V. DISCUSSION

During the replication of flaviviruses, viral RNA genome and the three structural proteins assemble with lipid bilayer of ER to generate immature viral particles, which then bud into the ER lumen. The virions are exported by the secretory pathway from ER through the Golgi apparatus, TGN and secretory vesicles to the extracellular compartment. During the transport, the prM glycoprotein on the surface of immature virions is cleaved by subtilisin-like proprotein convertases into soluble pr peptide and an envelope-bound M protein. Cleavage of prM results in the dissociation of the prM-E heterodimer, allowing the rearrangement of E glycoprotein into E homodimer and the acquisition of mature virion morphology with high infectivity (Rey et al., 1995; Heinz and Allison 2001; Kuhn et al., 2002). Cleavage of prM requires positively charged amino acids just proximal to the cleavage junction and the consensus sequence, Arg-Xaa-Lys/Arg-Arg, was thought to be required for efficient cleavage (Lindenbach and Rice 2001). However, there are additional charged residues located both within and proximal to the consensus sequence. Also, different serocomplexes within the genus flavivirus contain unique and highly conserved sets of charged amino acids in the cleavage proximal region. The contribution of these additional charged amino acids to the cleavage efficiency and the biological properties of the virus is not yet clear. In the a previous study on the influence of sequence variations of the prM cleavage junction on prM cleavage efficiency (Keelapang et al., 2004), the 13-amino acid sequence proximal to the pr-M junction of dengue virus serotype 2 was replaced with the homologous region of Japanese encephalitis virus, which contained three additional positively charged amino acids at the cleavage positions P8, P10 and P13, and lacked two negatively charged amino acids at the positions P3 and P7 when compared with dengue sequence. As a result, the pr-M junction of the chimeric virus, JEVpr/16681, contained seven positive net charges forming two furin consensus sequences (one nominal and one minimal). The analysis of JEVpr/16681 revealed that the pr-M junction of JEVpr/16681 was cleaved to the greater extent than the parent strain 16681, but the replication of this chimeric virus was substantially reduced in both

mosquito cells (C6/36) and mammalian cells (PS and Vero)(Keelapang et al., 2004). It was surprising that the extensive cleavage of prM protein did not lead to an increase of dengue virus replication as expected. Further analysis indicated that poor replication of JEVpr/16681 correlate with a delayed export of virions out of infected cells, but not with changes in virus infectivity, the ability to bind to heparin, or the changes in heparin-inhibitable infection of susceptible cells (Keelapang et al., 2004). At the present, the molecular mechanism(s) that is (are) involved in the retardation of virus export in JEVpr/16681-infected cells as well as the structural basis for the enhancement of prM cleavage remains unclear. It is possible that either one of the two types of changes of the charged residues at the pr-M junction (the addition of three positively charged residues at the positions P8, P10 and P13, and the loss of two negatively charged residues at the positions P3 and P7) could, in the absence of the other, result in an enhanced cleavage and virion export retardation. In this scenario, it is also possible that the presence of both of types of changes can have an additive effect. Alternatively, only one of these two types of changes is causing the observed enhanced prM cleavage, export retardation and reduced virus replication in JEVpr/16681. For the clarification of these possibilities, each of the two changes was introduced separately into the prM cleavage junction of the parent dengue virus strain 16681, resulting in the mutants 16681pr(+4, -0) and 16681pr(+7, -2). The former mutant lacked two glutamic acid residues at P3 and P7 whereas the latter contained three additional arginine residues at P8, P10 and P13. In addition, the influence of the two uncharged residues at the positions P6 and P9 was investigated by generating the mutant 16681pr(+7, -0) in which the P6 serine residue and P9 threonine residue of JEV sequence were substituted with the dengue virus counterparts. The three mutant viruses were viable. Subsequent comparisons of their properties (virus titer, focus size, and replication kinetics in C6/36 and PS cells) against those of the parent strain 16681 revealed that either the loss of two negative charges or the addition of three positive charges causes the reduction of virus replication. The effect of the two types of changes, when determined in the absence of the other, was more evident in PS cells than in C6/36 mosquito cells. However, the magnitude of changes as observed in the mutants 16681pr(+4, -0) and 16681pr(+7, -2) was clearly smaller than those observed with JEVpr/16681, indicating that, when present together as in JEVpr/16681, the two

types of changes cause an additive effect on virus replication. The comparison of the mutant 16681pr(+7, -0) and JEVpr/16681 revealed similar levels of changes, indicating that change of the two uncharged amino acids at P6 and P9 had no detectable effect on virus replication. These results provide an evidence for the influence of the combination of the addition of three positive charges and the loss of two negative charges at non-consensus positions within the 13-amino acid region proximal to the prM cleavage junction on dengue virus replication.

