EXPRESSION AND SUBCELLULAR LOCALIZATION OF WILD-TYPE AND MUTANT KIDNEY ANION EXCHANGER 1 FUSED WITH FLUORESCENT PROTEIN AND EPITOPE TAG

WANDEE UDOMCHAIPRASERTKUL

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (IMMUNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2006

ISBN 974-04-6836-5

Thesis

entitled

EXPRESSION AND SUBCELLULAR LOCALIZATION OF WILD-TYPE AND MUTANT KIDNEY ANION EXCHANGER 1 FUSED WITH FLUORESCENT PROTEIN AND EPITOPE TAG

Wandee Udomchaiprasertkul

Miss Wandee Udomchaiprasertkul, M.Sc.
Candidate

Pa-thai Yenchitsomanus

Prof. Dr. Pa-thai Yenchitsomanus, Ph.D.
Major-Advisor

Sansanee Noisakran

Lect. Dr. Sansanee Noisakran, Ph.D.
Co-Advisor

Nanyawan Rungroj

Dr. Nanyawan Rungroj, Ph.D.
Co-Advisor

V. Akkarapatumwong

Asst. Prof. Dr. Varaporn Akkarapatumwong, Ph.D.
Co-Advisor

Prof. Dr. M.R. Jisnuson Svasti, Ph.D.
Dean
Faculty of Graduate Studies

Prof. Dr. Kovit Pattanapanyasat, Ph.D.
Chair
Master of Science Programme in Immunology
Faculty of Medicine Siriraj Hospital

Thesis

Entitled

EXPRESSION AND SUBCELLULAR LOCALIZATION OF WILD-TYPE AND MUTANT KIDNEY ANION EXCHANGER 1 FUSED WITH FLUORESCENT PROTEIN AND EPITOPE TAG

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Immunology)
on
26 January, 2006

Wandee Udomchaiprasertkul
Miss Wandee Udomchaiprasertkul, M.S.
Candidate

Pa-thai Yenchitsomanus
Prof. Dr. Pa-thai Yenchitsomanus, Ph.D.
Chair

Sansanee Noisakran
Lect. Dr. Sansanee Noisakran, Ph.D.
Member

Pimchai Chaiyen
Asst. Prof. Dr. Pimchai Chaiyen, Ph.D.
Member

V. Akkarapatumwong
Asst. Prof. Dr. Varaporn Akkarapatumwong, Ph.D.
Member

Prof. Dr. M.R. Jisnuson Svasti, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

P. Sakolsatayadorn
Clin. Prof. Dr. Piyasakol Sakolsatayadorn, M.D., F.R.C.S.T.
Dean
Faculty of Medicine Siriraj Hospital
Mahidol University

ACKNOWLEDGEMENT

The success of this thesis is attributed to the extensive support and great assistance from my major advisor, Prof. Dr. Pa-thai Yenchitsomanus, who kindly gave invaluable suggestion, advice, and patience that helped to make this work passing through all the obstacles. I strongly believe that without his compassion and concern, I could not finish all what expected to be done for this thesis and my master degree.

I am extremely grateful to my co-advisors, Lect. Dr. Sansanee Noisakran, Asst. Prof. Dr. Varaporn Akkarapatumwong, and Dr. Nanyawan Rungroj for kind and invaluable advice, guidance, consultation, encouragement, and correction of this thesis. I would also like to express my great appreciation and thankfulness to the external thesis examination committee member, Asst. Prof. Dr. Pimchai Chaiyen, for the invaluable comment, suggestion, and correction of this thesis.

I am also indebted to Miss. Thitima Keskanokwong and Miss. Nunghathai Sawasdee, for their kindness in providing the plasmids, technical assistance and valuable suggestion, to Miss Atchara Paemanee, Miss Sita Kalayanarooj, Miss Piengpaga Ngaojanlar and Miss Araya Jintaviwat for their consultation, helpfulness, and support throughout my study. My sincere thanks and appreciations are expressed to all members of the Medical Molecular Biology Unit for their helpfulness and encouragement. I would like to thank to the National Center for Genetic Engineering and Biotechnology (BIOTEC), the National Science and Technology Development Agency (NSTDA), and the Faculty of Medicine Siriraj Hospital, Mahidol University, for providing the scholarship and financial support.

I would like to express my deepest appreciation and thankfulness to my parents, sisters and brother, and Mr. Thoranin Intarajak, for their love, understanding, support and encouragement throughout my graduate study. I would like to thank all my best friends; Hua, Yui, Mam, Jan and all friends at the Department of Immunology, Faculty of Medicine Siriraj Hospital, for their friendship and encouragement. Finally, I wish to thank many unnamed people who provided their time, interest, and resources.

Wandee Udomchaiprasertkul

EXPRESSION AND SUBCELLULAR LOCALIZATION OF WILD-TYPE AND MUTANT KIDNEY ANION EXCHANGER 1 FUSED WITH FLUORESCENT PROTEIN AND EPITOPE TAG

WANDEE UDOMCHAIPRASERTKUL 4536750 SIIM/M
M. Sc. (IMMUNOLOGY)
THESIS ADVISORS: PA-THAI YENCHITSOMANUS, Ph.D., SANSANEE NOISAKRAN, Ph.D., VARAPORN AKKARAPATUMWONG, Ph.D., NANYAWAN RUNGROJ, Ph.D.

ABSTRACT

Mutations of *anion exchanger 1* (*AE1*) encoding kidney anion exchanger 1 (kAE1) that exchanges Cl^-/HCO_3^- at basolateral membrane of kidney α–intercalated cells can result in distal renal tubular acidosis (dRTA). Interestingly, almost all *AE1* mutations do not cause abnormal anion transport function but usually result in impaired kAE1 trafficking to the cell membrane. *AE1* G701D is a common missense mutation causing autosomal recessive (AR) dRTA in homozygous or compound heterozygous condition, frequently observed in Thai and Southeast Asian populations. A compound heterozygous *AE1* G701D/SAO mutation is often found in Thai and other Southeast Asian patients with AR dRTA and ovalocytosis. The defects of mutant kAE1 proteins encoded from *AE1* G701D and SAO mutations are distinct. kAE1 G701D is functional if it can traffic to the cell surface, which occurs in red cells but this will depend on the presence of glycophorin A (GPA) in *Xenopus* oocytes. kAE1 SAO is, however, functionally inactive although it can traffic normally to the surface of both red cells and oocytes. In cultured mammalian cells, the individually expressed kAE1 G701D and kAE1 SAO showed trafficking defect with predominantly intracellular retention. However, it is not known what would happen if kAE1 G701D and kAE1 SAO are co-expressed in cultured mammalian cells, which mimic the compound heterozygous *AE1* G701D/SAO condition in human kidney cells. This study thus aimed to investigate the expression and co-expression of kAE1 SAO and kAE1 G701D in HEK293 cells by using green fluorescent protein (GFP) fusion and hemagglutinin A (HA) tagging to follow their expression and localization by confocal microscopy in both transiently and stably expressing cells. GFP-kAE1, GFP-kAE1 G701D, and GFP-kAE1 SAO fusion proteins were found to be expressed in HEK239 cells when they were initially analyzed by Western blot method. While GFP-kAE1 was co-localized with a surface marker-CD147, GFP-kAE1 G701D and GFP-kAE1 SAO were intracellularly retained when they were individually expressed. Co-expression of kAE1-HA with either GFP-kAE1 G701D or GFP-kAE1 SAO could rescue cell surface expression of both mutant proteins, consistent with previous observations. In co-expression of GFP-kAE1 G701D with kAE1 SAO-HA, both mutant proteins were intracellularly retained and seemingly without cell surface expression in both transiently and stably expressing HEK293 cells, indicating their severely impaired trafficking. This is most likely the explanation for the molecular mechanism of AR dRTA associated with compound heterozygous *AE1* G701D/SAO condition in patients for whom the trafficking impairment and intracellular retention of these kAE1 mutant proteins lead to the absence of functional kAE1 at the basolateral membrane of kidney α-intercalated cells.

KEY WORDS : KIDNEY ANION EXCHANGER 1(KAE1)/
DISTAL RENAL TUBULAR ACIDOSIS (DRTA)/
GREEN FLUORESCENT PROTEIN (GFP)/
SOUTHEAST ASIAN OVALOCYTOSIS (SAO)/
COMPOUND HETEROZYGOUS *AE1* G701D/SAO CONDITION.
130 P. ISBN 974-04-6836-5

การแสดงออกและตำแหน่งที่อยู่ภายในเซลล์ของโปรตีนแอนไอออนเอ็กเซ็งเจอร์-วัน ชนิดที่พบในไต ที่ปกติและผิดปกติติดด้วยโปรตีนเรืองแสงและอีพิโทปแทค (EXPRESSION AND SUBCELLULAR LOCALIZATION OF WILD-TYPE AND MUTANT KIDNEY ANION EXCHANGER 1 FUSED WITH FLUORESCENT PROTEIN AND EPITOPE TAG)

วันดี อุดมชัยประเสริฐกุล 4536750 SIIM/M

วท.ม. (วิทยาภูมิคุ้มกัน)

คณะกรรมการควบคุมวิทยานิพนธ์ : เพทาย เย็นจิตโสมนัส, Ph.D., ศันสนีย์ น้อยสคราญ, Ph.D., วราภรณ์ อัครปทุมวงศ์, Ph.D. นัญวรรณ รุ่งโรจน์, Ph.D.

