CHAPTER I
INTRODUCTION

Dengue virus infection is a major global public health problem, causing undifferentiated fever, dengue fever (DF) or dengue hemorrhagic fever (DHF). *Aedes aegypti* and *Aedes albopictus* mosquitoes serve as the major vectors for the transmission of dengue viruses between infected human in the urban setting. Dengue fever is a non-specific febrile illness characterized by severe headache, pain behind the eyes, muscles and joint pains, and a development of skin rashes. In a more severe manifestation of dengue infection, DHF, plasma leakage occurring in a small proportion of infected persons causes hemoconcentration and hypovolemic shock. There are four distinct serotypes of dengue virus circulating in the tropical countries around the world. Primary dengue virus infection normally induces specific protective antibodies only against the infecting serotype. During the secondary infection, cross-reactive antibodies derived from the primary infection may enhance viral entry into FcγR-bearing cells by antibody-dependent enhancement mechanism (ADE). The ADE is believed to be an underlying basis of DHF (Malaviage et al., 2004). Annually, half a million DHF cases around the world are reported. Appropriate treatments for DHF patients have been able to decrease the mortality rate to less than 1%.

Dengue virus belongs to the genus *Flavivirus* in the family *Flaviviridae*. The serotypic and phylogenetic characterizations allow classification of dengue viruses into serotypes 1, 2, 3 and 4. A dengue virion contains a single-stranded RNA genome about 11 kilobases, which replicates in the cytoplasm of the infected cells. The genomic RNA contains one long open reading frame which serves as the template for polyprotein synthesis. The large polyprotein with multiple membrane spanning regions is then cleaved into 3 structural proteins (C, prM and E) and 7 different non-structural proteins. The viral proteins and genomic RNA assemble in the ER before

budding. Immature virions are then transported through the secretory pathway to the extracellular environment.

On the surface of immature virions, the prM glycoprotein functions as a chaperone for the receptor-binding envelope protein, E, and is believed to protect the E protein from premature configuration changes induced by acidic environment during transport through the secretory pathway (Lindenbach et al., 2001). Just before particle release, prM is cleaved in the Golgi apparatus, leaving the M portion on the particle while the N-terminal portion, pr, is secreted. Proteolytic cleavage of prM allows homodimerization and rearrangement of the E protein so that the particle becomes fusion competent (Benette et al., 2004).

Like other enveloped viruses, cleavage of the prM glycoprotein is a critical step during dengue virus replication. This processing relies on a stretch of basic amino acid residues specifically recognized by furin, called the furin consensus sequence (Arg-Xaa-(Arg/Lys)-Arg, Xaa is any amino acid) or the minimal furin motif (Arg-Xaa-Xaa-Arg) at the cleavage site (Nathan et al., 2002). These amino acid sequences are found on the furin substrates including the prM protein of flaviviruses. Various studies have demonstrated that substitutions of Arg or Lys at P1, P2 and P4 are lethal to flaviviruses (Elshuber et al., 2003, 2005; Chaichoun, 2002). The changes of basic amino acids at these positions yield non-infectious particle and trypsin treatment of these immature particles is required to rescue their infectivity (Elshuber et al., 2003.). Therefore, the cleavage of flavivirus prM protein is important for virus infectivity.

The occurrence of conserved furin consensus sequence at the pr-M junction has been observed in several studies of dengue virus genome. While similar sequences are found in many other flaviviruses, the efficiency of cleavage of dengue virus prM has been shown to be lower than those of other viruses. When the 13-amino acid sequence just proximal to the pr-M junction of a dengue serotype 2 virus, strain 16681, was replaced with the homologous sequence from three other flaviviruses (Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), yellow fever virus (YFV)), the replacement significantly altered the prM cleavage level, especially in the chimeric virus JEVpr/16681 in which the level of prM cleavage was enhanced to 94% as compared with only about 60% as in the parental strain (Keelapang et al., 2004). This analysis reveals that certain amino acids within the 13-

amino acid stretch just proximal to the pr-M junction affect the level of prM cleavage of the extracellular dengue virus particles (Sittisombut et al., 2006). Interestingly, an enhanced prM cleavage in JEVpr/16681 is accompanied by the smaller focus size, reduced viral replication *in vitro* as well as a delayed export of infectious virus particles out of the infected cells (Keelapang et al., 2004).

A comparison of the 13-amino acid sequence just proximal to the pr-M junction of JEVpr/16681 virus and the parental strain reveals three types of amino acid substitutions: 1) the substitution of uncharged residues at positions P8, P10 and P13 with arginine, 2) the substitution of glutamic acid at positions P3 and P7 with uncharged residues, and 3) the substitution of two uncharged residues at positions P6 and P9 with other uncharged residues (Songjaeng, 2004). In a previous attempt to determine which of these changes affect the focus size and virus replication, each of the three types of amino acid substitutions was introduced into the full-length cDNA clone of a dengue serotype 2 virus, strain 16681 (Sriburi et al., 2001), generating three mutant viruses designated as 16681pr(+7,-2), 16681pr(+4,-0)HS and 16681pr(+7,-0), respectively (Songjaeng, 2004). The determination of focus size revealed that, similar to JEVpr/16681, all three mutants exhibited smaller focus sizes than that of the parent virus. On the other hand, the multi-step kinetics study revealed that two mutant viruses, 16681pr(+4,-0)HS and 16681pr(+7,-2), replicated to the lower levels than that of the parent strain but higher than JEVpr/16681 whereas another mutant, 16681 pr(+7,-0), replicated to the same level as JEVpr/16681 (Songjaeng, 2004). These results lead to a conclusion that the reduction of virus replication detected in JEVpr/16681 represents a combined effect of substitutions at uncharged residues at positions P8, P10 and P13 to arginine and the replacements of glutamic acid at positions P3 and P7 with uncharged residues.

In this study, the three mutant viruses, 16681pr(+7,-2), 16681pr(+4,-0)HS and 16681pr(+7,-2), were chosen to stand for each type of amino acid changes in examining the underlying basis of delayed export and enhanced prM cleavage of JEVpr/16681. Another single point mutant virus, 16681prE203A, was also included in a single-step kinetics study. Subsequently, three other mutants with dual substitution of the positions P8, P10 and P13 were constructed: 16681pr(+6,-2)P10, 13+ containing arginine at P10, P13, 16681pr(+6,-2)P8, 13+ containing arginine at P8

and P13, and 16681pr(+6,-2)P8, 10+ containing arginine at P8 and P10. Following the construction of mutant full-length cDNA clones, the mutant viruses were generated and expanded in a mosquito cell line, C6/36. They were then compared with the parent virus and JEVpr/16681 using the focus immunoassay and the single–step kinetics assay in order to dissect in more detail the role of the arginine substitution at positions P8, P10, and P13 of the pr-M junction in affecting the export of infectious particles out of the infected cells.