The influence of positively charged amino acids at non-consensus positions proximal to the furin cleavage junction is well documented. A previous study of hemagglutinin protein of H5N8 virulent influenza virus strains capable of causing systemic infection in avian species reveals that cleavage of the H_0 precursor, which contains two additional basic amino acids (lysine and arginine) at positions P5 and P6, respectively, proximal to the HA_1-HA_2 cleavage junction is influenced by these P5 and P6 basic residues (Walker et al., 1994). When either the P6 arginine, or the P5 arginine and P6 lysine residues were substituted with threonine and glutamic acid, respectively, cleavage of H_0 by endogenous SPC in CV-1 cells and by purified human furin in vitro was drastically reduced (Walker et al., 1994). Also, deletion of the P5 and P6 basic residues abolished cleavage of the H_0 precursor by both types of enzyme; however, this may also be due to an increase in the negative influence of the nearby carbohydrate moiety (Walker et al., 1994). In a study of cellular pro-albumin cleavage using an opposite approach, it was found that the P6 residue affected the cleavage efficiency of pro-albumin by human furin as there was a 5-fold increase in cleavage of pro-albumin upon the introduction of arginine at the P6 position (Brennan and Nakayama 1994; Rockwell et al., 2002). Similarly, a soluble HIV-1 envelope (Env) mutant precursor, which lacked the transmembrane and intracytoplasmic domains of gp41 (gp140), but contained two newly introduced basic residues (Arg-Arg or Lys-Lys) at the positions P5 and P6 upstream of the consensus sequence, was cleaved better than wild-type protein when expressed in 293T cell line (Binley et al., 2002). The enhanced cleavage occurs with either endogenous enzyme or with co-expressed furin. Thus, the influence of positively charged residues at the P5 and P6 cleavage positions on the cleavage of SPC target proteins has been observed in both viral and cellular precursor proteins.

Several SPCs specific targets contain negatively charged amino acids at the cleavage positions P3, P5 or P7 (Nakayama 1997; Molloy et al., 1999; Keelapang et al., 2004). The influence of these negatively charged residues located in the vicinity of SPC cleavage site on the cleavage efficiency is not yet clear. In the study of H5N8 hemagglutinin mutant by Walker et al. (1994), the reduction of cleavage efficiency at the HA_1-HA_2 junction followed the substitution of the P5 lysine residue with glutamic acid, but there was also a concomitant change of P6 arginine into threonine. Thus, the effect of glutamic acid at the P5 position alone cannot be established. For dengue virus, glutamic acid residue at the cleavage position P7 is highly conserved in all four dengue serotypes. At the P3 position, all dengue serotypes 2 and 4 strains contain glutamic acid whereas aspartic acid is present in serotypes 1 and 3. Comparison of 100 dengue virus sequences in Genbank database revealed very rare variation (2 out of 100 sequences) of the P3 acidic residue (Keelapang et al., 2004), indicating the importance of these negatively charged residues at the P3 and P7 positions in dengue virus evolution. Interestingly, both of these acidic residues are absent in other insect-borne flaviviruses, with an exception of certain strains within the TBE serocomplex in which glutamic acid can be found at the P7 position. Thus, the conservation of the P3 and P7 acidic residues appears to be restricted to dengue serocomplex. How these two residues contribute to the virus replicative ability is still unclear and further studies on their effect on prM cleavage are needed.

It is intriguing that the effect of mutations of dengue prM cleavage junction on virus replication kinetics is more evident in PS cell line, which is of mammalian origin, than in the mosquito-derived C6/36 cells. Previously, Keelapang et al. (2004) showed that markedly reduced replication of JEVpr/16681 occurred in both PS cells and Vero cells, suggesting that mammalian cells are more sensitive to the pr-M junction mutations than the mosquito cells. In mammals, the subtilisin-like proprotein convertases family is composed of seven members: furin, PC1/3, PC2, PACE4, PC4, PC5/6 and PC7/PC8/LPC/SPC7 (Nakayama 1997; Steiner 1998; Thomas 2002). The majority of these enzymes have broad tissue distribution. As the exceptions, PC1/3 and PC2 are detected in the neuroendocrine system whereas PC4 is restricted to testicular and ovarian germ cells (Thomas 2002). The heterogeneity of SPC in insects appears to be less extensive than in mammals. Analysis of the whole genome

