CHAPTER VI

DISCUSSION

Amino acid residue(s) other than the three basic residues at the furin consensus positions P1, P2 and P4 within the 13-amino acids sequence of dengue virus prM junction have been shown to affect the efficiency of prM cleavage as well as the export of infectious viral particles (Keelapang et al., 2004). In an attempt to delineate the residue(s) that is(are) responsible for these phenotypes, a number of pr-M junction mutant viruses were further characterized in this study. In the first part of the study, 16681pr(+4,-0)HS, 16681pr(+7,-0) and 16681pr(+7,-2) were used in the assessment of the effect of mutations within the 13-residue region on the efficiency of prM protein cleavage. By comparing the levels of prM and M proteins in the extracellular particles of these mutant viruses with those of the parental strain 16681Nde(+) and JEVpr/16681, it was found that the substitutions of arginine residues at P8, P10 and P13 (as in 16681pr(+7,-2)) did not result in high level of prM protein cleavage whereas the absence of the P3 and P7 negatively charged residues (as in 16681pr(+4,-0)HS) did. In 16681pr(+4,-0)HS, the absence of P3 and P7 negatively charged amino acid residues improved prM protein cleavage efficiency to a level that is comparable to that of JEVpr/16681. This evidence agreed well with a previous finding showing the cleavage suppressive effect of the P3-glutamic amino acid residue (J. Junjhon, personal communication). Therefore, either one or both the P3 and P7 negatively charged residues suppress the cleavage of prM proteins in a dengue serotype 2 virus, strain 16681Nde(+). The positively charged residues at P8, P10 and P13 do not appear to affect prM cleavage.

The suppressive effect of the P3 glutamic acid residue on furin cleavage has been observed previously in a study of furin cleavage of short peptide substrates *in vitro* (Holyoak et al., 2004). In an enzyme kinetics study using fluorogenic peptide substrates with various P3 residues, it was found that a peptide substrate carrying an

amino acid sequence similar to that of dengue serotype 2, strain 16681, was cleaved at lower V_{max} and K_{cat}/K_m (Jean et al., 1995). For the substrate AC-Arg-Glu-Lys-Arg-MCA, the V_{max} of 37±2 mM/h and K_{cat}/K_m of 5.84×10^{1} M^{-1} S^{-1} were much lower than those of a counterpart, AC-Arg-Ser-Lys-Arg-MCA (V_{max} of 1,597 ± 2 mM/h and K_{cat}/K_m of 8.07×10^{3} $M^{-1}S^{-1}$) (Jean et al., 1995). It has been proposed that the substrates with a glutamic acid residue at position P3 will experience an electrostatic repulsion, resulting in substantial decrease of the enzyme activity (Jean et al., 1995). Alternatively, the P3 glutamic acid residue on substrate bind potassium ion which exerts an inhibition effect on furin enzyme (Holyoak et.al., 2004). These *in vitro* results are compatible with our results on dengue virus prM protein cleavage study, which indicates that prM protein with P3 glutamic acid residue of dengue serotype 2 virus, strain 16681, is cleaved to a less extent than the mutant prM protein in which the P3 position has been replaced with an serine residue. In case of the P7 conserved glutamic acid at the pr-M junction, its effect on the efficiency of prM protein cleavage is only minimal (J. Junjhon, personal communication). These results suggest that the cleavage suppressive effect of P3 glutamic acid is stronger than that of the P7 glutamic acid.

The 13-amino acid sequences just proximal to the pr-M cleavage site of JEV, TBEV and YFV are able to change viral replication, focus size, and cleavage of prM protein when they were transferred into the homologous region of dengue serotype 2 virus, strain 16681 (Keelapang et al., 2004). The changes that these sequences induced are quite variable; for example JEV sequence enhances the pr-M cleavage efficiency to a greater degree than the sequence from TBEV and YFV. This variation quite likely reflects differences in the amino acid sequence within this pr-M junction. An alignment of the pr-M junction sequence within dengue antigenic complex shows that the P3 position is always aspartic acid in DENV-1 and -3, and glutamic acid in DENV-2 and -4. When compared to those of the members of JE antigenic complex and TBE antigenic complex, the P3 position is not negatively charged. Instead, a serine residue is found in the JE antigenic complex and other uncharged residue in the TBE antigenic complex. Thus, the presence of negatively charged residue at the P3 position is highly conserved only in dengue serocomplex. To date, the only flavivirus that contains negatively charged residue at the P3 position