บทคัดย่อ

มิวเตชั่นของยีน *anion exchanger 1* (*AE1*) ซึ่งถอดรหัสเป็นโปรตีน anion exchanger 1 ในไต หรือ kAE1 ซึ่งทำหน้าที่ในการแลกเปลี่ยนไอออน Cl^- กับ HCO_3^- ในเซลล์ขับกรดซึ่งบุท่อส่วนปลายของเนปฟอน (nephron) ในไต มีผลทำให้เกิดโรคไตผิดปกติในการขับกรด (distal renal tubular acidosis หรือ dRTA) เป็นเรื่องที่น่าสนใจว่ามิวเตชั่นของยีน *AE1* นั้นส่วนใหญ่ไม่ได้ทำให้เกิดความผิดปกติในการแลกเปลี่ยนไอออน แต่มีผลทำให้การเคลื่อนที่ไปยังเยื่อหุ้มเซลล์ผิดปกติ โดยปกติแล้ว มิวเตชั่นชนิด G701D ทำให้เกิดโรคไตผิดปกติในการขับกรดที่มีการถ่ายทอดลักษณะแบบด้อย ในภาวะที่มีมิวเตชั่นชนิดนี้ชนิดเดียวแบบโฮโมซัยโกต (homozygous) และในภาวะที่มีมิวเตชั่นของยีนร่วมกันสองชนิดแบบคอมเปาด์เฮ็ทเทอโรซัยโกต (compound heterozygous) ซึ่งทั้งสองแบบนี้พบได้บ่อยในคนไทยและในประเทศเพื่อนบ้านแถบใต้ โดยในภาวะที่มีมิวเตชั่นของยีนเดียวกันสองชนิดของ G701D กับ SAO (compound heterozygous G701D/SAO) พบได้บ่อยในผู้ป่วยไทยและแถบเอเชียอาคเนย์ ซึ่งทำให้เกิดโรคไตผิดปกติในการขับกรดที่มีการถ่ายทอดลักษณะแบบด้อย ความผิดปกติของโปรตีน kAE1 ในไต ที่เกิดจากมิวเตชั่น G701D และ SAO นั้นแตกต่างกัน โปรตีนผิดปกติชนิด G701D จะทำหน้าที่แลกเปลี่ยนไอออนได้ปกติ เมื่อสามารถเคลื่อนที่มาที่เยื่อหุ้มเซลล์ได้ ซึ่งจะเกิดขึ้นในเม็ดเลือดแดง แต่ในไข่กบ (*Xenopus* oocytes) นั้นจะต้องมีการแสดงออกร่วมของ glycophorin A (GPA) ด้วย แต่สำหรับโปรตีนผิดปกติชนิด SAO นั้น เมื่อศึกษาในเม็ดเลือดแดงและไข่กบ โปรตีนผิดปกติไม่สามารถทำหน้าที่แลกเปลี่ยนไอออน ถึงแม้ว่าจะสามารถเคลื่อนที่มาที่เยื่อหุ้มเซลล์ได้ก็ตาม ส่วนในเซลล์เพาะเลี้ยงจากสัตว์เลี้ยงลูกด้วยนม โปรตีนผิดปกติชนิด G701D และ SAO มีความผิดปกติในการเคลื่อนที่ทำให้โปรตีนค้างอยู่ภายในเซลล์ อย่างไรก็ตาม ขณะนี้ยังไม่ทราบว่าจะมีผลอย่างไร เมื่อมีการแสดงออกร่วมกันของโปรตีนผิดปกติชนิด G701D และ SAO ในเซลล์เพาะเลี้ยงสัตว์เลี้ยงลูกด้วยนม ซึ่งเป็นการจำลองภาวะที่มีมิวเตชั่นของยีนเดียวกันสองชนิดของ G701D กับ SAO ในเซลล์ไตของผู้ป่วย ดังนั้นเป้าหมายของงานนี้คือเพื่อศึกษาการแสดงออกและการแสดงออกร่วมของโปรตีนผิดปกติชนิด G701D และ SAO ในเซลล์เพาะเลี้ยงจากไตของมนุษย์ (HEK293 cells) โดยการติดด้วยโปรตีนเรืองแสงสีเขียว (GFP) และ hemagglutinin A (HA) เพื่อติดตามการแสดงออกและที่อยู่ของโปรตีนโดยใช้กล้องคอนโฟคอล โดยทำการศึกษาในเซลล์ที่มีการแสดงออกของโปรตีนแบบชั่วคราวและถาวร ผลการทดลองพบว่าโปรตีนปกติ โปรตีนผิดปกติชนิด G701D และ SAO ที่ติดด้วย GFP มีการแสดงออกใน HEK293 cells เมื่อศึกษาด้วยวิธี Western blot เมื่อมีการแสดงออกของโปรตีนแต่ละชนิดพบว่าในขณะที่โปรตีนปกติที่ติดด้วย GFP อยู่ที่เดียวกันกับโปรตีน CD147 ที่ผิวเซลล์นั้น โปรตีนผิดปกติชนิด G701D และ SAO ที่ติดด้วย GFP อยู่ภายในเซลล์ เมื่อมีการแสดงออกร่วมกันของโปรตีน kAE1 ปกติที่ติดด้วย HA กับโปรตีนผิดปกติชนิด G701D หรือ SAO ที่ติดด้วย GFP พบว่ามีการแสดงออกของโปรตีนทั้งสองที่เยื่อหุ้มเซลล์ ซึ่งสอดคล้องกับผลของการศึกษาที่มีผู้รายงานก่อนหน้านี้ เมื่อมีการแสดงออกร่วมกันของโปรตีนผิดปกติชนิด G701D ที่ติดด้วย GFP และ SAO ที่ติดด้วย HA พบว่าโปรตีนอยู่ภายในเซลล์และดูเสมือนว่าไม่มีอยู่ที่ผิวเซลล์ ทั้งในเซลล์ที่มีการแสดงออกของโปรตีนแบบชั่วคราวและถาวร ชี้ให้เห็นว่ามีความผิดปกติในการเคลื่อนที่อย่างรุนแรง ซึ่งน่าจะเป็นคำอธิบายสำหรับกลไกระดับอณูของโรคไตผิดปกติในการขับกรดที่มีการถ่ายทอดแบบลักษณะด้อยอันเกิดจากมิวเตชั่น G701D กับ SAO ของ *AE1* นั้นเกิดจากความผิดปกติในการเคลื่อนที่ไปผิวเซลล์และอยู่ในเซลล์ของโปรตีนผิดปกติทั้งสองทำให้ปราศจากโปรตีนที่ทำหน้าที่แลกเปลี่ยนไอออนที่เซลล์ขับกรดซึ่งบุท่อส่วนปลายของเนปฟอน nephron ของไต

130 หน้า. ISBN 974-04-6836-5

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

AD	=	autosomal dominant
AE1	=	anion exchanger 1
APS	=	ammonium persulfate
AR	=	autosomal recessive
bp	=	base pair
CA	=	carbonic anhydrase
cDNA	=	complementary deoxyribonucleic acid
C-terminal	=	carboxyl terminal
C-terminus	=	carboxyl terminus
˚C	=	degree Celsius
dATP	=	deoxyadenosine-5’-triphosphate
dCTP	=	deoxycytidine-5’-triphosphate
dGTP	=	deoxyguanosine-5’-triphosphate
dTTP	=	deoxythymidine-5’-triphosphate
dNTP	=	dATP, dCTP, dGTP, dTTP
del	=	deletion
DMEM	=	Dulbecco’s modified eagle’s medium
DMSO	=	dimethysulfoxide
dRTA	=	distal renal tubular acidosis
eAE1	=	erythroid anion exchanger 1
EDTA	=	ethylenediaminetetraacetic acid

LIST OF ABBREVIATIONS (cont.)

ER	=	endoplasmic reticulum
FBS	=	fetal bovine serum
GFP	=	green fluorescence protein
GPA	=	glycophorin A
h	=	hour (s)
HA	=	hemagglutinin
H^+-ATPase	=	hydrogen adenosine triphosphatase
H^+/K^+-ATPase	=	hydrogen/potassium adenosine triphosphatase
H_2DIDS	=	4,4'-diisothiocyanohydrostillbene-2-2'-disulfonate
HEK	=	human embryonic kidney
Ig	=	immunoglobulin
kAE1	=	kidney anion exchanger 1
kb	=	kilobase
kDa	=	kilo Dalton
LB	=	Luria-Bertani
MDCK	=	Madin-Darby canine kidney
mg	=	milligrame (s)
µg	=	micrograme (s)
min	=	minute (s)
ml	=	milliliter (s)
µl	=	microliter (s)
mmol	=	millimole

LIST OF ABBREVIATIONS (cont.)

nmol	=	nanomole
mM	=	millimolar
µM	=	micromolar
NaOAc	=	sodium acetate
ng	=	nanogram (s)
nM	=	nanomolar
nt	=	nucleotide (s)
N-terminal	=	amino terminal
N-terminus	=	amino terminus
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
Pfu	=	*Pyrococcus furiosus*
pmol	=	picomole
PMSF	=	phenylmethysulfonyl fluoride
RNase	=	ribonuclease
rpm	=	revolutions per minute
sec	=	second (s)
SDS	=	sodium dodecyl sulfate
SAO	=	Southeast Asia ovalocytosis
TAE	=	tris-acetate-EDTA
Taq	=	*Thermus aquaticus*
TEMED	=	N, N, N', N'-tetramethyl-ethylenediamine

LIST OF ABBREVIATIONS (cont)

TM	=	transmembrane
U	=	unit (s)
UV	=	ultraviolet

CHAPTER I
INTRODUCTION

Anion exchanger 1 (AE1) regulates red cell morphology and chloride/bicarbonate (Cl^-/HCO^-_3) exchange in red cells and acid-secreting ∝-intercalated cells of renal collecting duct. AE1 defect may result in abnormality of red cells and/or distal renal tubular acidosis (dRTA). dRTA is a disease characterized by failure of the kidney to appropriately produce acid urine in the presence of systemic metabolic acidosis, due to failure of hydrogen ion (H^+) secretion in the distal nephron, leading to hypokalemia, bone abnormality and nephrocalcinosis (1). Mutations in *SLC4A1* or *AE1* gene encoding kidney AE1 (kAE1) have been found to be associated with both autosomal dominant (AD) and autosomal recessive (AR) dRTA. In Southeast Asia, dRTA is usually recessive and caused either by homozygosity of a single *AE1* mutation or by compound heterozygosity of two different *AE1* mutations.