sequence of *Anopheles gambiae* reveals only three members of the furin-like proprotein convertase family (furin 1, dfurin 2, and amontillado) in anopheles mosquito (Riehle et al., 2002). Similarly, there are only two SPC enzymes (dfurin1 or dKLIP-1, and dfurin2) in drosophila genome (Roebroek et al., 1993; Chen and Raikhel 1996). The tissue distribution and target sequence specificity of this insect SPCs have not yet been studied extensively. In a study on the cleavage of pro-vitellogenin by *Aedes aegypti* vitellogenin convertase (a possible furin homolog in *Aedes* mosquito), it was found that *Aedes aegypti* vitellogenin convertase, drosophila dKLIP-1, and human furin shared up to 92-97 % of the amino acid sequence of the catalytic domain (Chen and Raikhel 1996). Vitellogenin convertase appeared to cleave pro-vitellogenin at the sequence Arg-Ala-Lys-Arg-Arg-Pro-Lys-Arg, which closely resembled the furin consensus sequence (Chen and Raikhel 1996). Both of these results suggest close structural and functional similarities between insect SPCs and mammalian SPCs. Due to the lack of additional evidences, it is not yet clear as to why the effect of prM cleavage junction mutations is more evident in PS cells than in C6/36 cells. It is possible that there are differences in the level of active SPC enzymes and the relative distribution of various SPC isoforms in various cells and tissues. It is also possible that there are inherent differences in the fine cleavage specificity between mosquito SPC enzymes and their mammalian counterparts. For example, the mosquito SPCs may have evolved to accommodate a wider range of substrate sequences than mammalian SPCs and thus become less discriminatory to the mutants 16681pr(+4, -0) and 16681pr(+7, -2) than the mammalian enzymes. One of the results of this change may be manifested by the reduction of the need for a large number of SPC isoforms in mosquito and other insects. If this possibility were true, one would expect that it would take more drastic mutations of the pr-M junction to affect prM cleavage and dengue virus replication in mosquito cells than in mammalian cells, providing that other factors influencing the virus replication are comparable. Other possible explanation may involve differences of factors other than the SPCs, such as type of virus specific receptor(s), cellular site of virus-receptor interaction, fusion and the requirement of these factors for of receptor-binding and fusion-competent E glycoprotein that have been freed of prM by SPC action. It is also

possible that more than one of these factors are contributing to the differences in viral mutant replication in C6/36 and PS cells.

Detailed structural analysis of murine furin provides the atomic basis for the requirement of the consensus sequence, Arg-Xaa-Lys/Arg-Arg, on the substrate protein for the cleavage by SPCs (Henrich et al., 2003). Each of these basic residues is recognized by separate subsites in the substrate binding pocket of the enzyme. The majority of interaction between the basic residues at the P1, P2 and P4 positions of the substrate with the corresponding subsites occurs through charge-charge interaction as the subsites (and also the surrounding surface) are rich in acidic residues (Henrich et al., 2003). Based on the orientation of short soluble inhibitor within the enzyme, the side chain of the P3 residue projects out into the solvent and does not interact with any enzyme subsites. This is consistent with the lack of side chain preference at this position (Henrich et al., 2003). However, many proproteins contain basic residues at the P3 position; this may reflect the favorable interaction of the basic side chain with a nearby glutamic residue (Glu257) on the surface of the enzyme (Henrich et al., 2003). Similar interaction is also possible with the basic residue at the P5 position. Based on this structural model it would be likely that the P3 glutamic acid side chain in dengue pr-M junction is unfavorable to the substrate-enzyme interaction due to the repulsion between the acidic P3 side chain and the Glu 257 residue. Indeed, the acidic residue at the P3 cleavage position is quite uncommon among proproteins; two examples of substrate with P3 acidic residue include HIV gp160 protein (Val-Gln-Arg-Glu-Lys-Arg) and ectodysplasin-A (Arg-Val-Arg-Arg-Glu-Lys-Arg) (Rockwell et al., 2002). Based on this consideration, it is expected that the cleavage of the pr-M junction in 16681pr(+4, -0) is more efficient that that of 16681Nde(+) parent strain due to the removal of the two acidic residues from the substrate. The structural model of furin also provide evidence for the preference of the basic side chain of the P6 cleavage position as this side chain can interact with two surface-located Glu230 and Asp233 residues (Henrich et al., 2003).

