

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENT	iii
ABSTRACT	iv
LIST OF TABLES	xiv
LIST OF ILLUSTRATIONS	xv
ABBREVIATIONS	xviii
 I. INTRODUCTION	 1
II. LITERATURE REVIEW	
A. Dengue virus	4
1. Structure and composition of virion	4
2. Genome structure	4
3. Viral proteins	5
3.1. Viral structural proteins	5
3.2. Viral non-structural proteins	10
4. Synthesis and processing of viral proteins	11
B. Subtilisin-like proprotein convertases	12
1. Members of subtilisin-like proprotein convertase family in mammalian	12
2. Structure and function of subtilisin-like proproteins convertases	13
3. Biochemical and enzymatic feature of furin	16
4. Target of subtilisin-like proprotein convertase in the surface proteins of other viruses	17

	PAGE
III. MATERIALS AND METHODS	23
1. <i>E. coli</i>	23
2. Cell lines	23
3. Recombinant plasmids containing dengue virus genome	23
4. Oligonucleotide primers for PCR-based site-directed mutagenesis	27
5. PCR-based site-directed mutagenesis	31
6. Preparation of mutant plasmid	33
6.1 Preparation of plasmid DNA by small-scale boiling lysis	34
6.2 Midi-preparation of plasmid DNA by the alkaline lysis method	35
7. Nucleotide sequence analysis for confirmation of mutated sequence	36
8. Construction of full-length mutant cDNA clones	38
9. Generation of mutant viruses	41
9. 1 Production of genome-length capped RNA by <i>in vitro</i> transcription	41
9. 2 Introduction of capped RNA transcripts into cells by transfection	41
9. 3 Detection of mutant dengue viruses	42
9. 3.1 Dot blot immunoassay	42
9. 3.2 Reverse transcriptase polymerase chain reaction (RT-PCR)	42
9.3.3 Virus titration by focus immunoassay	45

	PAGE
IV. RESULTS	46
V. DISCUSSION	88
VI. SUMMARY	101
REFERENCES	104
APPENDICES	118
Appendix A. Titration of K204 dengue mutant virus set	119
Appendix B. Titration of R205 dengue mutant virus set	120
Appendix C. Burst size of the K204R dengue mutant virus	121
Appendix D. Burst size of the R205K dengue mutant virus (transfection I)	123
Appendix E. Percent of small and large foci of K204R mutant virus	124
Appendix F. Percent of small and large foci of R205K mutant virus (transfection I)	125
Appendix G. Percent of small and large foci of R205K mutant virus (transfection II)	126
Appendix H. Nucleotide sequence analysis at prM-M junction of dengue mutant clones by using primer C1204	127
Appendix I. Nucleotide and amino acid sequence around the prM-M cleavage site of K204R and R205K viruses compare with dengue serotype 2 strain 16681	131
Appendix J. Properties of amino acids	133
Appendix K. Reagents	135
Appendix L. Instruments	144
CURRICULUM VITAE	145

LIST OF TABLES

Table	Page
1. Sequences around the cleavage site of precursor proteins	19
2. Oligodeoxyribonucleotide primers for site-directed mutagenesis employed to be changed at amino acid residue 204	29
3. Oligodeoxyribonucleotide primers for site-directed mutagenesis employed to be changed at amino acid residue 205	30
4. Parameter of PCR based site-directed mutagenesis for change at residues 204 and 205	32
5. Oligonucleotide primers for polymerase chain reaction and nucleotide sequence analysis	37
6. Oligoribonucleotide primers for reverse transcriptase-polymerase chain reaction	44
7. PCR-based mutagenesis of the codons 204 and 205 within pBK S1SP6-1547Δ402 <i>Pst</i> I subclone	48
8. Construction of mutant 5'half-genomes	57
9. Comparison of the dengue K204R and R205K mutant viruses	82

LIST OF ILLUSTRATIONS

Figure	Page
1. Schematic diagram of the composition of immature and mature flaviviruses	7
2. Members of the mammalian family of Subtilisin-like proprotein convertase compare with Kex2 (Kexin)	15
3. Three plasmids for the construction of the full-length dengue cDNA clone	26
4. Construction of the full-length dengue serotype 2 strain 16681 cDNA clone	40
5. Nucleotide sequence of the 1.3-kb <i>Pst</i> I fragments (nt 214-1531)	49
6A. Two insertion patterns of the mutant <i>Pst</i> I fragment (nt 212 – 1535) within the 5'half-genome	58
6B. Determination of the orientation of the ligated mutant <i>Pst</i> I fragment (nt 212–1535) in 5'half-genomes by digesting 5'half genomes with by <i>Sph</i> I and <i>EcoR</i> I	59
7. Analysis of mutant 5'half-genomes (pBK S1S6-4497Δ402 <i>Pst</i> I); K204 mutation set	60
8. Analysis of mutant 5'half-genomes (pBK S1S6-4497Δ402 <i>Pst</i> I); R205 mutation set	61
9. Checking mutant full-length dengue cDNA clones (pBK S1SP6-10723 Δ402 <i>Pst</i> I) by restriction enzyme digestion; K204 mutation set	63