Several *AE1* mutations associated with AD and AR dRTA have been studied for anion transport function and trafficking of AE1 protein in red cells, *Xenopus* oocytes, and kidney cells lines. The AD dRTA mutant kAE1 proteins all retain substantial anion transport activity in mutant red cells and when they are expressed in *Xenopus* oocyte (2-6). This result suggests that the molecular mechanism of AD dRTA dose not result from a simple reduction in anion-transport activity of mutant kAE1. The AD dRTA mutations, R589H, R589C, R589S and R901X, caused impaired kAE1 trafficking to the cell surface of human embryonic kidney 293 (HEK293) cells resulting in intracellular retention (2, 7, 8). Moreover, the kAE1 R901X also exhibited mistargeting to apical surface in MDCK cells (9). Co-expression of wild-type kAE1 and R589H, or R901X, resulted in intracellular retention of wild-type protein. This is caused by dominant negative effect due to hetero-oligomer formation of the mutant and wild-type kAE1 proteins and impaired trafficking of the hetero-oligomers. Alternative mechanisms proposed for the domiant disease include mistargeting of the mutant protein to the apical membrane and/or altered targeting of the normal protein by interaction with the mutant protein(10). The *AE1* mutation associated

with AR dRTA was first reported in two Thai sister (11) who had hemolytic anemia and dRTA in which red cell anion transport was normal. They were found to carry a homozygous *AE1* G701D mutation, namely *band 3 Bangkok I.* The G701D mutation is accompanied by two polymorphisms, M31T and K56E. In *Xenopus* oocyte, AE1 G701D and kAE1 G701D showed impair anion transport and trafficking to the oocyte surface. Co-expression of the erythroid AE1 chaperone, glycophorin A (GPA), rescued both AE1 and kAE1 G701D mediated Cl^- transport and AE1 surface expression in the oocytes (11). Therefore, the anion transport function and surface expression of eAE1 in the patient's red cells, which normally contain GPA, are normal. The genetic and functional data both suggest that the homozygous *AE1* G701D mutation causes recessively transmitted dRTA in this kindred with apparently normal erythroid anion transport (11, 12).

In the case of AR dRTA, the molecular mechanism is not associated with the dominant negative effect. The novel compound heterozygous *AE1* G701D/S773P mutation, recently reported in the Thai patient with AR dRTA (13), was studied to elucidate the molecular mechanism of AR dRTA caused by *AE1* mutations (14). In HEK293 cells and non-polarized LLC-PK1 cells, the kAE1 G701D is retained intracellularly and it is retained in the Golgi in both non-polarized and polarized Madin-Darby canine kidney (MDCK) cells (15). The kAE1 S773P protein exhibits lower expression and more rapid turnover compared to the wild-type kAE1. The kAE1 S773P is mis-folded and is targeted for degradation by the proteasome. Both kAE1 S773P and G701D exhibit defective trafficking to the cell surface and show predominant immunolocalization in the ER of both HEK 293 and LLC-PK1 cells. However, kAE1 S773P can form homodimer, as well as a heterodimer with either wild-type kAE1 or kAE1 G701D. Hetero-oligomers of wild-type kAE1 with kAE1 S773P or G701D, in contrast to the dominant kAE1 R589H mutant, are delivered to plasma membrane (14). Therefore, the molecular mechanism of AR dRTA caused by *AE1* mutation is explained by that the heterodimer of the mutant and wild-type kAE1 proteins is able to traffic to the cell surface which the wild-type kAE1 seems to show a 'dominant-positive effect' in rescuing the recessive mutant kAE1 trafficking, in contrast with the dominant mutant kAE1 resulting in a 'dominant-negative effect' when heterodimerized with the wild-type kAE1 (12).

Two cases of AR dRTA and Southeast Asian ovalocytosis (SAO), a 27-bp deletion in exon 11 of *AE1* gene leading to loss of 9 amino acid residues at the positions 400-408, resulted from compound heterozygous *AE1* SAO/G701D mutations were originally reported in two Thai families from southern Thailand (16). These patients were not anemic but have little morphological changes of red cells in addition to SAO, and had a decrease of red cell sulfate flux of about 40%. The AE1 SAO mutation in the heterozygous condition is not sufficient to cause dRTA (5, 16). The transport function and surface expression of SAO was studied in red cells, oocytes, and mammalian cell lines. The specific anion transport activity in SAO reds cells was reduced of that in normal red cells (5, 17). AE1 SAO is not functional as an anion transporter but is inserted stably into the plasma membrane of oocytes (18). In HEK cells, expression levels and stabilities of SAO proteins were significantly reduced, and no mutant protein was detected at the cell surface (19). However, the co-expression of AE1 SAO and AE1 G701D in mammalian cells to mimic the compound heterozygous AE1 SAO/G701D mutations have not been studied. To examine the protein trafficking of compound heterozygous G701D/SAO, the experimental study in this thesis would be performed by fusing green fluorescent protein (GFP) with either wild-type or mutant kAE1, which allows to directly determine protein expression and trafficking in the cultured cell lines. The use of GFP fusion for study wild-type AE1 in K562 erythroleukemia cells has recently been reported (20). Fluorescence signal could be detected predominantly at the surface of K562 cells transfected with plasmid constructs expressing GFP-eAE1 or GFP-kAE1. It has been demonstrated in this report that amino-terminal GFP-tagging dose not affect the targeting or chloride transport properties of eAE1 and kAE1 (20). This has supported that it is possible to use the GFP fusion for the study of wild-type and mutant AE1 proteins in kidney cell line.

To study the co-expressions between either the wild-type kAE1 and mutant kAE1 SAO or G701D, or the mutant kAE1 SAO and G701D to mimic either the heterozygous or compound heterozygous conditions, the wild-type or mutant kAE1 would be tagged with GFP or hemaglutinin (HA). These fusion proteins would then be studied for expression and trafficking of the proteins by transfection or co-transfection into HEK293 cells. The results obtained from this study would provide a

better understanding in the molecular mechanism of AR dRTA caused by compound heterozygous G701D/SAO AE1 mutations.

CHAPTER II
OBJECTIVES

The objectives of the study in this thesis are:

1. To construct and express the wild-type and mutant kAE1 fused with GFP,
2. To study subcellular localization of wild-type and mutant kAE1 fused with either GFP or HA and,
3. To study subcellular localization of the co-expressed mutant kAE1 SAO and G701D proteins.

CHAPTER III
LITERATURE REVIEW

3.1 AE1 and dRTA

Primary hereditary distal renal tubular acidosis (dRTA) is a disease characterized by failure of the kidney to generate sufficiently acidic urine in the setting of spontaneous systemic metabolic acidosis (complete dRTA) or after an imposed acid load (incomplete dRTA)(21). The other associated abnormalities include hypokalemia leading to muscle weakness, and metabolic bone disease (rickets or osteomalacia). The inadequate urinary acidification is usually accompanied by hypocitraturia, hypercalciuria, and nephrocalcinosis, leading to interstitial disease, urinary concentration defects, nephrolithiasis, and ultimately to chronic renal insufficiency. Normally, in the proton-secreting α-intercalated cell in the renal collecting duct of the kidney, the proton or hydrogen ions (H^+) to be secreted into urine are dissociated from carbonic acid, generated from the hydration of carbon dioxide to carbonic acid through the enzymatic action of cytoplasmic carbonic anhydrase II (Figure 3.1). The hydrogen ions are secreted into tubular lumen through the action of an H^+-ATPase and an H^+/K^+-ATPase at the apical membrane of the α-intercalated cell. The bicarbonate (HCO_3^-) generated by the same process leaves the α-intercalated cell by kidney anion (Cl^-/HCO_3^-) exchanger 1 (kAE1) on the basolateral membrane and enters the blood stream(20). The dRTA disease can be caused either by a defect of the H^+-ATPase, or by an abnormality of kAE1, occured from mutations of the responsible genes. Mutations in human *solute carrier family 4, member 1* (*SLC4A1*) or *anion exchanger 1* (*AE1*) encoding kidney AE1 (kAE1) may cause dRTA. The abnormality of kAE1 that mediates anion (Cl^-/HCO_3^-) exchange at the basolateral membrane of the α-intercalated cells can result in a defect in bicarbonate extrusion across the membrane leading to the intracellular accumulation of bicarbonate. To maintain intracellular acid-base and electroneutal balances, the cells would reserve hydrogen ion instead of secreting it through the apical membrane. Consequently, the intracellular accumulation of both HCO_3^- and H^+ would inhibit the dissociation of carbonic acid (H_2CO_3).

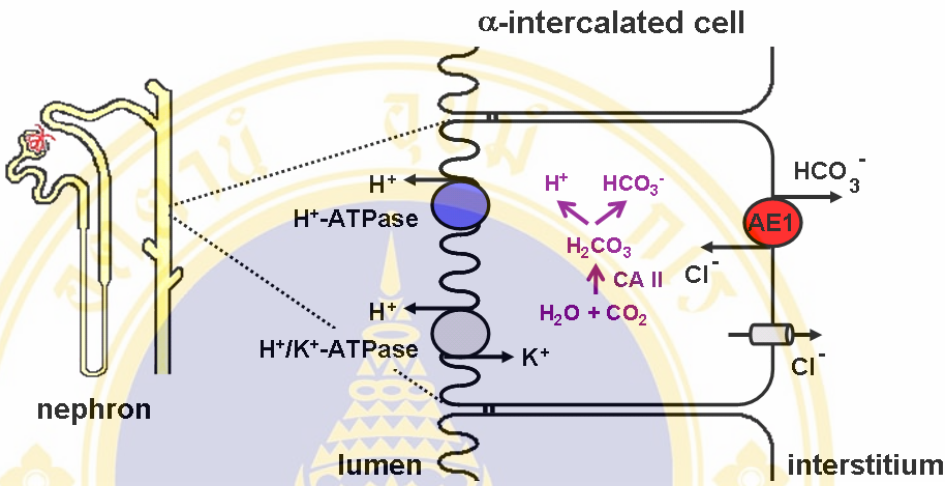


Figure 3.1 Schematic diagram of the α-intercalated cell in the collecting tubule and transporters involving in acid-base regulation [redrawn from Karet *et al.* (1)].

H^+-ATPase and H^+/K^+-ATPase are located at the apical membrane while kidney anion (Cl^-/HCl_3^-) exchanger 1 (kAE1) at the basolateral membrane of the α-intercalated cell.

The reduction or failure of hydrogen ion secretion through the apical membrane into the tubular lumen due to the defect of basolateral kAE1 will eventually result in dRTA (12).

3.1.1 Molecular and cellular biology of human AE1

(1) *AE1* gene

AE1 is encoded by *AE1* or *SLC4A1* gene which is located on chromosome 17q21-22 (22). Human *AE1* encompasses approximately 20 kb and contains 20 exons and 19 introns. The erythroid AE1 (eAE1) transcript contains all sequences of exons 1-20. An additional promoter is located within erythroid intron 3 and this promoter is used in kidney to yield the kAE1 transcript containing part of intron 3 (K1 exon) together with exons 4-20. This transcript yields an N-terminally truncated isoform of erythroid AE1 namely kAE1 which is expressed in the distal nephon of the kidney (23). The diagram of human *AE1* gene as well as eAE1 and kAE1 isoforms is shown in Figure 3.2.

