containing ~40 members (Kuhn, 2013; Strauss and Strauss, 1994). Alphaviruses such as Chikungunya virus, Venezuelan equine encephalitis virus (VEEV) and Eastern equine encephalitis virus cause serious human diseases including arthritis and encephalitis (Griffin, 2013; Schwartz and Albert, 2010). There are currently no antiviral therapies for human alphavirus infections, and an approved human vaccine is only available for VEEV (Griffin, 2013; Paessler and Weaver, 2009). The alphaviruses Sindbis virus (SINV) and Semliki Forest virus (SFV) are less pathogenic for humans and have been intensively studied to characterize the entry, biogenesis, structure and assembly of members of this genus, and to identify potential targets for antiviral therapies.

Alphavirus particles contain a nucleocapsid (NC) core consisting of the RNA genome packaged with the viral capsid protein (reviewed in Kuhn (2013)). This central core is surrounded by the virus lipid bilayer containing the E1 and E2 transmembrane glycoproteins (Li et al., 2010; Voss et al., 2010). Alphaviruses infect cells by receptor-mediated endocytosis and low pH-triggered membrane fusion, which releases the NC into the cytoplasm (Kielian, 2014). Following release, the NC is disassembled and the RNA is translated to generate the viral RNA replication complex (Kuhn, 2013). During virus biogenesis the structural proteins are translated as a polyprotein from a subgenomic RNA, with the capsid protein located at the N-terminus of the polyprotein, followed by the E2 precursor protein (termed PE2/p62) and the E1 membrane fusion protein. The capsid protein is a chymotrypsin-like autoprotease that folds co-translationally and cleaves itself from the polyprotein (Aliperti and Schlesinger, 1978; Hahn et al., 1985; Nicola et al., 1999). The membrane proteins PE2 and E1 are then co-translationally translocated into the endoplasmic reticulum, form a heterodimer, and are transported through the secretory pathway to the plasma membrane. During transport PE2 is processed into the mature E2 protein by furin (deCurtis and Simons, 1988; Zhang et al., 2003). Virus particles bud from the plasma membrane.

Alphaviruses have a roughly spherical structure that is highly organized and contains 240 copies each of the capsid, E2 and E1 proteins (Cheng et al., 1995; Kuhn, 2013; Paredes et al., 1993; Tang et al., 2011; Zhang et al., 2011). The capsid protein forms a $T=4$ icosahedral lattice in which the N-terminal basic region (~residues 1–113) associates with the viral RNA in the interior, and the protease domain (residues 114–264) forms 12 pentameric and 30 hexameric capsomers on the outside of the capsid shell (Choi et al., 1991, 1997; Tang et al., 2011; Zhang et al., 2002, 2011). The E2/E1 membrane protein dimers are arranged in a $T=4$ icosahedral lattice composed of trimeric spikes. The cytoplasmic tail of each E2 protein interacts with a hydrophobic pocket on the

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C-terminal domain of the capsid protein. This 1:1 interaction of E2 and capsid is specifically required for virus budding (Jose et al., 2012; Lee et al., 1996; Zhao et al., 1994).

Cytoplasmic NCs are assembled in alphavirus-infected cells and are detectable by electron microscopy at ~6 h post-infection (Brown et al., 1972; Kuhn, 2013). Biochemical and morphological studies indicate that the structure of cytoplasmic NC differs from that of the NC in released virus particles (Coombs et al., 1984; Lamb et al., 2010), suggesting that the NC organization changes through its association with the envelope protein lattice during budding (Jose et al., 2012; Pletnev et al., 2001). However, the structure of the cytoplasmic NC is sensitive to purification (Mukhopadhyay et al., 2002), complicating its interpretation. In addition, only a fraction of the cytoplasmic capsid protein actually buds into virus. Thus, it is not clear if NC undergoes a maturational step, and if so, how such maturation might take place.

Here we characterized the unique phenotype of SINV and SFV capsid mutants with insertions at the subunit interface of capsid pentamers and hexamers in the viral nucleocapsid. The mutants showed strongly temperature-sensitive growth, but even at the permissive temperature the insertion destabilized the cytoplasmic nucleocapsids to sucrose gradient sedimentation. In contrast, nucleocapsids released from budded mutant virus particles were stable to gradient sedimentation. Thus, the capsid insertion mutants revealed a biochemical difference between nucleocapsids in the infected cell cytoplasm and those in virus particles. Our data also suggest that the transition from the cytoplasmic NC state to that in virus particles involves the interface between capsid proteins.