Current structural model of murine furin does not provide the explanation for the effect of the positively charged side chain located more proximal to the P6 cleavage position. So, the structural basis for the effect of the arginine side chain at the positions P8, P10 and P13 of dengue pr-M junction on virus properties remains

obscure. Keelapang et al. (2004) suggested that this reflect the formation of the minimal furin cleavage motif, Arg-Xaa-Xaa-Arg, at the cleavage position P13 and P10. The presence of a tandem repeat of the furin consensus motifs has been shown to enhance cleavage efficiency in both HIV gp140 precursor (Binley et al 2002) and H_0 precursor of certain H5N1 and H5N3 avian influenza virus strains (Rohm et al., 1995; Subbarao et al., 1998; reviewed in Horimoto and Kawaoka 2001). However, in these cases the enhanced cleavage occurs at the nominal physiologic site, rather than at the cleavage-distal or minimal site (Basak et al., 2001; Fenouillet and Gluckman 1992; Morikawa et al., 1993; Brakch et al., 1995; Decroly et al., 1996). Structurally, it is unlikely that the substrate binding subsites can accommodate these additional basic residues together with the basic residues of the consensus positions. It remains possible that the additional basic residues, which form the minimal furin consensus motif in JEVpr/16681 and 16681pr(+7, -0), contribute to the enzyme-substrate interaction by interacting with the acidic side chains on the surface of enzyme, particularly those located nearby the substrate binding subsites, resulting in either the stronger attraction or tighter binding between the enzyme and the substrate.

In a study of HIV gp140, it was possible to enhance the cleavage by increasing the content of the basic residues in the cleavage junction (Binley et al., 2002). The three sequences that result in greatly enhanced cleavage are Arg-Arg-Arg-Arg-Arg-Arg, Arg-Arg-Arg-Lys-Lys-Arg and Arg-Arg-Arg-Arg-Lys-Arg (Binley et al., 2002). As mentioned above, it is quite likely that the additional basic residues at the P3, P5 and P6 positions strengthen the substrate-enzyme interaction by interacting through charge-charge interaction with the acidic residues on the enzyme surface (Henrich et al., 2003). As the surface of murine furin is quite enriched with acidic residues and there is strong conservation of the primary sequences of SPCs among animal species, it may be possible to enhance the cleavage of dengue prM and to affect virus properties from the level observed with JEVpr/16681 and 16681pr(+7, -0) by further increasing the basic residues between the cleavage positions P7 and P13. As expected, the mutant 16681pr(+9, -0), which contained two more basic residues at P7 and P11 on top of those already present in JEVpr/16691, was severely affected in its replication in both RNA-transfected C6/36 cells and infected C6/36 and PS cells. Currently, the efficiency of prM cleavage of the mutant 16681pr(+9, -0) is not yet

known, but is expected to be even more efficient than JEVpr/16681. The results reinforce the previous observations by Keelapang et al. (2004) that the basic residues at more cleavage-proximal locations can affect prM cleavage and viral phenotype in dengue virus.

One of the weak points of this study is the lack of structural data on the cleavage of prM in the four dengue mutants that had been generated. It is thus not possible to directly correlate the mutations with the actual cleavage of the prM and the changes in virus replication. Another interesting area that needs additional studies is how the enhanced cleavage of prM, as observed in JEVpr/16681 and possibly in 16681pr(+7, -0) and 16681pr(+9, -0), affects virus export from virus-infected cells. Keelapang et al. (2004) suggested either that enhanced binding between the SPC enzymes with substrate containing additional basic residues resulted in the retention of the viral particles in the *trans*-Golgi network, or that the virus envelope has the greater propensity to fuse with the Golgi membrane. These possibilities remain speculation that need to be verified in the future.

In conclusion, this study revealed that an alteration of two uncharged amino acids at the cleavage positions P9 (amino acid position 197) and P6 (amino acid position 200) of the 13-amino acid region proximal to the prM cleavage did not affect the replication of JEVpr/16681. An absence of the two negatively charged residues at the cleavage positions P7 (amino acid position 199) and P3 (amino acid position 203) of dengue pr-M junction region had some effect in reducing dengue replication as well as the increase of three positive charges at the cleavage positions P8 (amino acid position198), P10 (amino acid position 196) and P13 (amino acid position 193). Taken together, the results indicated that both of the increase of three positive charges and the absence of two negative charges within the 13-amino acid region proximal to the pr-M junction additionally contributed to the reduction of virus replication of JEVpr/16681. In addition, further reduction of dengue virus replication occurred when the net positive charge of the 13-amino acid region proximal to the pr-M junction was increased by an *in vitro* mutagenesis to the level of nine positive charges.