(2) Structure and function of AE1

AE1 is a member of human anion exchanger protein family which regulates Cl^- /$HCO3^-$ exchange in the red cells and α-intercalated cells of the renal collecting duct(24). Erythroid AE1 (eAE1) isoform is expressed on red blood cell membrane at a high copy number of 1.2×10^6. Kidney AE1 (kAE1) isoform is located on the basolateral membrane of the α-intercalated cells of the collecting duct of the kidney. Human eAE1 contains 911 amino acids with a single site of N-glycosylation at Asn 642 (25). kAE1 lacks the amino-terminal 65-amino acid residues of eAE1 and has a molecular weight of 95 kDa. It has a long amino-terminal cytoplasmic domain, ~400 amino acids, followed by ~500 amino acids of polytopic transmembrane domains, and a short carboxy-terminal cytoplasmic tail (~33 amino acids) (26). The cytoplasmic amino-terminal domain is involved in protein-protein interactions with several structural proteins and metabolic enzymes. In red cells, the amino-terminal cytoplasmic domain (43 kDa), residues 1-359, functions in anchoring membrane to the underlying cytoskeleton through interactions with ankyrin, band 4.1, band 4.2 and other proteins (23). The high resolution (0.26 nm) crystal structure of cytoplasmic

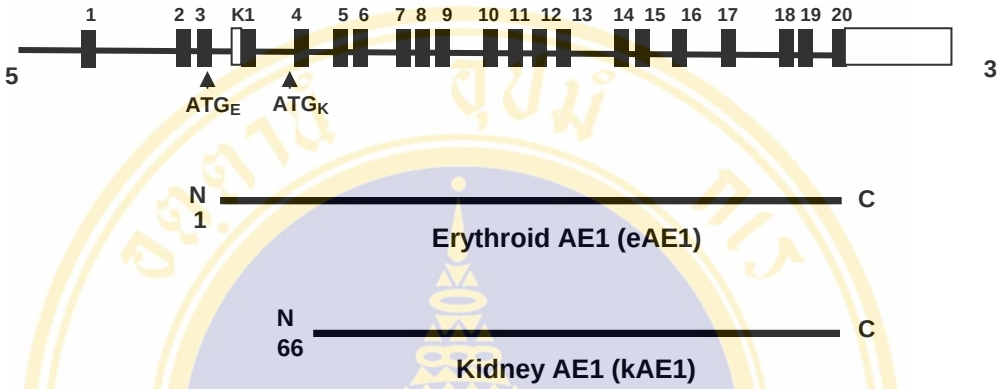


Figure 3.2 Schematic diagram of human *AE1* gene and encoded proteins [redrawn from Yenchitsomanus *et al.* (27)].

Exons are represented by open and filled boxed. Exon numbers are indicated above the exons. The same gene encodes both erythroid AE1 (eAE1) and kidney kAE1 (kAE1) isoforms by using alternative promoters and different start codons (ATG_E and ATG_K), resulting in different length of polypeptides. The eAE1 contains 911 amino acids whereas the kAE1 consists of 846 amino acids.

amino-terminal domain of band 3 (cdb3) determined by X-ray crystallography has been reported (28) (Figure 3.3). The carboxy-terminal membrane domain (52-kDa) has been proposed to span the membrane 12-13 times and functions in anion exchange (23). Howerver, a clear picture of the C-terminal region remains elusive. The AE1 inhibitors are pyridoxal phosphate and 4',4'-diisothiocyanodihydrostilbene-2,2'-disulfonate (H_2DIDS). Stilbene disulfonate binds to an external site in the membrane domain, inhibiting anion transport. The isothiocyanate moiety covalently binds to the ε amino group of Lys539 in the transmembrane 5 (TM5). The second isothiocyanate group of H_2DIDS and pyridoxal phosphate can crosslink to Lys851 in nominal TM13. In the cell membrane, intact AE1 exists as a complex of homodimers and ankyrin-associated homotetramers. The absence of residues 1-65 in kAE1 should result in deletion of a central strand of the major β-sheet but the effect on the remaining folded structure is still unknown. The AE1 membrane domain is operationally defined as the region from around $Tyr_{359}Lys_{360}$ to Val_{872} in the C-terminal region of the protein. The data from several studies indicate that Glu_{681} in TM8 is the likely proton-binding site for proton-sulfate co-transport and contributes to the determination of anion selectivity and pH sensitivity of the anion translocation pathway of human AE1 (23). The 33-amino-acid cytoplasmic carboxyl-terminal tail of human AE1 also implicates in the anion-exchange activity. The carbonic anhydrase II (CAII) binds to LDADD motif (residues 886-890) in the cytoplasmic carboxyl-terminus to facilitate efficient chloride/bicarbonate exchange activity(29). The current structural model of human AE1 (Figure 3.4) and a model of transmembrane domain topology are proposed (Figure 3.5).

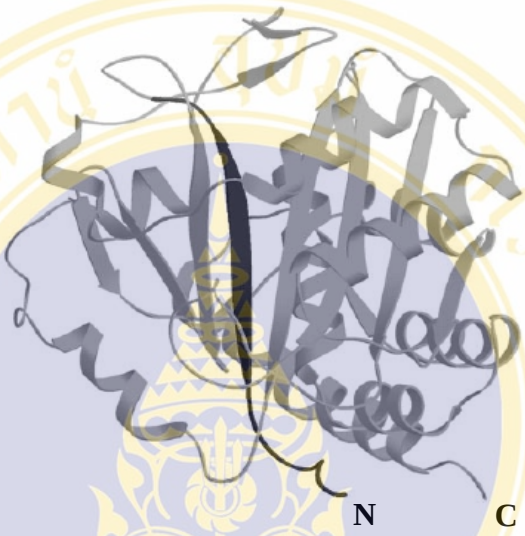


Figure 3.3 Structure of the cytoplasmic amino-terminal domain of human AE1 (cdb3) [taken from Zhang *et al*. (28)].

Ribbon diagram of the monomer of cytoplasmic amino-terminal domain of eAE1. The dark strand in the middle is the β-strand (65 amino acids) which is deleted in kAE1.

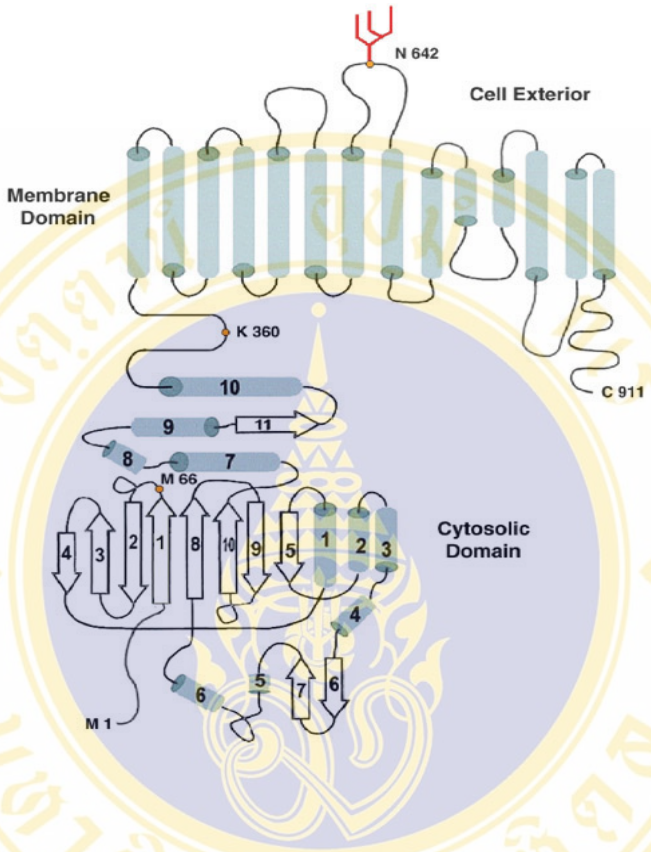


Figure 3.4 Structural model of human AE1 [taken from Zhang *et al.* (28)].

The protein contains two domains, cytoplasmic and membrane domains. The amino-terminal cytoplasmic domain (M1-K360) comprises eleven β-strands and ten helical segments, which belongs to the α+β-fold class. The carboxyl-terminal membrane domain (G361-V911) spans the membrane 12 times. N642 on the forth extracellular loop of membrane domain represents N-link glycosylation site.

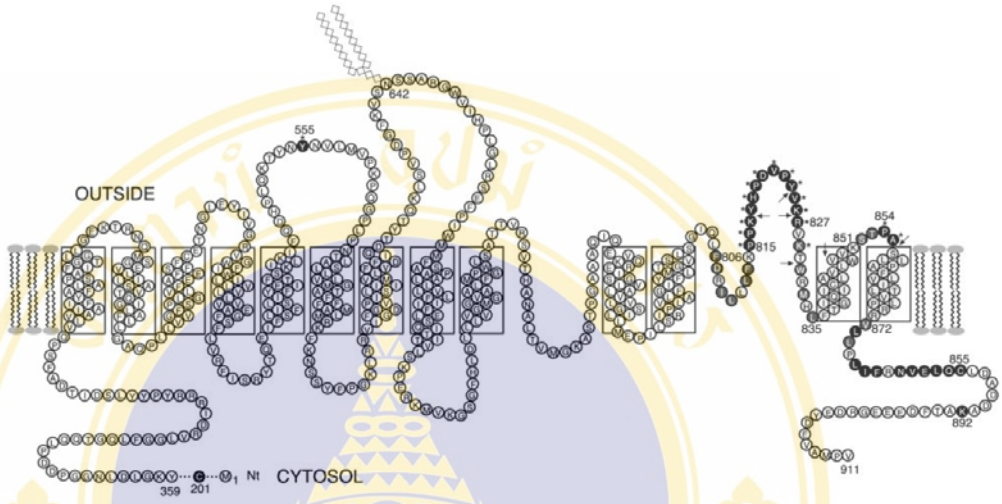


Figure 3.5 The topology model of the transmembrane domain of human AE1 [taken from Zhu *et al.* (30)].