LIST OF ILLUSTRATIONS

Figure	Page
10. Checking mutant full-length dengue cDNA clones (pBK S1SP6-10723 Δ 402 <i>Pst</i> I) by restriction enzyme digestion; R205 mutation set	64
11. A full-length cDNA clone, pBK S1SP6-10723 Δ 402 <i>Pst</i> I R205S, linearized by digesting with <i>Xba</i> I	65
12. Analysis of the capped <i>in vitro</i> transcripts of a mutant full-length cDNA clone containing the R205S mutation at the prM-M cleavage junction	66
13. Release of K204 mutant dengue virus set following transfection	66
14. Kinetics of virus production following transfection by transcripts of parental dengue strain 16681 (#81.2) into C6/36 cells	70
15 Kinetics of virus production following transfection of five different K204 mutant transcripts into C6/36 cells	71
16A. Release of mutant dengue viruses from C6/36 monolayers following transfection with five different R205 mutant transcripts	73
16B. Release of R205K mutant dengue virus, set 5 (2), from transfected C6/36 monolayers in the second experiment	74
17. Kinetics of virus release into culture media following transfection of C6/36 cells with five different R205 mutant transcripts	76
18. Proportion of small and large foci of the K204R mutant virus following transfection into C6/36 cells	78
19. Nucleotide sequences surrounding the prM-M cleavage site of K204R cDNA and K204R virus as compared with the parent cDNA clone	79

LIST OF ILLUSTRATIONS

Figure	Page
20. Proportion of R205K mutant viruses generating small and large infected PS foci during 56 days of transfection into C6/36 cells	81
21. Representative foci of the K204R and R205K mutant viruses, as observed in infected PS clone D cells following immunoperoxidase staining	83
22. Nucleotide sequence surrounding the prM-M cleavage site of K204R cDNA and K204R virus compared with the wild type	86
23. Schematic representation of the substrate binding region of the enzyme furin	91
24. Arrangement of E and prM protein on a triangular surface of the subviral particle of tick-borne encephalitis virus	100

ABBREVIATIONS

A	adenine
Å	Angstrom (10^{-10} m)
Ala	alanine
Arg	arginine
Asp	aspartic acid
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	degree celsius
cDNA	complementary DNA
C protein	capsid protein
CoCl₂	cobalt dichloride
CS	conserved RNA sequence
Cys	cysteine
DAB	3, 3' diaminobenzidine
DF	dengue fever
DHF	dengue hemorrhagic fever
dNTP	deoxyribonucleoside triphosphate
DEPC	diethylpyrocarbonate
dsDNA	double-stranded deoxyribonucleotide
DSS	dengue shock syndrome
DTT	dithiothreitol
E	envelope protein
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide

ER	endoplasmic reticulum
ffu	foci forming unit
g	gravity
G	guanine
hr	hour
HEPES	<i>N</i> -(2-hydroxyethyl) piperazine- <i>N'</i> -(2-ethanesulfonic acid)
His	histidine
HIV	human immunodeficiency virus
Ig	immunoglobulin
k	kilo (10^3)
k_{cat}	turnover number
K_M	Michaelis constant
kb	kilobase
kDa	kilo-Dalton
KOH	potassium hydroxide
LB	Luria-Bertani medium
LPC	lymphoma pro hormone convertase
Lys	lysine
M	membrane protein
M	molar
mAb	monoclonal antibody
MESA	MOPS-EDTA-Sodium acetate buffer
MgCl₂	magnesium chloride
min	minute
mL	milliliter
mM	millimolar

mol	mole
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
μg	microgram
μl	microliter
μM	micromolar
NaCl	sodium chloride
ng	nanogram
nm	nanometer
NS	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	premembrane protein
RNA	ribonucleic acid
RNase	ribonuclease
rNTP	ribonucleoside triphosphate
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
sec	second
Ser	serine
SPCs	subtilisin-like proprotein convertases
T	thymine

TAE	tris-acetate-EDTA buffer
TE	tris-EDTA buffer
TGN	<i>trans</i> -Golgi network
T_m	melting temperature
Tris-HCl	tris-hydrochloride buffer
u	unit
UV	ultraviolet
Val	valine
VC	vitellogenin convertase
V_g	vitellogenin
(v/v)	volume:volume ratio
(w/v)	weight:volume ratio