V. DISCUSSION

The main objective of this study is to determine the requirement for basic amino acid in the prM-M cleavage site by proprotein convertase during the multiplication of dengue serotype 2 strain 16681 in a mosquito cell line, C6/36. In *Aedes aegypti* mosquito, it was documented that the physiological proprotein convertase for a precursor protein, pro-vitellogenin, was pro-vitellogenin convertase expressed in the fat body. In this mosquito, the pro-vitellogenin convertase cleaves precursor proteins after dibasic motif of the general consensus Arg(P4)-Xaa(P3)-Arg/Lys(P2)-Arg(P1)↓- (Chen and Raikhel, 1996). Sequence alignment indicates that all of the four amino acids characteristics of proprotein convertase enzyme are conserved in pro-vitellogenin convertase: Asp_{268}, His_{309}, Asn_{410} and Ser_{483}. It is likely that the deduced amino acid sequence of pro-vitellogenin convertase has a high similarity to a domain structure characteristic of subtilisin-like proprotein convertases of mammals. However, the internal hydrophobic domain near the *N*-terminus of mosquito pro-vitellogenin convertase is similar to the internal signal sequence of *Drosophila* convertases dKLIP-1, dfurin1 and dfurin2. In drosophila, Dfurin1 was found only in the embryo, whereas dfurin2 was identified at all developmental stages (Roebroek *et al* 1992). On the other hand, dKLIP-1 was detected in the central nervous system and the fat body of adults (Chen and Raikhel, 1996). Because of the similarity between drosophila and mosquito, it is likely that there are more than one species of proportein convertase in mosquito. It is possible that proteolytic cleavage of the flavivirus prM-M junction in C6/36 mosquito cell, which was derived from larvae of *Aedes albopictus* mosquito (Singh, 1967; Igarashi, 1978), occurs by the action of pro-vitellogenin convertase or other closely related enzyme(s) that are expressed in early developmental stage.

In this study, genomic length cDNA clones, *in vitro* transcription and the expression system were used to analyze the effects of mutation in the prM internal cleavage site on dengue serotype 2 virus production within transfected C6/36 cells. Ten full-length mutant cDNA clones of the dengue serotype 2 strain 16681 virus were constructed by using PCR-based site-directed mutagenesis and propagated in *E. coli*. Linearized cDNA clones were transcribed to genomic-length capped RNA that was transfected to C6/36 (*Aedes* mosquito cell line). From the results of this study, infectious viruses were produced only when strongly dissociating basic amino acid residues replaced the arginine (P1) or lysine (P2) residues. However, the kinetic of virus production from transfected cells appears to be quite different. The K204R virus, which contained substituted arginine at the P2 position, had similar kinetics of virus production from transfected cells when compared with the parent virus. The amino acid sequence in the prM-M cleavage site of the K204R virus, Arg(P4)-Glu(P3)-Arg(P2)-Arg(P1)↓-, was the same as the junction sequence in prM protein of many other naturally occurring flaviviruses, Arg(P4)-Xaa(P3)-Arg/Lys(P2)-Arg(P1)↓-. Actually, the P2 position of the majority of flavivirus members, with an exception of the dengue virus serocomplex, contains arginine residue instead of lysine (Table 1). In other viral glycoproteins known to be cleaved by proprotein convertases, both arginine and lysine residues were found at this P2 position (Table 1) (Denault and Luduc, 1995; Nakayama, 1997; Molloy, 1999). Thus, our data are in agreement with the observation that both arginine and lysine are found at the P2 position of many proprotein convertase substrates.