Diagrammatic representation of AE1 topological model according to the traditional 13-span transmembrane model. Amino acids 359-911 are indicated. The branched structure at Asp 642 represents N-linked glycosylation. The protein comprises a long amino-terminal domain and a short carboxyl terminal part located in cytoplasm.

3.1.2 Trafficking of AE1

In order to carry out their physiological functions, ion transport proteins must be targeted to the appropriate domains of cell membrane. The attribution of these transport proteins is important especially in polarized epithelial cells. The cell surface membranes of polarized epithelial cells are divided into apical and basolateral domains, separated from one another by tight junctions. The basolateral domain participates in contacts with the basement membrane and neighboring cells, whereas the apical domain generally confronts a lumen(31). Various membrane proteins are sorted to the basolateral domain through interaction of tyrosine-bases targeting motifs in their cytoplasmic domain with adaptor-protein complexes. The motif YDEV in C-terminus of AE1 conforms to the consensus motif -YxxΦ- (where Y is tyrosine, x is any amino acid, and Φ is a bulky hydrophobic residues), which is associated with localization to coated pits and clathrin-mediated endocytosis, and also in basolateral sorting. Normally, kAE1 expresses at the basolateral membrane of α-intercalated cells. These data indicated that a tyrosine-based motif in C-terminus of AE1,-YxxΦ-, has an importance role in polarized distribution of AE1 at the cell surface. Generally, the –YxxΦ- motif interacts with μ subunits of adaptor-protein (AP) complexes (AP-1, AP-2, AP-3 and AP-4), especially AP-1B which is specific to polarized epithelial cells. The sorting of AE1 depended on AP-1B was investigested in LLC-PK1 cells, which lack the μ1B subunit and thus cannot form AP-1B complexes. kAE1 still had a basolateral localization; therefore, AP-1B may not be necessary for kAE1 trafficking to the basolateral membrane. This observation suggests that another adaptor complex, such as AP-4 or other unidentified components, may direct kAE1 to the basolateral membranes (9). The amino acid sequence of C-terminus of AE1 and the motifs are shown in Figure 3.6

Moreover, the residues $Y_{904}DEV_{907}$ motif of kAE1 have been investigated for the role in basolateral targeting of kAE1. In polarized MDCK cells, the mutation of tyrosine 904 altered kAE1 localization and resulted in the intracellular retention of the mutant kAE1 but the mutant V907A did not change kAE1 basolateral localization. This contrasts with the apical localization of the mutant R901X and Y904A (9).

Figure 3.6 Amino acid sequence of C-terminus of human AE1.

Amino acid residues from position 872 to 911 of the C-terminus of AE1 are shown. Boxes represent known or putative interacting motifs for carbonic anhydrase II (CA-II), AP and PDZ.

In addition, R901X is mistageted to both apical and basolateral membrane in polarized LLC-PK1 cells (32). These data confirmed that Y904 is essential for the basolateral targeting of kAE1 and supported the previous report that AE1 targeting is not depend on the presence of AP-1B which interacts with -YxxΦ- motifs. Additionally, a nearby motif in the C-terminal tail, $D_{902}EYDE_{906}$, has been proposed as a basolateral localization signal for kAE1 (33).

The last five amino acids at C-terminus of AE1 has been proposed to encode a putative Post-Synaptic-Density-95/Discs-large/Zona-Occludens-1 (PDZ) interacting motif (AMPV) (34)which involved in the assembly of supramolecular complexs that perform localized signaling function at particular subcellular locations (35). Deletion of these residues induced intracellular retention of AE1 protein in HEK293 and LLC-PK1 cells. These indicate that the last five amino acids are importance for proper trafficking of AE1 to the cell surface. However, kAE1 Δ11 proteins were localized at both apical and basolateral surface in polarized LLC-PK1 cells, suggesting that the C-terminal PDZ-binding motif is not absolutely required for the basolateral localization of kAE1 but still play some role in the efficient trafficking and/or retention of kAE1 at the basolateral membrane (32). Taken together, these trafficking studies of kAE1 protein indicate C-terminus of kAE1 is importance for efficient kAE1 trafficking from its site of synthesis in the ER to the correct surface of plasma membrane.

The importance of the N-terminus of kAE1 in protein targeting has recently been described (32). In stably transfected MDCK cells expressing the AE1 membrane domain (residues 361-911) but lacking the N-terminal domain, the protein was localized to the apical membrane. Therefore, an element within the N-terminal sequence is required in addition to the –YxxΦ- motif of C-terminus for the basolateral targeting of kAE1. $Y_{359}KGL_{362}$ is a potential tyrosine-targeting motif in the N-terminus of kAE1 (32). These data suggested that the determinant within the kAE1 N-terminus co-operates with that within the C-terminus for kAE1 basolateral localization.

3.1.3 *AE1* mutations associated with red cell abnormalities

Mutations in eAE1 may result in hereditary spherocytosis (HS), are inherited hemolytic anemia that causes erythrocytes to become spheroidal in shape and osmotically fragile. The erythrocytes are trapped and destroyed in the spleen leading to splenomegaly. HS can be caused by defects in erythrocyte proteins that are involved in interactions between the cytoskeleton and the erythrocyte membrane: spectrin, ankyrin, band 4.2 or the anion exchanger, AE1 (band 3). Approximately 20% of HS cases are due to mutations in *AE1* (36). The *AE1* mutations that give rise to HS are located in both the membrane domain and the cytoplasmic domain of eAE1. These usually occur from nonsense, frameshift, and 11 missense mutations of *AE1* (36, 37) (Table 3.1). The missense mutations may cause a greater decrease in AE1 expression in red cells, probably due to a dominant-negative effect.

Certain mutations locating in the cytoplasmic domain of AE1 presumably alter the cytoskeleton binding sites for band 4.2, α- and β-spectrin and ankyrin. Band 3 Montefiore (Glu40Lys) produced the 88% reduction in binding to protein 4.2 (38). Band 3 Fukuoka (Gly130Arg) which is associated with autosomal recessive HS, when present as a homozygous mutation caused a slight reduction of band 3 (9%) and a substantial reduction in protein 4.2 (45%) (39). Band 3 Tuscaloosa (Pro327Arg) associated with autosomal dominant HS produce an 29% reduction in protein 4.2. Pro327 is located in a highly conserved region of band 3, its substitution with Arg may disrupt the secondary and tertiary structure of the cytoplasmic domain of band 3, which may lead to the altered binding of protein 4.2 (40). In band 3 Nachod, five highly conserved amino acid residues are deleted from the putative ankyrin binding site (amino acid residues 117-121) disrupting ankyrin binding (41).

Seven HS missense mutations (L707P, R760Q, R760W, R808C, H834P, T837M, and R870W) locating in the membrane domain of the human AE1 caused mutant AE1 to misfold and to be retained intracellularly (42). However, there was no change in the oligomeric state or in the half-life of the mutant AE1. The HS mutant AE1 was retained in a pre-medial Golgi compartment. During erythoid development HS mutant AE1 would be destroyed before the reticulocyte stage during the elimination of the ER and Golgi. This would lead to decreased AE1 levels observed in the mature red cell and the resulting HS phenotype (42).

Table 3.1 ***AE1*** **mutations associated with hereditary spherocytosis (36).**

Band 3 variant	Mutation
A. **Affects protein 4.2 binding**	
Montefiore	Glu40 → Lys
Fukouka	Gly130 → Arg
Tuscaloosa	Pro327 → Arg (Lys56 → Glu)
B. **Affects ankyrin binding**	
Nachod (Hradec Kralove II)	Aberrant splicing: deletion of codons 117–121
C. **mRNA instability**	
Genas	G89 → A (exon 2)[a]
Neapolis	Aberrant splicing: insertion of intron 2: stop codon (+19) after exon 2 or deletion of exon 2: truncated protein using alternative ATG
Kagoshima	Deletion A in codon 19
Fukayama I	Deletion AC in codon 38
Foggia	Deletion ACCCAC → ACCAC (codon 54 or 55)
Fukayama II	Insertion A in codon 61
Bohain	Deletion T in codon 81
Hodonin (Prague IV)	Trp81 → Stop
Napoli I	Insertion TCT → TTCT (codon 100)
Osnabruck I (Lyon)	Arg150 → Stop
Worcester	Insertion G into codons 170–172
Campinas	Aberrant splicing: stop codon (+13) after exon 7
Princeton	Insertion C into codons 273–275
Noirterre	Gln330 → Stop
Bruggen	Deletion C in codon 419
Bicetre II	Deletion G in codons 454–456
Pribam (Prague VI)	Aberrant splicing: stop codon (+7) after exon 12
Evry	Deletion T in codon 496
Smichov (Prague VII)	Deletion C in codon 616
Trutnov	Tyr628 → Stop
Hobart	Deletion G in codons 646–647
Osnabruck II	Deletion of codon 663 or 664
D. **Band 3 protein instability**	
Boston	Ala285 → Asp
Benesov (Prague V)	Gly455 → Glu
Coimbra	Val488 → Met
Bicetre I	Arg490 → Cys
Milano	Insertion of 23 amino acid residues at codon 498
Dresden	Arg518 → Cys
Most (Prague VIII)	Leu707 → Pro
Okinawa	Gly714 → Arg
Kumamoto (Prague II)	Arg760 → Gln
Hradec Kralove	Arg760 → Trp
Chur	Gly771 → Asp
Napoli II	Ile783 → Asn
Jablonec	Arg808 → Cys
Nara	Arg808 → His
Prague	Duplication (nucleotides 2455–2464)
Birmingham	His834 → Pro
Philadelphia	Thr837 → Met
Tokyo	Thr837 → Ala
Prague III	Arg870 → Trp
Vesuvio	Deletion ACC → AC in codon 894
Tambau	Met663→Lys

Amino acid and nucleotide numbering as described by Tanner et al 1988.
[a]Nucleotide numbering from the first residue of exon 1.