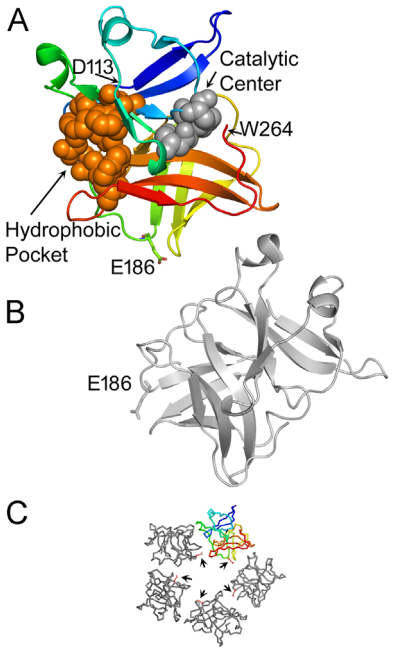

Fig. 1. Location of E186 in the SINV capsid–capsid interface. The fitting of the SINV capsid protease domain into the viral nucleocapsid structure is described in Ref. Zhang et al. (2002) and (PDB: 1LD4). A capsid pentamer from that structure is shown in panel C, with E186 shown as a red stick structure (arrows). The capsid protein shown in color and the gray protein located clockwise to it in the pentamer in C are displayed in the larger cartoon structures in A and B respectively, with E186 indicated on each. The colored subunit (A) is displayed in blue (starting at the N-terminal D113) to red (C-terminal W264). The residues that form the catalytic triad (H141, D163, and S215) are shown as space filling structures in gray. The residues that form the hydrophobic pocket into which the E2 cytoplasmic tail inserts (Lee et al., 1996; Skoging et al., 1996) are shown as space filling structures in orange. Figure was generated using PyMOL (DeLano, 2002).

Results

Characterization of SINV capsid 186-TC insertion mutant

In earlier work we introduced tetracysteine (TC) tags into various positions on the SINV capsid protein (Zheng and Kielian, 2013). The tag was well-tolerated at several positions just N-terminal to the protease domain, allowing for live cell imaging studies. Insertions at several positions within the protease domain decreased virus growth by 1–7 logs. Unique among these mutants, insertion of the tag after capsid residue E186 in the protease domain produced strongly temperature-sensitive (ts) growth. This mutant was termed 186-TC. Examination of the structure of the SINV capsid protein showed that E186 is located at the tip of a flexible loop just before the A2 β-strand in the capsid protease domain (Fig. 1) (Choi et al., 1991). In addition, based on cryo-EM reconstruction of SINV and fitting studies (Tang et al., 2011; Zhang et al., 2002), E186 lies within the capsid–capsid interface of pentamers and hexamers on the surface of the virus nucleocapsid (Fig. 1).

To quantitatively assess the temperature-sensitive phenotype, BHK-21 cells were electroporated with WT or 186-TC viral RNA and the kinetics of progeny virus production at 37 °C and 28 °C were determined. When the electroporated cells were incubated at 37 °C, the titers of the 186-TC mutant were ~4–6 logs lower than those of WT SINV at all time points (Fig. 2A). However, when the cells were incubated at 28 °C efficient production of 186-TC was observed at 10 h post-electroporation and by 40 h the titer of 186-TC was less than 1 log lower than that of WT SINV (Fig. 2B). Thus growth of the 186-TC mutant was strongly temperature-sensitive. Mutant virus stocks prepared by 30 h growth at 28 °C were shown to maintain the ts phenotype when tested on fresh cells, and thus we did not observe significant generation of revertants within the time span of our experiments.

Growth properties of SINV capsid 186 insertion mutants

To test whether the temperature-sensitive phenotype of 186-TC is dependent on the specific sequence introduced, we generated SINV mutants with a variety of insertions at the same position. Substitution of all of the cysteine residues in the TC motif with serine (186-TSer) or replacement of the 12 residue TC motif with a 13 residue motif of a different sequence (186-LAP) resulted in strongly temperature-sensitive virus growth in each case (Table 1). To determine the effect of the length of the insertion, we tested insertions that contained only a half or a quarter of the TC sequence (1/2 TC, 1/4TC). Both of these mutants had a temperature -sensitive growth phenotype, but growth at the non-permissive temperature progressively improved as the size of the insertion was reduced. Together our data demonstrate that the temperature-sensitive phenotype was independent of specific residues or amino acid sequence and that smaller insertions produced a milder phenotype.

Specific infectivity of 186-TC at 37 °C vs. 28 °C

To address whether the entry of 186-TC is temperature-sensitive, the specific infectivities of 186-TC and WT SINV were compared at 37 °C and 28 °C. [^{35}S]-labeled viruses were prepared by growth at 28 °C, and then incubated with BHK cells at 37 °C or 28 °C to allow endocytic entry and virus fusion. Further infection was then blocked with 20 mM NH_4Cl and primary infection was quantitated after incubation at either temperature, and normalized to virus radioactivity (Table 2). The specific infectivity of the 186-TC mutant was slightly less than that of WT SINV at 37 °C (~31% of WT), and was also slightly lower than the WT at 28 °C (~ 62% of WT). Thus, the specific infectivity suggested that entry was not responsible for the 4–6 log reduction in mutant virus titer at 37 °C vs. 28 °C. This experiment also suggested that, once

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