

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENT	iii
ABSTRACT	iv
LIST OF TABLES	x
LIST OF ILLUSTRATIONS	xi
ABBREVIATIONS	xiii
CHAPTER	
 I. INTRODUCTION	 1
II. LITERATURE REVIEW	
A. Biology of dengue virus	5
1. Viral proteins	6
1.1. Viral structural proteins	6
1.2. Viral non-structural proteins	8
2. Dengue attachment and entry into host cell	9
3. Structure of mature virion and viral assembly	11
B. Subtilisin-like proprotein convertases	14
1. Members of subtilisin-like proprotein convertase (SPCs) family in mammalian cells	14
2. General structure of subtilisin-like proprotein convertases	14
3. Furin/SPCs localization and trafficking	16
4. Biochemical and enzymatic features of furin	17
5. Implications of furin cleavage and flavivirus infectivity	19
 III. MATERIALS AND METHODS	
1. Virus and cell lines	22
2. Antibodies	22
3. Plasmids and competent cells	22

4. Mutagenesis of dengue pr-M junction cDNA clones	24
4.1. Mutagenesis of the subclone plasmid	24
4.2. Mini- and midi-preparations of plasmid from cultured <i>E. coli</i>	28
4.3. Screening of the subclone plasmids	28
4.4. Preparation of 1.3 kb Pst I fragment for the construction of mutant 5' half-genome cDNA clones	29
4.5. Construction and characterization of mutant 5' half-genome plasmids	29
5. Construction of full-length cDNA clones containing mutations of the pr-M junction	30
6. <i>In vitro</i> transcription and transfection of full-length RNA into C6/36 cells	31
7. Detection of virus replication	33
7.1. Virus titration by focus immunoassay	33
7.2. Determination of focus size by 4-step focus immunoassay	34
7.3. Extraction of viral genomic RNA and amplification of the prM gene by RT-PCR	35
7.4. Nucleotide sequence analysis	38
8. Determination of virus replication kinetics	38
IV. RESULTS	40
V. DISCUSSION	75
VI. SUMMARY	83
REFERENCES	88
APPENDICES	102
Appendix A	103
Appendix B	104
Appendix C	106
Appendix D	114
CURRICULUM VITAE	115

LIST OF TABLES

TABLE	PAGE
1. Comparison of pr-M junction of insect-borne flaviviruses and pr-M junction chimeras.	21
2. Oligonucleotides for site-directed mutagenesis and mutant plasmid construction.	25
3. Specific primers for reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequence analysis.	37
4. Screening of the pBK (S1SP6-1547) Δ 402 Pst I subclone mutant plasmids.	42
5. Screening of the 5' half-genome, pBK (S1SP6-4497) Δ 402 Pst I, for the introduced mutations in dengue pr-M junction.	47
6. Generation of mutant full-length cDNA clones following ligation of the 5' half-genome plasmids with the 3' half-genome sequence.	51
7. Expanded stock of prM mutant viruses.	60
8. Alteration of focus size in prM mutant and chimeric viruses.	62

LIST OF ILLUSTRATIONS

FIGURE	PAGE
1. Three recombinant plasmids for the generation of prM mutant dengue viruses.	23
2. Construction of the full-length dengue strain 16681 pr-M junction mutant cDNA clone.	26
3. Screening for intended mutations in the subclone plasmids by restriction enzyme digestion and agarose gel electrophoresis.	41
4. Purified 1.3 kb Pst I fragment of the four prM mutant subclone plasmids.	43
5. Determination of the orientation of the ligated 1.3 kb Pst I fragment (nt 212–1535) in 5' half-genomes with Sph I and EcoR I.	45
6. Determination of the orientation of the ligated 1.3 kb Pst I fragment in the 16681pr(+7, -2) 5' half-genome by Sph I and EcoR I double digestion and agarose gel electrophoresis.	46
7. Characterization of the mutant 5' half-genomes by restriction enzyme digestions and agarose gel electrophoresis.	48
8. Determination of the orientation of the ligated 6.2 kb Kpn I fragment in the full-length cDNA plasmid clone by Xba I and Hind III double digestion and agarose gel electrophoresis.	50
9. Characterization of the full-length cDNA clones containing intended mutations of the pr-M junction by restriction enzyme digestions and agarose gel electrophoresis.	52
10 A. A full-length mutant cDNA clone linearized by digesting with Xba I.	53
10 B. Analysis of the capped <i>in vitro</i> transcripts of a mutant full-length cDNA clone containing mutation at the pr-M cleavage junction.	53
11. Production of infectious dengue viruses from C6/36 cells transfected with various amounts of capped, <i>in vitro</i> transcripts of a full-length dengue cDNA clone (16681 clone #5.2).	55