In contrast to K204R mutant virus, the R205K virus showed a reduced virus production from transfected C6/36 monolayer at the early transfection period (days 14-28 after transfection). When reverted viruses replaced the original R205K mutant in the late transfection period (days 35-56), an increase in virus titer was observed. Increased production of the reverted virus, which contains natural (parental) prM-M

junction, may reflect higher efficiency of prM-M cleavage by the mosquito proprotein convertase against cleavage junction containing arginine at the P1 position as compared with lysine. Our observation agrees well with the fact that arginine is highly conserved at the P1 position among flaviviruses (Table 1) (Henchal and Putnak, 1990). Moreover, most, if not all, viral glycoproteins and cellular proprotein precursor contain arginine at the P1 position of proprotein convertase target sequence (Table 1) (Denault and Luduc, 1995; Nakayama, 1997; Molloy, 1999), indicating different evolutionarily conserved constraint between the P1 and P2 positions of cellular target sequence and proprotein convertases.

In contrast to reversion, it is possible that changes observed with the R205K virus at the late transfection period is due to contamination with the parent 16681 virus. However, reversion is more likely for several reasons. First, the parent virus was intentionally excluded in the transfection experiment of the 205 mutant set. Second, only one nucleotide change at the second P1 codon position (from adenine to guanine) is required for the amino acid change from lysine to arginine. Third, while there were six intended nucleotide substitutions in R205K mutant when compared with the 16681 parent strain, base change detected in the R205K revertant in the late transfection period was observed at only one base position (710), but not in the other five positions: 708, 711, 712, 713 and 714. The last evidence strongly suggests that cross contamination by the 16681 parent virus is highly unlikely.

The structural basis for the requirement of arginine at the P1 position and arginine or lysine at the P2 position of the dengue prM-M cleavage junction can be extrapolated from studies of other proteins. In subtilisin-like proprotein convertases, there are subsites in the substrate-binding region containing negatively charged amino acids, which may interact with the positively charged residue of substrates (Nakayama, 1997). It was suggested that interactions between the positive and negative charges in these subsites determined the affinity of the substrate for

subtilisin-like proprotein convertases.Creemers *et al* (1993) studied the binding of furin and a precursor substrate, von-Willebrand factor, by site-directed mutagenesis of several amino acids in the substrate-binding region of furin. They found that mutation of two negatively charged amino acids-Asp199 and Asp47-strongly inhibited enzyme activity. In a three-dimensional structure model of the catalytic domain of human furin constructed on the basis of the crystal structure of subtilisin BPN' (bacterial protease Nagase) and termitase, it was predicted that these two acidic amino acids provided critical negatively-charged side chain within the S1 and S2 subsites of the substrate-binding region required for charge-charge interaction with the positively charged amino acids at the substrate P1 and P2 positions (Creemers *et*

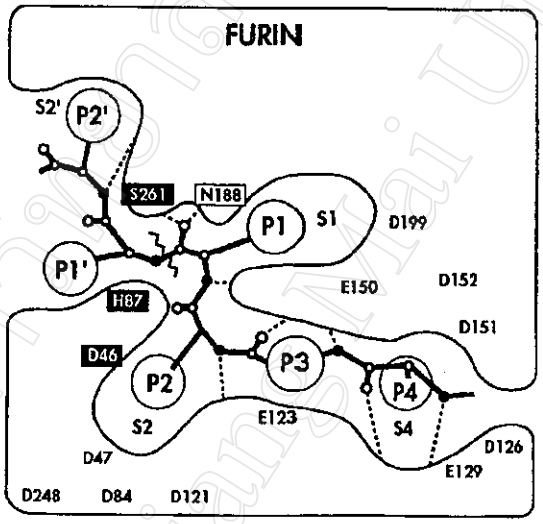

Figure 23. Schematic representation of the substrate binding region of the enzyme furin (Creemers *et al*, 1993). The positions of negatively charged residues, which was predicted to be in or near the substrate-binding region of furin, are indicated around the binding pockets S1, S2 and S4. The position of the residues of the catalytic triad, Asp_{46}, His_{87} and Ser_{261}, and the oxyanion-binding site Asn_{188} are boxed. The cleavage site of hexapeptide substrate (P4-P3-P2-P1-P1'-P2'), carboxyl-terminal of P1, is indicated by a zig-zag line.

al, 1993). In this model, mutation of these critical negatively charged residues alters the specificity of furin for multiple basic amino acid residues of the target substrate.