at the pr-M junction is the cell fusing agent virus (CFAV) (Crabtee et al., 2003). In the case of P7 position, it is also conserved and is found to be glutamic acid in DENV-1 to DENV-4. As in the P3 position, the P7 position is not negatively charged in members of JE antigenic complex and TBE antigenic complex (i.e. histidine and glycine, respectively). The absence of the negatively charged amino acid residues at the P3, and possibly P7, positions may enhance prM protein cleavage during the replication of JEV and TBEV. The influence of the negatively charged amino acids in controlling the prM protein levels in extracellular particle is further supported by the finding that high amount of prM protein is found in extracellular particles of cell fusing agent virus (CFAV) (Crabtee et al., 2003). This virus carried a furin consensus amino acid sequence and glutamic reside at P3 on prM protein similar to that of DENV-2 and -4. The conservation of the negatively charged residues at positions P3 and P7 within dengue antigenic complex suggests that the low efficiency of pr-M cleavage is evolutionarily beneficial to dengue virus. It is of interest to determine the functional consequences of the retention of some prM proteins on extracellular particles.

In the second part of the study, the influence of the charged residues in the 13-amino acid region just proximal to the pr-M junction on the export of infectious viral particles was examined. The mutants with known pr-M cleavage efficiency, i.e. 16681pr(+4,-0)HS, 16681prE203A and 16681pr(+7,-2) were tested in the single-step kinetics experiment against the parent virus, strain 16681Nde(+), and JEVpr/16681. In the single-step kinetic studies, the changes in the extracellular infectious virus titers of the parent virus and JEVpr/16681 were similar to those in previous report (Keelapang et al., 2004). Changes in the titers of infectious cell-associated and extracellular particles of 16681pr(+7,-2) were similar to those of JEVpr/16681, indicating that there was a delay in the export of infectious particles of 16681pr(+7,-2) out of PS cells. This is likely due to the presence of three positively charged residues at positions P8, P10 and P13 of the pr-M junction. However, it is evident that the differences in the levels of cell-associated vs. extracellular virus titers in 16681pr(+7,-2) were not as pronounced as in JEVpr/16681. The difference may reflect the higher numbers and types of mutations in JEVpr/16681 as JEVpr/16681 contains three types of amino acid changes, i. e. positively charged amino acid residues at P8, P10 and

P13; negatively charged amino acid residues at P3 and P7; and uncharged amino acid residues at P6 and P9, whereas the 16681pr(+7,-2) carries only positively charged amino acid residues at P8, P10 and P13. The combination of these amino acid changes that are present in JEVpr/16681 may result in a greater influence on particle export than a single type of change in 16681pr(+7,-2).

The influence of the loss of the negatively charged residues at positions P3 and P7 was assessed with the mutants 16681pr(+4,-0)HS and 16681prE203A. The lack of P3 and P7, or only P7, negatively charged residues in these two mutants did not affect the particle export as the changes in the cell-associated and extracellular virus titers were not different form the parent strain. While the data on the influence of the P6 and P9 uncharged residues were not available, a previous study (Songjaeng, 2004) revealed that the replication kinetics in PS cells of 16681pr(+7,-0), which is different from JEVpr/16681 only at the P6 and P9 residues, was very similar to JEVpr/16681. This result suggests that the influence of the uncharged P6 and P9 residues on the export of infectious particles is minimal. Thus, these data strongly indicate that delay in export of JEVpr/16681 infectious particles is due to the arginine residues at P8, P10 and P13.

Currently, the underlying basis for the marked differences between the extracellular and the cell-associated infectious virus titers at 20-24 hr after infection that has been observed in JEVpr/16681, but not 16681pr(+7,-2) remains unclear. It is unlikely that differences in the P8 (AGG vs. CGA in JEVpr/16681 and 16681pr(+7,-2), respectively, P10 (CGG vs. CGC) and P13 (CGG vs. AGG) arginine codons for arginine and the influence of codon preference are involved at the level of protein synthesis because the cell-associated titers of these two viruses are similar. However, this may have to be rigorously assessed by the measurement of the total prM proteins that are synthesized in infected cells. The other possible explanation is the additive effect of various mutations in JEVpr/16681 on the export of viral particles, or the ability of the other mutations to exert its effect only on top of the presence of the three basic residues at P8, P10 and P13. This latter effect will be observed only in JEVpr/16681, but not 16681pr(+7,-2).