12. Production of the 16681pr(+4, -0), 16681pr(+7, -2), 16681pr(+7, -0) viruses from C6/36 cells transfected with capped, <i>in vitro</i> transcripts.	57
13. Production of the 16681pr(+9, -0) mutant viruses from C6/36 cells transfected with capped, <i>in vitro</i> transcripts.	58
14. Alteration of focus size of the pr-M junction mutant dengue viruses 16681Nde(+), JEVpr/16681, 16681pr(+4, -0), 16681pr(+7, -2), 16681pr(+7, -0) and 16681pr(+9, -0).	63
15. Amplification of the 2.3 kb region (nucleotides 134-2,504) of the prM mutant viruses by RT-PCR and analysis by agarose gel electrophoresis.	65
16. The semi-nested PCR products containing the pr-M junction (nucleotides 134-1518) of the prM mutant viruses.	66
17. Confirmation of the intended mutations of the 13-amino acid region proximal to the pr-M junction of dengue prM mutant viruses.	67
18. Kinetics of virus production from C6/36 cells infected with the prM mutant viruses, 16681pr(+4, -0), 16681pr(+7, -2) and 16681pr(+7, -0).	69
19. Kinetics of virus production from PS cells infected with the mutant viruses 16681pr(+4, -0), 16681pr(+7, -2), and 16681pr(+7, -0).	70
20. Kinetics of virus production from C6/36 cells infected with the prM mutant virus 16681pr(+9, -0).	73
21. Kinetics of virus production from PS cells infected with the prM mutant virus 16681pr(+9, -0).	74