Southeast Asian ovalocytosis (SAO) is an asymtopmatic hereditary condition that is widespread in the south of Thailand, Malaysia, the Philippines, Indonesia and, Papua New Guinea,, where malaria is endemic (26). The ovalocytic red cells afford some protection against cerebral malaria in children and cause increased red-cell rigidity, but the abnormality dose not cause hemolytic anemia, and by itself dose not give rise to morbidity (20). SAO results from the heterozygous presence of a 9-amino-acid deletion (residues 400-408) in AE1 and is linked to the relatively common band 3 Memphis mutation (K56E) (5). Despite this deletion of nine amino acids residues, AE1 SAO is not degraded in the internal membranes of the cell, but inserts into the erythrocyte membrane (36). SAO red cells are much more rigid than normal red cells probably due to the 9 amino acid deletion removes a flexible region of the protein causing a non-specific interaction of the cytoplasmic domain of AE1 SAO with the cytoskeleton. The expression and functional study of AE1 SAO in red cells and *Xenopus* oocytes have been examined. The specific anion transport activity per AE1 molecule of the normal protein in SAO red cells is reduced of that in normal red cells (5, 17). The expression of AE1 SAO has been studied in *Xenopus* oocytes. AE1 SAO is not functional as an anion transporter but is inserted stably into the plasma membrane of oocytes (18). Additionally, expression of AE1 SAO in *Xenopus* oocytes dose not require normal AE1 or GPA and AE1 SAO is translocated to the oocytes plasma membrane more readily than normal AE1, supporting the view that movement through ER and Golgi apparatus may also be enhanced in the red cells (36). Not surprisingly, all the known individuals with SAO are heterozygous for AE1 SAO. Individuals homozygous for AE1 SAO have never been detected and this condition is probably lethal (36).

3.1.4 AE1 mutations associated with dRTA

Reported *AE1* mutations associated with AD and AR dRTA and their locations on protein are shown in Figure 3.7 and Tables 3.2 and 3.3.

(1) Autosomal dominant dRTA (AD dRTA)

Some mutations of *AE1* gene have been found to be associated with dRTA. The mutations associated with autosomal dominant (AD) dRTA are missense mutations in codon 589 of the *AE1* gene, resulting in substitutions of arginine by either histidine (R589H), cysteine (R589C) or serine (R589S) (1, 3, 4, 13). Other mutations associated with AD dRTA are S613F, A858D (1, 3, 4, 13), R901X (1), A888L (43), and a novel mutation, G609R (6). These mutations have been studied for anion transport function either in red cells or *Xenopus* oocytes, and protein trafficking in kidney cell lines. However, the anion transport function in the red cells and *Xenopus* oocyte could not explain the abnormality associated AD dRTA. Instead of losing the anion transport function, AE1 S613F mutation is associated with upregulation of the anion transport activity in red cells and normal chloride transport activity in *Xenopus* oocytes (3). The AE1 R589H mutation showed a modest reduction for sulfate uptake into red cells, and for chloride transport and chloride/bicarbonate exchange in *Xenopus* oocytes (3, 4). In addition, co-expression of eAE1 and eAE1 R589H or kAE1 and kAE1 R589H in *Xenopus laevis* oocytes did not cause a dominant-negative effect on chloride transport activity (4). Recently, impaired trafficking of kAE1 R589H (as well as R589C and R589S) but not eAE1 R589H was demonstrated in transfected HEK 293 cells (8). The kAE1 R589H proteins have a low level of cell surface expression in HEK293 cells and retained in ER. Co-expression of kAE1 R589H with kAE1 or eAE1 caused a reduction in the cell surface expression of kAE1 and eAE1 in HEK293 cells, indicating a dominant negative effect on the ability of wild-type protein to reach the cell surface because of hetero-dimerization of mutant and wild-type proteins (7, 8). When stably expressed in non-polarized and polarized MDCKI cells, both kAE1 R589H and kAE1 S613F were also retained internally (32). This means that R589H and S613F mutations locating in the transmembranes 6 and 7 (TM6-7) of kAE1 do not cause gross misfolding of mutant proteins, since they have significant transport activity in both *Xenopus* oocytes and red blood cells.

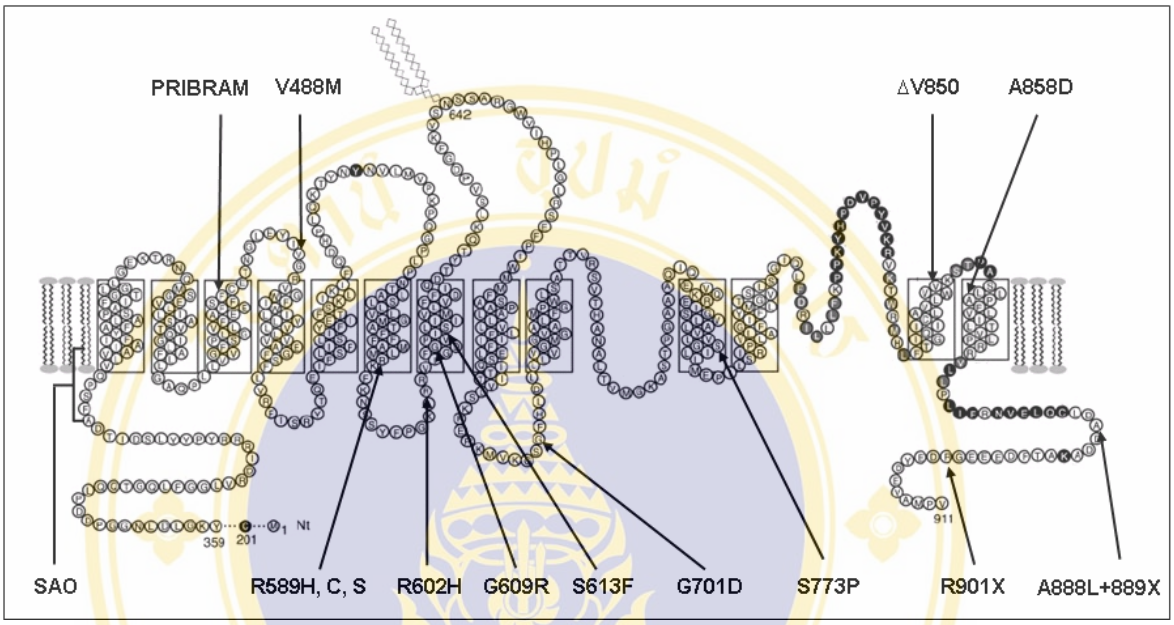


Figure 3.7 ***AE1*** **mutations associated with dRTA and their locations on the AE1 protein [taken from Yenchitsomanus *at al.* (12)].**
Circles with alphabets represent amino acid residues of AE1 protein between position 359 and 911 and the locations of mutations associated with dRTA are pointed

Table 3.2 *AE1* mutation associated with AD dRTA.

Mutation		Reference
Protein	DNA	
Band 3^{PRIBRAM}	IVS12+1G>T	(44)
R589H	1766G>A	(1, 3, 4)
R589C	1765C>T	(3, 45)
R589S	1765C>A	(1)
G609R	1825G>A	(6)
S613F	1838C>T	(3)
A858D[1]	2573C>A	(3)
A888L	20-bp deletion in exon 20	(43)
R901X	13-bp duplication in exon 20	(1)

[1]Heterozygous A858D mutation causes incomplete dRTA; complete dRTA is occurred from its compound heterozygous conditions with other recessive mutations. Thus, the assignment of A858D mutations under AD dRTA may be questionable.

Table 3.3 *AE1* mutation associated with AR dRTA.

Mutation		Reference
Protein	DNA	
SAO	ΔAla400-Ala408	(46)
V488M	1462G>A	(47)
R602H	1805G>A	(13)
G701D	2102G>A	(5, 11, 16, 48)
S773P	2317T>C	(13)
ΔV850	2548-2550delGTG	(5)

In addition, TM6-7 do not appear to be essential for AE1 chloride transport. It is possible that localized misfolding in TM6-7 region increases the aggregation of the mutant proteins or alters their interactions with ER chaperones. This would affect their exit from ER, and may then lead to their degradation by the ER-associated degradation (ERAD) system (10).

A missense mutation, A858D, was reported in compound heterozygous either with SAO or AE1 with a deletion of valine at position 850 (ΔV850). The A858D/SAO red cells were small and elliptocytic whereas the A858D/ΔV850 patients both showed bizarre red-cell morphology with microcytes, elliptocytes and poikilocytes. Moreover, the chloride influx activity and cell surface expression of mutant AE1 in *Xenopus* oocyte were decreased. The simple heterozygotes (A858D/N) cause incomplete dRTA. The compound heterozygous A858D/SAO and A858D/ΔV850 had more severe form of dRTA, and also anaemia (10). The dominant A858D mutation might cause the disease by mis-tergeting of the mutant protein to the apical instead of the basolateral membrane of the α-intercalated cells (5). Since heterozygous A858D mutation causes incomplete dRTA; complete dRTA is occurred from its compound heterozygous conditions with other recessive mutations, the assignment of A858D mutations under AD dRTA may be questionable.

The AE1 R901X mutation (band 3 Walton) is caused by a duplication of a region from codon 896 to codon 900 resulting in creation of a premature stop codon at position 901 and deletion of the carboxyl-terminal 11 residues (1). Transport studies showed that eAE1 R901X had normal sulfate transport activity, and kAE1 R901X had normal chloride transport activity in *Xenopus* oocytes. The mutant protein is able to traffic to the cell surface of erythrocytes and oocytes, while mutant protein was retained in ER but was also present in late endosome/lysosomes when expressed in non-polarized MDCK cells (10). The AE1 R901X mutant protein gives rise to AD dRTA by inhibiting the movement of normal AE1 protein to the cell surface. This results from the association of the normal and mutant proteins in hetero-oligomers causing the intracellular retention of the normal along with the abnormal proteins (2, 20). In polarized cells, the R901X was mistargeted to the apical membrane (9, 32). From this result, it was demonstrated that dominant dRTA mutations also cause aberrant targeting of kAE1 in polarized kidney cells and provide additional

explanation for the origin of dominant dRTA. It was also demonstrated that C-terminal 11 residues of kAE1 contain a tyrosine-dependent basolateral targeting signal that is not recognized by μ1B-containing AP-1 adaptor complexes (9, 32).

A missense *AE1* mutation, G609R, causing dominant dRTA was recently reported in a Caucasian family (6). The AE1 G609R mutation has a substitution of G by A at nucleotide 1825, which resulting in substitution of glycine (GGG) at position 609 by arginine (AGG). This study demonstrated that kAE1 G609R have a normal anion transport function in *Xenopus* oocytes and mutant proteins were expressed at the cell surface. Mutant kAE1 was localized at subapically and at the apical membrane, as well as the basolateral membrane in polarlized MDCK, in contrast to the normal localization of wild-type kAE1 at basolateral membrane. It is proposed that Gly609 may play an importance role in correct protein targeting. Gly609 is located close to two residues where missense mutations have been reported, codons Arg589 and Ser613, this region of AE1 may be involved in the protein trafficking or sorting process (6).

