I. INTRODUCTION

Dengue viruses are mosquito-borne human pathogens that represent a serious public health threat. Mosquitoes, such as *Aedes aegypti* or *Aedes albopictus*, transmit the viruses. Annually, there are an estimated 50-100 million cases of dengue fever (DF), and 250,000 to 500,000 cases of dengue hemorrhagic fever (DHF) in the world. Over half of the world's population live in areas at risk of infection and these are popular tourist destinations too (Gubler, 1997; Gubler, 1998; Gubler and Clark, 1995; Rigau-Perez et al., 1998). All of the four serotypes of dengue virus, DEN 1, DEN 2, DEN 3 and DEN 4, can cause classical dengue fever and dengue hemorrhagic fever (Rigau-Perez et al., 1998) and infection with one serotype induces protective immunity to that virus but not to the others. Diseases caused by dengue viruses range from a mild form of DF to a more severe DHF/dengue shock syndrome (DSS), which results in considerable morbidity and mortality, especially among children in the tropical and subtropical regions of the world (Monath, 1994; Rigau-Perez et al., 1998; Kurane and Takasaki, 2001).

DF is an acute infectious disease caused by the dengue viruses. It is characterized by biphasic fever, myalgia, headache, and pain in various parts of the body, rash, lymphadenopathy, and leukopenia (Bhamarapravati et al., 1967; Burke et al., 1988; Henchal and Putnak, 1990). In most cases, DF is self-limited (Kurane and Takasaki, 2001; Lei et al., 2001), however, there is a risk of progressive development into DHF or dengue shock syndrome (DSS). DHF is a severe febrile disease characterized by abnormalities in hemostasis and increased vascular permeability, and severe progression may result in DSS (Lei et al., 2001). DSS is a form of hypovolemic shock that is associated clinically with hemoconcentration and which might lead to death if appropriate care is not given (Lei et al., 2001). Although DF is distinct from DHF/DSS by traditional classification, the various clinical manifestations after dengue virus infection show a continuum from mild to severe reactions, just as in many other viral diseases. The mechanisms involved in the pathogenesis of dengue virus infection, especially the manifestation of DHF/DSS, remain unresolved. An explanation of the pathogenesis of dengue virus infection

must account for specific characteristics of clinical, pathologic, and epidemiological observations (Lei et al., 2001).

Dengue viruses serotypes 1-4 are members of the family *Flaviviridae*. The genome of dengue viruses and other flaviviruses consist of a single molecule of positive-stranded RNA, which is 10.5-11 kb in length. The viral genome encodes three structural proteins [capsid (C), premembrane/membrane (prM/M), envelope (E)], and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). During the replication, the viral genome is translated into a long polyprotein and subsequently is cleaved by host- and virus-encoded proteases (Rice, 1996). The viral RNA genome and three of structural proteins assemble with lipid bilayer of endoplasmic reticulum (ER) to generate immature viral particles, which bud into the ER lumen and are exported through the secretory pathway; i.e. from ER, Golgi apparatus, *trans*-Golgi network (TGN) and secretory vesicles to the extracellular compartment. The surface of immature virions consist of 180 molecules each of the two glycoproteins E and prM, which associate by non-covalent bond interactions at the molecular ratio of 1:1 in the form of prM-E heterodimer (Wengler and Wengler, 1989; Zhang et al., 2003). This heterodimer form prevents premature acid-induced conformational changes of the E protein during virion maturation of immature virion in the *trans*-Golgi network and secretory vesicles (Heinz et al., 1994). During the transport, the prM protein of immature virions is cleaved into soluble pr protein and the envelope-bound M protein by subtilisin-like proprotein convertases, especially the furin protease (Murray et al., 1993, Stadler et al., 1997), resulting in the dissociation of the prM-E heterodimer. The dissociated E proteins rearrange into E homodimer (Rey et al., 1995; Heinz and Allison, 2001; Kuhn et al., 2002; Zhang et al., 2003), allowing the virions to acquire to the mature virion morphology with high infectivity.