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่

Copyright© by Chiang Mai University

All rights reserved

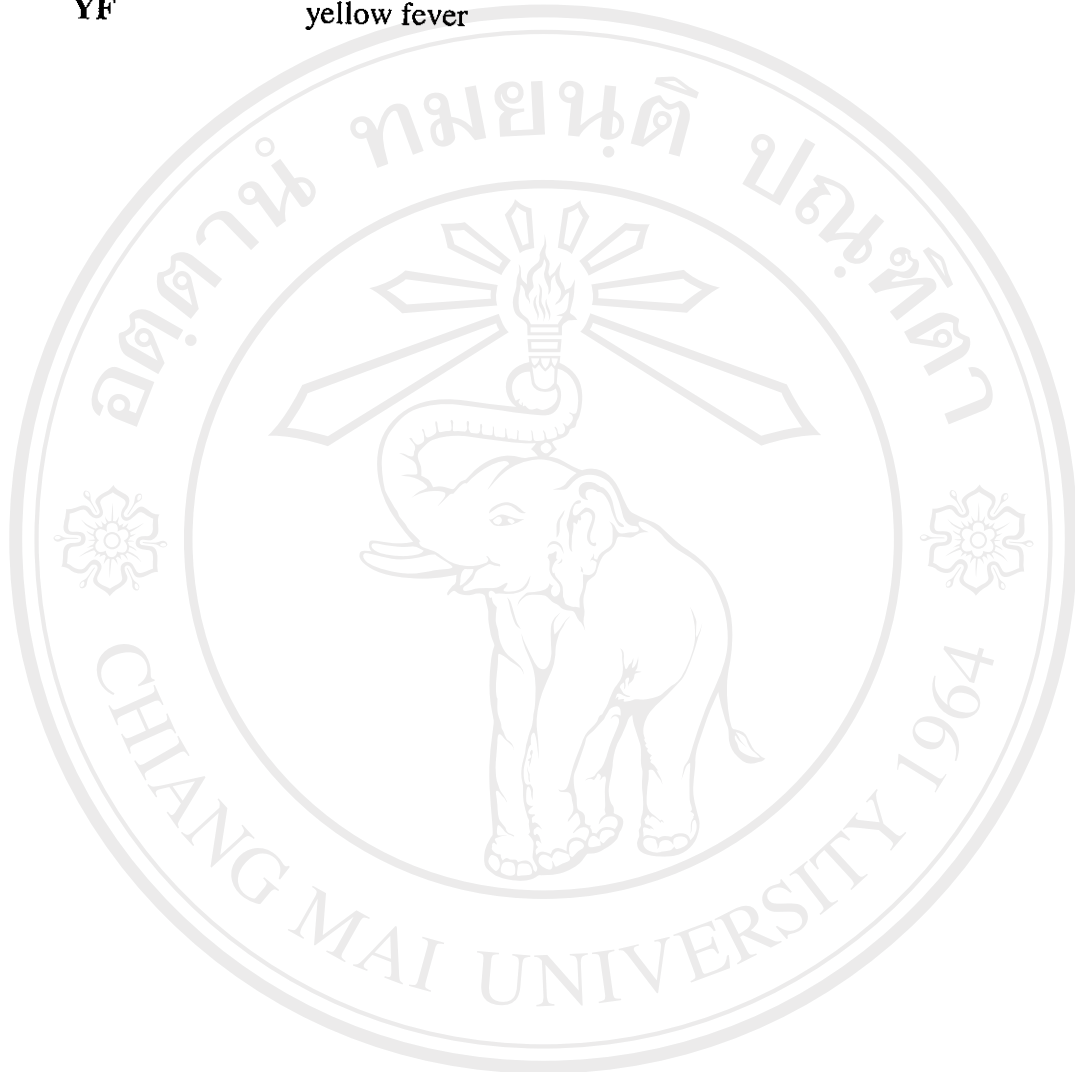
ABBREVIATIONS

°C	degree Celsius
(v/v)	volume: volume ratio
(w/v)	weight: volume ratio
μg	microgram
μl	microliter
μM	micromolar
A	adenine
Å	Angstrom (10^{-10} m)
Ala or A	alanine
anC	anchor capsid protein
Arg or R	arginine
Asp or D	aspartic acid
bp	base pair
BSA	bovine serum albumin
C protein	capsid protein
C	cytosine
cDNA	complementary DNA
CS	conserved RNA sequence
cryoEM	cryoelectron microscopy
Cys or C	cysteine
DAB	3, 3' diaminobenzidine
DEN	dengue virus
DEPC	diethylpyrocarbonate
DF	dengue fever
DHF	dengue hemorrhagic fever
dNTP	deoxyribonucleoside triphosphate
dsDNA	double-stranded deoxyribonucleic acid
DSS	dengue shock syndrome
DTT	dithiothreitol

E protein	envelope protein
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
EtBr	ethidium bromide
FFU or ffu	foci forming unit
g	gravity
G	guanine
Gly or G	glycine
gm	gram
HEPES	<i>N</i> -(2-hydroxyethyl) piperazine- <i>N'</i> -(2-ethanesulfonic acid)
His or H	histidine
HIV	human immunodeficiency virus
hr	hour
Ig	immunoglobulin
JEV or JE	Japanese encephalitis virus
k	kilo (10^3)
kb	kilobase
k_{cat}	turnover number
kDa	kilo-Dalton
K_M	Michaelis constant
LB	Luria-Bertani medium
Lys or K	lysine
M protein	membrane protein
M	molar
MESA	MOPS-EDTA-Sodium acetate buffer
Met or M	methionine
min	minute
mg	milligram
ml	milliliter
mM	millimolar
mol	mole

MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
ng	nanogram
nm	nanometer
NS protein	non-structural protein
nt	nucleotide
PACE	pair amino acid convertase enzyme
PBS	phosphate-buffered saline
PC	prohormone convertase
PCR	polymerase chain reaction
pmol	picomole
poly(A)⁺	polyadenylated
prM protein	premembrane protein
RNA	ribonucleic acid
RNase	ribonuclease
rNTP	ribonucleoside triphosphate
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
sec	second
Ser or S	serine
SPCs	subtilisin-like proprotein convertases
T	thymine
TAE	tris-acetate-EDTA buffer
TBEV	tick-borne encephalitis virus
TE	tris-EDTA buffer
TGN	<i>trans</i> -Golgi network
Thr or T	threonine
T_m	melting temperature
Tris	tris(Hydroxymethyl)aminomethane
Tris-HCl	tris(Hydroxymethyl)aminomethane hydrochloride
u	unit
UV	ultraviolet

Val or V	valine
VC	vitellogenin convertase
Vg	vitellogenin
YF	yellow fever



ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่

Copyright© by Chiang Mai University
All rights reserved