(2) Autosomal recessive dRTA (AR dRTA)

The principal mutations reported in Southeast Asia and Papua New Guinea in relation to AR dRTA are SAO Δ400-408, G701D, and ΔV850 (5, 16). The other reported recessive mutations include R602H (13) and a novel mutation, S773P (14). The homozygous AE1 G701D mutation, frequently observed in Thai pediatric patients with dRTA (11, 49), causes AR dRTA with normal erythroid anion transport (11). AE1 G701D loss-of-function was accompanied by impaired trafficking to the *Xenopus* oocyte surface. Co-expression of AE1 G701D and erythroid AE1 chaperone, glycophorinA (GPA), rescues the mutant protein trafficking and transport function. Moreover, there was no dominant negative behavior of kAE1 G701D when co-expressed with eAE1. AE1 G701D trafficking in erythroid precursors is likely rescued by the presence of GPA, explaning the normal sulfate transport activity in erythrocytes. Heterozygous SAO mutation dose not cause dRTA (13). Two cases of AR dRTA and SAO resulted from compound heterozygous G701D/SAO mutations were originally reported in two Thai families from southern Thailand (16). These patients were not anemic but have little morphological changes of red cells in addition

to SAO, and had a decrease of red cell sulfate flux of about 40%. In compound heterozygous G701D and SAO, there was loss of cell surface expression and no chloride transport activity in *Xenopus* oocyte (5). The recessive mutations had greatly decreased anion transport function in red cells and *Xenopus* oocytes when present as compound heterozygotes (ΔV850/A858D, ΔV850/SAO or A858D/SAO) (5).

A novel *AE1* S773P mutation, which locates within tenth transmembrane segment in the C-terminal region of AE1, reported in Thai family with AR dRTA in a compound heterozygous (S773P/G701D) (13) was studied in HEK293 cells and LLC-PK1 cells (14). kAE1 S773 protein exhibits lower expression and more rapid turnover compared to the wild-type kAE1. The kAE1 S773P was mis-folded and targeted to the proteasome for degradation. Both kAE1 S773P and G701D exhibited defective trafficking to the cell surface and showed predominant immunolocalization in the ER of both HEK293 and LLC-PK1 cells. However, kAE1 S773P could form homodimer, as well as a heterodimer with either wild-type kAE1 or kAE1 G701D. Hetero-oligomers of wild-type kAE1 with kAE1 S773P or G701D, in contrast to the dominant kAE1 R589H mutant, were delivered to plasma membrane. Thus, in the heterozygous state, sufficient kAE1 would be present in the basolateral membrane of α-intercalated cells to retain adequate bicarbonate transport into the blood.

In the recent study in MDCK cells, it was found that the kAE1 S773P and G701D, showed distinct trafficking defects (15). The mis-folded kAE1 S773P was largely retained in the ER in non-polarized MDCK cells, whereas it was predominantly targeted to the basolateral membrane in polarized MDCK cells. In contrast, kAE1 G701D was retained in the Golgi in both non-polarized and polarized cells. The co-expression of kAE1 S773P and G701D showed some co-localization of S773P with G701D in the Golgi, but kAE1 S773P could still traffic to the basolateral membrane of MDCK cells. Also, no kAE1 G701D was detected at the cell surface, suggesting that kAE1 S773P did not assist the kAE1 G701D to traffic to the cell surface as did the wild-type kAE1, despite their ability to oligomerize in the MDCK cells. This result suggests that in the patients with compound heterozygous *SLC4A1* G701D/S773P mutations, only the mis-folded S773P/S773P homodimers which may not properly function can reach the basolateral membrane of the kidney α-intercalated cells, resulting in the development of dRTA (15).

Recently, SAO mutation has been studied for stability and trafficking in HEK293 and MDCK cells. In HEK cells, expression level and stability of SAO protein were significantly reduced, and no mutant protein was detected at the cell surface. This result suggests that erythroid-specific factors missing in HEK cells may be required for cell-surface expression. Although misfolded SAO protein could form heterodimer with the normal protein, as well as homodimer, in polarized MDCK cells, kAE1 SAO was retained intracellularly. When kAE1 SAO was co-expressed with kAE1 in MDCK cells, kAE1 SAO was largely retained intracellularly; however, it also co-localized with kAE1 at the cell surface. These finding indicates that in the kidney of heterozygous SAO patients, homodimer of kAE1 and heterodimer of kAE1 SAO and kAE1 traffic to the basolateral membrane of α-intercalated cells, while homodimers of kAE1 SAO are retained in ER and rapidly degraded. This results in sufficient cell surface expression of kAE1 to maintain adequate bicarbonate reabsorption and proton secretion without dRTA (19).

3.2 Expression and functional studies of AE1 in transfected cells

Expression and functional studies of AE1 mutant proteins in several cell types and cell lines would lead to understand the molecular pathology and mechanism of AE1 mutation causing dRTA. The African claw frog egg, *Xenopus* oocyte, is the first heterozygous system used to study the functional expression of recombinant AE1 (50, 51). Since *Xenopus* oocyte does not express endogenous AE1 and its large size, it provides an ideal expression model for transport function of ion transporters. Anion transport function of AE1 has been determined by many methods such as chloride and bicarbonate fluxes mediated in oocytes.

To date, several types of cultured mammalian cell line such as human embryonic kidney (HEK293), human erythroleukemia cell (K562), green monkey kidney fibroblast (COS-7), Madin-Darby canine kidney (MDCK), inner medullary collecting duct (IMCD) and porcine kidney (LLC-PK1) cells were used for study of biosynthesis, trafficking, properties and function of AE1 proteins by oligosaccharide processing determination, immunofluorescence techniques, cell surface biotinylation and anion transport assay (5-9, 14, 19, 32, 34).

Transiently transfected HEK 293 cells have also been widely used to study the expression, function, properties and trafficking of AE1. These cells have been used due to their cells anion exchange activity allowing for functional assays with no interference from endogenous anion exchangers. Although HEK293 cells successfully express both AE1 and kAE1 at plasma membrane, a major disadvantage is that these cells are unable to polarized into distinct apical and basolateral membrane domains. In addition, post-translational modifications of AE1 and kAE1, such as complex N-glycosylation and pamitoylation do not occur in HEK293 cells. Therefore, AE1 expressed at the plasma membrane in transfected HEK293 cells retains a high mannose oligosaccharide at Asn642 in extracellular loop 4 (which appears to have no adverse effect on bicarbonate-chloride exchange conducted by AE1) (10). Interestingly, moving the acceptor site to position 555 has been found to allow N-glycosylation processing to complex form to occur.

MDCK cells generate and maintain two morphologically and functionally distinct plasma membrane domain, the apical and basolateral junction domain. Polarized MDCK cells are useful for studying the AE1 trafficking responsible for the polarized localization. Human kAE1 has now been transiently expressed in MDCK and LLC-PK1 cells and stably expressed in MDCK cells. kAE1 was progressively converted from a core glycosylated form to a complex glycosylated form in both non-polarized and polarized MDCK cells. Immunofluorescence staining showed that kAE1 was predominantly expressed at the cell surface in non-polarized MDCK cells and localized to the basolateral membrane in polarized MDCK cells and LLC-PK1 cells (10).

The most commonly used techniques for the study of the cellular localization of proteins in cultured cells is immunofluorescence by using antibody specific against protein. However, some proteins have proved difficult to raise antibodies against them (particularly membrane proteins) or else the antibodies which are available have very limited use. Epitope tags are artificial epitopes (also known as antigenic determinants) engineered into a protein sequence by placing sequence encoding the epitope within the same open reading frame of the protein encoded by the gene sequence. Epitope tags, short amino acid sequences, are fused to the N-terminus or C-terminus of the protein. The most commonly used epitope tags are c-myc, hemagglutinin (HA), 6xHis,

and FLAG. These epitopes, and the antibodies which detect them, have been well characterised that useful for applications such as immunocytochemistry, Western blotting, and immunoprecipitation (52).

Another technique is to tag the protein with green fluorescent protein (GFP) which enables the tagged protein to be visualized in live cells using fluorescence microscopy, without the need to use antibodies to detect it (53). Various recombinant strategies may be used to fuse the GFP tag to the amino terminus or carboxyl terminus of the target protein. These constructs may be introduce into the cell or organism using standard methods for transient or stable expression. The protein expression and transport is then followed using conventional fluorescence microscopy. Because detection does not require permeabilization or fixation of the cells, there is little probability of introducing artifacts. Moreover, expressed fusion proteins are rarely toxic to cells (54). To gain understanding of the mechanism of protein trafficking and the subcellular localization, GFP have enabled enhanced the monitoring of protein trafficking through dual labeling (53).

3.3 The study of protein expression by fluorescent protein fusion technique

3.3.1 Properties of green fluorescent protein

Green fluorescent protein (GFP) has established a universal method for introducing a fluorescent tag into nearly any biological structure. GFP fusion protein technology is applicable to essentially any protein, beginning with the gene, to visualize it in living cells. GFP fusion proteins are currently being used as tools in research on a wide variety of problems (55) such as the study of promoter activation, gene expression, protein trafficking and cell lineage determination (56). It has been efficiently used to image gene expression, to monitor cellular protein localization and its dynamics (57).

The GFP from *Aequerea victoria* is one member of a small but important class of proteins that exhibit strong visible fluorescence without the requirement of cofactors or other enzymes (58). GFP is a 27 kDa protein, comprised of 238 amino acids, in which GFP chromophore consists of a cyclic tripeptide derived from ser-tyr-gly (amino acids 65–67) in the primary protein sequence (59) and is only fluorescent when embedded within the complete GFP protein. The crystal structure of GFP has revealed a tightly packed β-can enclosing an α-helix containing the chromophore (60) (Figure 3.8). GFP is fluorescent either as a monomer or as a dimer. GFP absorbs both UV and blue light and emits green light (55) without the use of a substrate and may thus be viewed in living or fixed tissue (57). GFP fluoresces with maximum at approximately 509 nm. Additionally, detection of GFP can be performed in living cells and tissues as well as fixed samples.