The delay of infectious virus export out of infected PS cells appears to be unique to the chimeric virus JEVpr/16681. In a previous single-step kinetics

experiment of JEV, strain Nakayama, in PS cells (Uchil and Satchidanandam, 2003), the accumulation of infectious JEV particles in the culture media did not differ from that of 16681 in this study. It was found that the eclipse period of 12 hours was rapidly followed by an exponential period, and by 18 hours after adsorption the infectious JEV virus titers had reached the plateau level. This finding suggests that the markedly delayed accumulation of JEVpr/16681 virus at 20 and 24 hours after infection is due to the combination of introduced JEV pr-M junction and the genetic background of strain 16681, but not the sequences of JEV or 16681. It is intriguing that several chimeric viruses, which had been generated by exchanging the prM+E proteins in the genus *Flavivirus*, usually displayed reduced replication. For example, while JEV replicates to greater titers than dengue virus in BHK, LLC-MK_2 and C6/36 cells (Chambers et al., 2006), the Japanese encephalitis virus/dengue 2 chimeric virus with prM-E coding region from a dengue serotype 2 virus, strain NGC, on the JEV genetic background replicates at the lower levels than JEV, strain Nakayama, in the two mammalian cell lines employed (Chambers et al., 2006). Conceivably, the reduction of replication in flavivirus chimeric viruses may be due to several distinct mechanisms, most of which remain to be delineated. The delay in virus export in JEVpr/16681 chimeric virus may represent one among many possible mechanisms underlying the deleterious effect of chimerization.

Sequence comparison reveals that the residues at positions P8, P10 and P13 of dengue pr-M junction always contain polar side chains, and the positively charged residues have not been reported. Based on the results of our study, an explanation for the absence of positively charged residues at these positions may be due to the undesired influence of the basic residue on the export of infectious virus out of the infected cells. As mentioned previously, this effect was not observed during the replication of JEV despite the presence of the basic residues at the homologous positions of JEV prM protein. It is quite likely that complete cleavage of prM in JEV may nullify the effect of these P8, P10 and P13 basic residues on viral export. On the other hand, other region(s) in the prM protein of JEV may compensate for the negative influence of these three basic residues on virus export. The regular function of these P8, P10 and P13 residues during the replication of dengue virus and JEV remains to be defined in future studies.

The results of single-step kinetics experiments indicated that arginine residue(s) at P8, P10 and P13 may affect the export of infectious particles of JEVpr/16681. It is, however, not clear whether all three arginine residues are required to exert the export delaying effect. The addition of the furin minimal amino acid sequence adjacent to furin consensus sequence enhanced gp140 processing by furin-like serine protease(s), but the secretion level of gp140 was reduced (Binley et al., 2002). In the pr-M junction of JEVpr/16681, the presence of arginine residues at positions P10 and P13 is similar to the minimal furin motif of HIV-1 gp140 and it is possible that this minimal furin motif at P10 and P13 is responsible for the delayed export of infectious particles of JEVpr/16681. In an attempt to assess this hypothesis, additional prM mutant viruses with two arginine residues were constructed, i.e. 16681pr(+6,-2)P8,10+, 16681pr(+6,-2)P8,13+ and 16681pr(+6,-2)P10,13+. The single-step kinetics experiment was then used to study the effect of the double positively charged amino acids on export of infectious particles. As expected, the cell-associated infectious particles of the three additional prM mutant viruses were detected at the same levels as the parent strain. Moreover, the accumulation of the extracellular infectious particles of 16681pr(+6,-2)P10,13+ was about 10 folds lower that strain 16681. Surprisingly, similar reduction of the extracellular virus level was observed with 16681pr(+6,-2)P8,10+, but not 16681pr(+6,-2)P8,13+. This result suggests that two nearby arginine residues at (P8 and P10) as well as (P10 and P13) are able to exert the down-modulating influence on particle export. It is also clear that the reduction of infectious extracellular virus titers 16681pr(+6,-2)P8,10+ and 16681pr(+6,-2)P10,13+ was not as great as that of 16681pr(+7,-2) and JEVpr/16681. It is possible that the presence of all three arginine residues at P8, P10 and P13 have stronger affect than each of the double arginine residues alone. Alternatively, it is also possible that only the P10 arginine residue alone is responsible for the delayed export in 16681pr(+6,-2)P8,10+ and 16681pr(+6,-2)P10,13+. This alternative possibility cannot yet be excluded at the present. The construction and testing of 16681pr(+5-2)P10+ will be needed to clarify this point.