In a more detailed study, Cameron *et al* (2000) systematically described the inhibition of furin activity by positively charged amino acids at various substrate positions by using positional scanning acetylated synthetic *L*-hexapeptide combinatorial library. The hexapepide library consisted of 120-peptide mixture with carboxyl-terminal acetylation, divided into six groups corresponding to each position within the hexapeptide (P1-P6). In a designated position, there were twenty peptides, each of which contained only one of the twenty L-amino acids at the designated position, whereas other five non-designated positions contained any amino acid with an exception of cysteine (Box).

Example of 20 inhibitory peptides for the P1 position:

- Ac-XXXXXA-NH_2	- Ac-XXXXXC-NH_2	- Ac-XXXXXD-NH_2	- Ac-XXXXXE-NH_2
- Ac-XXXXXF-NH_2	- Ac-XXXXXG-NH_2	- Ac-XXXXXH-NH_2	- Ac-XXXXXI-NH_2
- Ac-XXXXXK-NH_2	- Ac-XXXXXL-NH_2	- Ac-XXXXXM-NH_2	- Ac-XXXXXN-NH_2
- Ac-XXXXXP-NH_2	- Ac-XXXXXQ-NH_2	- Ac-XXXXXR-NH_2	- Ac-XXXXXS-NH_2
- Ac-XXXXXT-NH_2	- Ac-XXXXXV-NH_2	- Ac-XXXXXW-NH_2	- Ac-XXXXXY-NH_2

Inhibitory activity of the hexapeptides against furin was determined by pre-incubating each of the 120 inhibitory peptides with purified mouse furin at pH 7.0, 37°C for 30 min and then adding pERTKR-MCA substrate for another 30 min. Proteolytic cleavage of the substrate was monitored by an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Inhibition of substrate cleavage by each inhibitory peptide was defined by the inhibition constant (K_i): $K_i = K_{i(app)}/(1+ [S]/K_m)$, where K_m for furin was 8 μM. When 1 mg/mL of inhibitory peptides was employed, it was found that peptide with arginine, lysine or histidine at the P1 position exerted inhibition greater than average inhibition by other 17 amino acids (Cameron *et al*,

2000). However, at 0.5 mg/mL of inhibitors the effect of arginine, lysine or histidine at the P1 position against furin was not as great as at 1 mg/mL. At the P2 and P3 positions, only arginine or lysine showed the greater inhibition than the other amino acids against furin. In contrast, at the P4, P5 and especially P6 positions, many amino acid residues showed greater than average inhibition, but no clear distinction could be made on the basis of size, hydrophobicity, or charge (Cameron *et al.*, 2000). Our finding that at the position P2 only the K204R mutant is capable of efficient multiplication agrees well with Cameron's data of strong inhibition of furin activity by inhibitory peptide containing arginine or lysine at this position. On the other hand, we found that the multiplication of R205K mutant was less efficient than the wild type virus and the R205H mutant virus was not detectable after the transfection whereas they were detected at a similar levels of inhibition of furin activity by peptides containing either arginine, lysine, or histidine at the P1 position. Such difference may be due to: 1) the variation in the structure of mosquito proprotein convertase responsible for cleavage of dengue prM-M junction in C6/36 cells and mouse furin employed in the study of Cameron *et al* (2000); 2) the difference in concentration of prM target in C6/36 cells and the concentration of inhibitory peptide employed by Cameron *et al* (2000); 3) the difference in the read out systems (prolonged virus multiplication in cells with continuous supply of enzyme(s) vs. short term cleavage by limited quantity of an enzyme). These results suggest similar requirement of mosquito proprotein convertase present in C6/36 for positively charged amino acids at the P2 position of the prM-M junction of dengue polyprotein as the mammalian cell-derived homologs, but there is some dissimilarity for the P1 position.