The cleavage site of furin specific consensus sequence, Arg-Xaa-Lys/Arg-Arg or Arg-Xaa-Xaa-Arg (Xaa can be any amino acid) within the prM protein of dengue and other flaviviruses, is located at the pr-M junction. Activation cleavages are often carried out by the cellular protease furin, an enzyme that is concentrated in the *trans*-Golgi network but also cycles between endosomes and the plasma membrane (Molloy et al., 1999; Plaimauer et al., 2001). In naturally occurring substrates, including not only viral surface proteins but also a large number of cellular proproteins, furin

cleaves after the conserved sequence motif Arg-Xaa-Lys/Arg-Arg, but mutagenesis studies have revealed that the minimum consensus sequence recognized by this enzyme is Arg-Xaa-Xaa-Arg (Molloy et al., 1992; Nakayama, 1997). A mutant tick borne encephalitis (TBE) virus with specific mutation of an amino acid within the consensus sequence of the pr-M junction is not infectious (Allison et al., 1995; Stadler et al., 1997; Elshuber et al., 2003), possibly due to the failure of prM cleavage. For dengue virus, however, not all of the prM protein is cleaved and up to 40-60 molecules of prM remains uncleaved in the mature virions released from infected mosquito cell despite the presence of the consensus furin cleavage sequence at the pr-M junction (Keelapang et al., 2004). Failure to cleave all dengue prM proteins in the presence of intact consensus sequence for furin does not appear to affect the infectivity of dengue virions, suggested that certain amino acids surrounding the consensus sequence may affect the efficiency of prM cleavage, but not virus infectivity. In a recent study, Keelapang et al. (2004) constructed a full-length cDNA clone in which the 13-amino acid sequence just proximal to dengue pr-M junction was replaced with the homologous region derived from the pr-M junction of Japanese encephalitis (JE) virus. The 13-amino acid sequence just proximal to the pr-M junction of JEVpr/16681 mutant virus shares the furin consensus sequence with the parent strain 16681, but differs by the presence of three additional positively charged amino acids resulting in an increase from four positive charges to seven positive charges, and the absence of two negative charges. In addition, the side chains of two non-charged amino acids at the amino acid positions 197 (P9) and 200 (P6) of JEVpr/16681 prM also differ from the parent virus. Analysis of the resultant JEVpr/16681 chimeric virus revealed that the cleavage of prM in JEVpr/16681 was clearly enhanced as compared with the parent dengue virus strain 16681. This indicates that the cleavage of prM protein in dengue virus, while requires the presence of furin consensus sequence, can be affected by other amino acids at the pr-M junction proximal region. Unexpectedly, while the prM protein of JEVpr/16681 is cleaved to the greater extent than the parent strain, the replication of this chimeric virus is substantially reduced when compared with the parent virus (Keelapang et al., 2004).

This study was intended to further determine whether the reduction of JEVpr/16681 replication may be due to each or the combination of changes of the

amino acid sequence at the 13-amino acid region proximal to the pr-M junction: (1) the different side chains of two uncharged amino acids at the amino acid positions 197 (P9) and 200 (P6), or (2) the absence of the two negative charges at the amino acid positions 199 (P7) and 203 (P3), or (3) the presence of three additional positive charges at the amino acid positions 193 (P13), 196 (P10) and 198 (P8). In addition, it was interesting to investigate whether dengue replication would be reduced to a lower level than in JEVpr/16681 if the dengue pr-M junction encoded more positively charged amino acids than those present in the JEVpr/16681 strain. The strategy of Sriburi et al. (2001) was used in this study to construct dengue full-length cDNA clones containing four different mutations and to initiate virus replication in C6/36 cells by transfection. Production of the viral proteins and infectious virus was then monitored in the supernatant of transfected mosquito cell line for up to two weeks to explore the effect of these mutations on virus replication. Production of viruses from C6/36 cells transfected with *in vitro* transcripts RNA, focus size in PS cell, virus titer and kinetic of infection in both cell lines were then analyzed in parallel to the dengue parent strain and JEVpr/16681 chimeric virus.