The mechanism involved in the effect of positively charged amino acids proximal to the pr-M junction on the export of viral particles is not yet known. This, however, has a parallel example in HIV-1 gp140 containing the tandem repeat of the

minimal amino acid sequences (Binley et al., 2002). The addition of the furin minimal amino acid sequence adjacent to furin consensus sequence enhanced gp140 processing by furin-like serine protease(s), but the secretion level of gp140 was reduced (Binley et al., 2002). It has been proposed that the positively charged amino acids in the form of furin minimal motif in the recombinant HIV-1 gp140 affect the secretion of gp140 through the formation of furin-gp140 stable complexes, which are perhaps trapped within the transfected cells (Binley et al., 2002). In the case of pr-M junction, the positively charged amino acids at P10-P13 may result in binding enhancement of the prM protein to the high negatively charged amino acid stretch on the surface of furin active site in the same manner, increasing the affinity of the furin-prM interaction. This idea appears to be the most likely explanation of the effect of the arginine substitutions at P8, P10 and P13 of the pr-M junction to export of JEVpr/16681 particles. The increase in positively charged amino acid residues adjacent to pr-M cleavage site did not enhance prM cleavage efficiency as reported in HIV-1 gp140, and the influence of these basic residues cannot operate through an enhanced fusion of viral particles with complete prM cleavage to intracellular membrane as has been suggested previously (Keelapang et al., 2004).

An additional explanation for the delay in the export of JEVpr/16681 particles is the change in the tertiary structure of prM protein, leading to its destruction (or the viral particles) in ER and/or lysosome. To examine this possibility, the amino acid sequences of the entire prM coding region of the parent strain and prM mutant viruses were analyzed using the software package PredictProtein (WWW.predictprotein.org). The two methods employed in the prediction of the protein structure were PHDsec (Rost et al., 1994; Rost et al., 1996) and PHOFec (Rost et al., 2003), which give the accurate score in more than 76% of proteins with known structure (Baxevanis et al., 2005). In the prM protein of dengue serotype 2 virus, strain 16681Nde(+), a loop is predicted in the secondary structure of the 13-amino acid region proximal to the pr-M cleavage junction (a high score of 0.9). This loop region is accessible by water (a score of 100%). The prM protein contains helices (19.9%), strand (24.1%) as well as loops (56%). About 59.0% of the amino acid residues on prM protein are accessible by solvent. Analysis of the prM protein of the mutant viruses revealed only slight differences from that of the parent virus; there is

no major effect of the substitutions on the secondary structure and solvent accessibility. The lack of major change from structural predictions of prM protein agrees well with the previous observations that the mutation exerts minimal effect on the infectivity of JEVpr/16681 chimeric virus, and that comparable levels of cell-associated viruses were observed among mutant viruses as compared with the parent virus. Structural alteration of prM protein appears unlikely to be a responsible mechanism affecting the export of JEVpr/16681 infectious particles.

This study has many limitations. The export of the viral particles out of infected cells was assessed only by comparing the levels of infectious cell-associated virus and extracellular virus. The influence of the introduced mutation on the export of non-infectious particles cannot be measured. Pulse labeling-chase of the viral structural protein may be employed to determine the rate of progression of all viral particles into the extracellular environment irrespective of the infectivity. Repetition of the comparison of the export of the second set of mutant virus will be needed to increase the reliability of the data. In addition, co-localization of the viral structural protein with markers of the components of the secretory pathway will help in visualizing the effect of the introduced mutation at various subcellular organelles along the secretory pathway.