In accordance with our finding, similar requirement for the positively charged amino acid at position P2 of the prM-M cleavage junction was observed previously in TBE/dengue 4 chimeric virus. Pletnev *et al* (1993) used a tick-borne encephalitis

virus – dengue serotype 4 virus (TBEV/DEN-4) chimeric clone to test the effects of mutations in the prM internal cleavage site, which is the Arg(P4)-Thr(P3)-Arg(P2)-Arg(P1)↓-Ser(P1') of tick-borne encephalitis virus. Substitution of valine for the serine at P1' residue resulted in viruses that showed a reduction in the growth in simian and mosquito cells. While in a double mutant, the substitution of valine for the arginine residue at the P2 position and the serine residue at the P1' position, abolished virus production.

It has been documented that changes in the target sequence of subtilisin-like proprotein convertase in viral glycoproteins may affect viral virulence. In the case of avian influenza virus outbreak in Hong Kong, sequence analysis of genes encoding hemagglutinin (HA) from 16 isolates revealed a consistent alteration in the viral genomes to generate a second consensus furin site at the HA1–HA2 junction (-Arg-Lys-Lys-Arg↓- to -Arg-Glu-Arg-Arg-Arg-Lys-Lys-Arg↓-), which contains many highly positive charges in the cleavage site. Cleavage by furin and other ubiquitous subtilisin-like proprotein convertases had been shown to be responsible for systemic infection caused by highly pathogenic strains of avian influenza virus (Subbarao *et al*, 1998). The variation at cleavage site of Hong Kong H5N1 viruses that accounts for difference in pathogenicity and/or multiplication of the virus was determined by Hatta *et al* (2001). They generated recombinant infectious viruses using a plasmid-based reverse genetic system to determine the molecular basis for difference in virulence among Hong Kong H5N1 viruses. The plasmid cDNA were constructed in which a human RNA polymerase I promoter and mouse polymerase I terminator flanked cDNA for full-length RNAs of A/Hong Kong/483/97. To identify the effect of sequence change at the HA1-HA2 junction on virulence, they generated mutant viruses of which the amino acid sequence Pro-Gln-Arg-Glu-Arg-Arg-Arg-Lys-Lys-Arg↓Gly was changed to Pro-Glu- - - - -Arg-Glu-Thr-Arg↓Gly (dash indicates a deletion). When tested in mice, this HA mutant virus was highly attenuated (virus

dose lethal to 50% of mice, MLD_{50}, $>10^5$ pfu), and none of the infected mice showed any signs of disease (Hatta *et al*, 2001). The results strongly suggest that the variation of the HA1-HA2 junction of influenza virus hemagglutinin affect virus virulence. These findings may be applicable to dengue virus; however, no natural variant of the prM-M junction was found in our limited search of GenBank database. It will be interesting to introduce additional basic residues into the prM-M junction of dengue virus and explore the phenotypic changes that occur in the mutant virus both *in vitro* and *in vivo*.

For many viruses in the *Paramyxoviridae* family, cleavage of viral glycoprotein by furin cleavage and other proprotein convertases is absolutely required for their activity and thus determines the extent of virus pathoginicity. Measles virus is the prototype member of the morbillivirus genus in the *Paramyxoviridae* family of negative-stranded RNA viruses. Virions contain envelope with two virus-encoded integral membrane glycoproteins, the viral attachment protein hemagglutinin (H) and the fusion protein (F), which form spike-like projections on the outer surface. The F protein is synthesized as an inactive precursor molecule F_0, which is cleaved intracellularly by proprotein convertases to generate two polypeptide subunits, F_1 and F_2, held together by disulfide bonds. Infected cells exposing cleaved F protein on the surface fuse with adjacent cells at neutral pH, thereby causing syncytium formation. The multibasic cleavage site at which the protein of measles virus is activated consists of five basic amino acids, Arg(P5)-Arg(P4)-His(P3)-Lys(P2)-Arg(P1)↓-, at the positions 108-112. Correct proteolytic cleavage after arginine 112 is essential, because changing this residue to leucine [clevage site Arg(P4)-His(P3)-Lys(P2)-Leu(P1)↓-] was shown to result in aberrant cleavage and loss of fusion ability (Alkathib *et al.*, 1994). Maisner *et al* (2000) generated mutant F protein of measles virus of which the sequence Arg(P5)-Arg(P4)-His(P3)-Lys(P2)-Arg(P1)↓- was changed by site-directed mutagenesis to Arg(P5)-Asn(P4)-His(P3)-Asn(P2)-

Arg(P1)↓-. When they expressed this mutant in Vero cells or 293 cells, the mutant remained in the uncleaved form, whereas the wild type F protein was cleaved. This aberrant cleavage appears to have abolished the ability of the F protein to cause syncytium formation (Alkathib *et al.*, 1994; Maisner *et al.*, 2000). In the case of dengue and other flaviviruses, it is known that E protein is involved in fusion of virus envelope with cell or endosomal membrane, but E needs to be in appropriate configuration provided by the cleavage of prM to be active in fusion. Thus, even though prM is not involved directly in fusion as in the case of measle virus F protein, it would be interesting how mutations of prM in our dengue mutants will indirectly affect fusion process mediated by E.

The studies of glycoprotein processing of the Ebola virus (subtype Zaire) by furin and other subtilisin-like proprotein convertases, correlated with other virus glycoprotein processing. The glycoprotein (GP) of Ebola virus is the only surface protein of virions with suggested function in receptor binding and fusion with cellular membranes. As seen in other viruses, the fusogenic property of Ebola virus glycoprotein requires posttranslational proteolytic processing. The maturation involves posttranslational cleavage of a precursor at the C-terminal end of the sequence Arg(P4)-Thr(P3)-Arg(P2)-Arg(P1)↓- (at positions 498-501) into the disulfide-linked fragments GP_1 and GP_2. The proprotein convertase furin has been identified as a cleavage enzyme. Volchkov *et al* (1998) modified the internal cleavage sequence by site-directed mutagenesis. In the first mutation, arginine at position 501 was substituted by lysine [Arg(P4)-Thr(P3)-Arg(P2)-Arg(P1)↓- to Arg(P4)-Thr(P3)-Arg(P2)-Lys(P1)↓-]. Besides this, in the second mutant the arginine residue at positions 500 and 501 were changed to asparagine and methionine, respectively [Arg(P4)-Thr(P3)-Arg(P2)-Arg(P1)↓- to Arg(P4)-Thr(P3)-Asn(P2)-Met(P1)↓-]. Both mutants were evaluated for proteolytic processing by transient expression in HeLa cell. Unlike wild-type glycoprotein, which was processed into subunit GP_1 and GP_2,

both mutants expressed only the uncleaved glycoprotein. This result indicates that glycoprotein is cleaved at the C-terminal side of arginine 501 and that the cleavage site has the classical consensus sequence Arg(P4)-Xaa(P3)-Arg(P2)-Arg(P1)↓- recognized by proprotein convertase. However, mutant Ebola viruses containing mutated furin cleavage sequence were viable. Ebola virus with uncleaved glycoprotein was able to mediate infection in various cell lines as efficiently as the wild type virus (Wool-Levis and Bates, 1999). Thus, in contrast to the situation in TBEV/dengue chimera and our dengue mutants, cleavage of the Ebola virus glycoprotein is not required for efficient replication in cell lines.

There are several possible explanations for eight prM-M junction mutants, which failed to grow after the transfection of C6/36. It is possible that the prM-M cleavage of these mutants was severely inhibited because of the high substrate specificity of proprotein convertase members of mosquito cells. Suboptimal cleavage of the flavivirus polyproteins and impairment of virus infectivity has been studied previously by using acidotropic amine inhibition of the prM-M cleavage. Randolph *et al* (1990) treated flavivirus-infected mammalian cells (BHK, KB, Vero cells) and *Aedes albopitus* mosquito cells with acidotropic amines (chloroquine and ammonium chloride) and the [^{35}S] methionine-labeled proteins of extracellular virus were detected by SDS-PAGE and autoradiography. With virus infected cells were treated with acidotropic amines and viruses from treated culture and untreated culture were purified by centrifugation through the 20%-50% sucrose gradient, it was found that the treatment with either chloroquine or ammonium chloride led to a marked decrease in amount of labeled M protein for the three flaviviruses (dengue serotype 2, St. Louis encephalitis virus and Powassan virus) studied. The result indicated that these compounds interfered with the processing of prM to M protein. When infectivity of dengue viruses derived from treated- and untreated cultures were compared by plaque assay on Vero cells, the specific activity of dengue virus in Vero cells decreased from

226.3 pfu/cpm in M-containing virus to 27.6 pfu/cpm in the prM-M containing virus (8-fold reduction). The result indicated that virions containing the prM protein had lower specific infectivity than virions containing M protein (Randolph *et al*, 1990). It is likely that in the eight non-viable prM-M junction mutants, prM-M cleavage may be so inefficient that the resultant viruses were devoid of M protein and the replication of such viruses is too low to be detectable.

Lack of prM-M cleavage may affect dengue virus replication in many ways. Some studies indicated that the processing of prM to M protein is a mechanism used for activation of the flavivirus fusion protein, E glycoprotein. A study by Guirakoo *et al* (1991) showed fusion inhibition of tick-borne encephalitis virus and Japanese encephalitis virus based on the "fusion from without assay" in C6/36 mosquito cells. They experimented with prM-containing (immature) virions which were grown in the presence of ammonium chloride. These particles did not cause fusion under the fusion-from without-assay conditions. The acquisition of fusion activity, therefore, also depends on the proteolytic cleavage of the prM protein. In similar study in the West Nile virus, the prM processing was found to be required for full infectivity and the rearrangement of oligomeric structures on the surface of virions (Wengler and Wengler, 1989).

Recent cryo-electron microscopy studies of a virus-like particle have provided evidence that the E protein dimers on its surface form an extensive network of specific lateral interactions, in which domain II of one E dimer contacts domains I and III of a neighboring dimer, resulting in a highly ordered outer shell with icosahedral symmetry (Ferlenghi *et al.*, 2001). These capsidless recombinant subviral particles are generated by co-expression of the prM and E protein in COS1 cells. Their structure was determined to a resolution of 19Å by image reconstruction of electron micrographs and this allowed fitting of the atomic structure of the E protein in to the lower-resolution structure obtained by the cryo-electron microscope. The

subviral particle, which has a diameter of about 30 nm (about two-thirds the size of the virion) contains 30 copies of the E dimer arranged in an icosahedral lattice with a triangulation number of 1 (T=1). Extra electron density not accounted for by the E protein fitting suggests that the M_protein (and probably the prM protein in immature virions) is likely to occupy positions at local threefold symmetry axes (Figure 24). At the pH of fusion, the E proteins on the surface of virus particles and recombinant subviral particles undergo dramatic irreversible structure change that involve not only the conformation of the individual protein subunits, but also the lateral intersubunit interactions that make up the icosahedral lattice structure (Ferlenghi *et al.*, 2001; Heinz and Allison, 2001). The information provided by cryo-electron microscope reveals that the uncleaved prM protein would restrict the lateral freedom of E, and prevent the loosening of the intermolecular E contact for conformational change. Based on these studies, it is quite likely that the underlying basis for non-viability of the eight prM-M junction mutants lays in the failure of E protein to undergo conformational change required for fusion activity of flaviviruses.

Figure 24. Arrangement of E and prM protein on a triangular surface of the subviral particle of tick-borne encephalitis virus, a model flavivirus (Ferlenghi *et al.*, 2001). E protein is shown in red (domain I), yellow (domain II) and blue (domain III). PrM is shown as violet circle in oval white structure.