The applied function of GFP is its ability to serve as a fluorescent molecular label for proteins of interest, allowing the localization and trafficking of the protein to be visualized *in vivo*. The study of membrane trafficking is one area of research in which GFP fusion proteins are having a significant impact (55). There is now abundance of characterized GFP fusion proteins that allows live fluorescent marking of many subcellular structures (61).

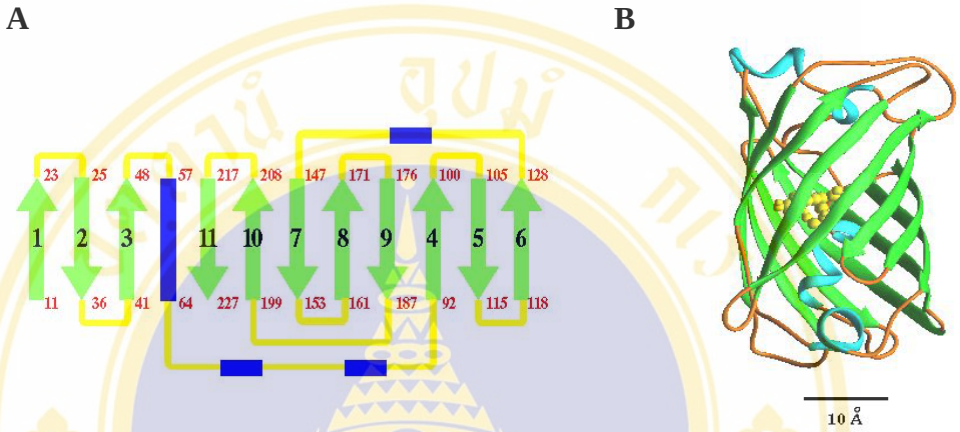


Figure 3.8 A topology diagram and structure of GFP [taken from Yang, *et al.* (62)].

(A) A topology diagram of the folding pattern in GFP. The sheet strands are shown in light green, α-helices in blue, and connecting loops in yellow. The positions in the sequence that begin and end each major secondary structure element are also given. The anti-parallel strands (except for the interactions between strand 1 and 6) make a tightly formed barrel.

(B) The GFP exhibits a unique cage-like structure eleven strands of sheet (green) form the walls of a cylinder. Short segments of helices (blue) cap the top and bottom of the 'β-can' (yellow) and also provide a scaffold for the fluorophore which is near geometric center of the can and shielding it from the solvent.

3.3.2 Expression and localization study by GFP fusion technique

GFP has served as a versatile marker in cell biology for monitoring physiological processes, gene expression and visualizing protein folding and trafficking. The utility of GFP in research applications derives from its intrinsic ability to generate fluorescence in live tissues or fixed tissues, in the absence of any cofactor(s). GFP is fused in-frame to a protein of interest, and the resulting chimeric protein is expressed in an appropriate cellular background for examining protein function and fate. Fortunately, GFP can be targeted to most subcellular sites, making it feasible to examine biochemical processes in real time. GFP fusion is used for study in several proposes include:

(1) Reporter of gene activation

GFP is used as the reporter protein that can be targeted to a particular cell type by fusing the gene encoding GFP to an exogenous cell type-specific gene promoter, by inserting GFP into the coding region of a promoter-driven transgene, or by inserting GFP into the coding region of an endogenous gene (knock-in approach) in order to study regulation of gene activation *in vivo*. Within a particular cell type, GFP can be targeted to the cytoplasm or, in the presence of an appropriate subcellular localization signal engineered into the reporter gene, to the nucleus, mitochondria, or other subcellular location (63). Typically, the gene for GFP is placed downstream of the target response element, allowing the kinetics and regulation of gene activation to be monitored. Because GFP is not an amplified reporter system, GFP is primarily suitable as a reporter for moderate to strong response elements. Examples of the use of GFP to monitor gene activation include: developmental gene regulation (64), gene regulation in neurobiology (63), transcriptional activation of tumor suppressor genes (65), and pharmacological analysis of protein response to treatment with test compounds (66).

(2) Fluorescent tracer

GFP has been used successfully to track the efficiency of gene delivery, both to cells in culture and to whole organisms. Typically, GFP is co-expressed with the gene of interest either bi-cistronically or on a separate delivery vector. In the latter case, the

ratio of target gene to GFP may be adjusted to ensure that all cells expressing GFP have a high probability of expressing the gene of interest. GFP has also proved to be a useful biological tracer to monitor cell lineage and to improve techniques for the production of genetically modified organisms. Examples of the use of GFP as a fluorescent tracker include: developing methods for gene therapy (67, 68), tracing cell lineage in the development of cell- and organ-transplant therapies (69-72), developing improved somatic nuclear transfer techniques for animal cloning (73), production of transgenic organisms (74, 75), and following tumor metastasis and neoplastic disease (76-78).

(3) Reporter of protein behavior

As a genetically encodable fluorescent tag, GFP has been used to monitor a variety of protein behaviors :

(3.1) Dynamic localization of proteins and protein tureover

Understanding the dynamic intracellular distribution of a protein can be the first clue toward unraveling its function. The use of GFP as a minimally invasive tool for studying protein dynamics and function has been stimulated by the engineering of mutant GFPs with improved brightness, photostability and expression properties. Cells that express proteins tagged with these GFPs can be imaged with low light intensities over many hours and so can provide useful information about changes in the steady-state distribution of a protein over time. The other two techniques, photobleaching and photoactivation that, when combined with time-lapse imaging, can uncover the kinetic properties of a protein by making its movement observable. Moreover, photoactivation have been used to elucidate the kinetics and regulation of protein turnover (79).

(3.2) Protein translocation

An application area for GFP involves tracking the movement of proteins within or between cells in response to extra cellular stimuli. The movement of a target protein *in vivo* may be rapid and transient, and therefore difficult or even impossible to detect in a traditional fixed-cell assay. With the aid of high-speed, live-cell fluorescence

imagers, previously intractable targets can be measured by quantifying the real-time redistribution of GFP-tagged proteins after treatment with test compounds (79). Live cellular translocation assays are becoming increasingly attractive in drug discovery for the development of pharmaceutical screens.

(3.3) Changes in cell and organelle morphology

GFP fusion proteins targeted to particular regions of the cell can be used as dynamic markers of general cell shape or organelle morphology. Agonist-induced changes in morphology can be quantified by image analysis. GFP reporters can monitor shape changes indicative of important cellular processes such as apoptosis (regulated cell death), proliferation, adhesion, migration and neurite outgrowth.

(3.4) Protein-protein interactions

Fluorescence resonance energy transfer (FRET) has been successful in detecting interactions between molecules tagged with fluorescent proteins, two GFP molecules or one GFP and secondary fluorophore, whose excitation and emission spectra overlap (80). If two fluorophores are in close proximity and right orientation, the exited donor molecule can transfer its energy to the nearby acceptor molecules through resonance interaction. The light from the excited acceptor molecule can now be observed. Examples of GFP-based FRET applications include assays to detect protease activity, transcription factor dimerization and calcium changes (81).

(3.5) Changes in local pH

A number of GFP variants have been optimized for use as pH sensors. For example, Miesenbock et al., 1998 (82) have generated pH-sensitive GFP with excitation profiles tailored to respond to pH changes occurring along exocytic and endocytic pathways. Such pH-sensitive GFP could prove useful in monitoring processes such as synaptic transmission and regulated secretion.

3.3.3 The use of GFP tagging for studying protein trafficking and function associated with human diseases

Several reports that used GFP tagging for studying trafficking and function of mutant protein causing human diseases are listed in Table 3.4. For example, the study of localization of connexin (Cx) (83). Cx is polypeptide subunits that make up gap junctions, in the keratinocyte, including Cx43, Cx32, Cx30, and Cx26. Mutations in Cx26 are a major cause of autosomal dominant and recessive forms of sensorineural deafness and hyperproliferative skin disease in humans. This report showed the subcellular localization and function of two GFP-tagged Cx26 point mutants that exhibit both phenotypes, G59A-GFP and D66H-GFP. D66H-GFP was retained within the brefeldin A-insensitive trans-Golgi network, whereas a population of G59A-GFP was transported to the cell surface of Hela cells. Neither G59A nor D66H formed gap junctions, suggesting they are loss-of-function mutations. When co-expressed with wild-type Cx26, both G59A and D66H exerted dominant-negative effects on Cx26 function. G59A also exerted a trans-dominant negative effect on co-expressed wild type Cx32 and Cx43, whereas D66H exerted a trans-dominant negative effect on Cx43 but not Cx32. The authors proposed that the severity of the skin disease is dependent on the specific nature of the Cx26 mutation and the trans-dominant selectivity of the Cx26 mutants on co-expressed connexins (83).

The use of GFP fusion protein technique for study AE1 in human K562 erythroleukemia cells has recently been reported (20). This study also demonstrated that confocal microscopy is a powerful tool for studying the targeting and interactions of recombinant human kAE1 protein. Fluorescence was detected predominantly at the surface of K562 cells transfected with GFP-eAE1 or GFP-kAE1. The two fusion proteins could stably be expressed in K562 cells. Using immunofluorescence staining, flow cytometry, chloride transport assay, they were demonstrated that amino-terminal GFP-tagging does not affect the targeting or chloride transport properties of eAE1 and kAE1. The result showed that eAE1 and kAE1 tagged with GFP expressed at the cell surface. The amount of chloride flux per molecule of K562 cell expressed GFP-eAE1 or GFP-kAE1 was similar to the amount of chloride flux per molecule of red cell band 3. GFP-tagging, therefore, provides a powerful and alternative means of studying the targeting of AE1 mutants in cultured mammalian cells.

Table 3.4 The use of GFP tagging for studying trafficking and function of mutant protein causing human diseases.

Human disease	Protein of interest	Finding	References
Hemochromatosis type IV	Iron exporter ferroportin (Fpn)	Defective in cell surface localization or have a decreased ability to be internalized and degraded in response to hepcidin.	(84)
Congenital long QT syndrome (LQTS)	KCNQ1	Abnormal cellular transport due to the assembly failure in this type of LQTS patient.	(85)
Cystic fibrosis (CF)	Cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel	Demonstration of important molecular site for inhibition or activation of CFTR by genistein.	(66)
Early-onset primary dystonia (DYT1)	Endoplasmic reticulum luminal protein torsinA	Striking redistribution of mutant torsinA from ER to nuclear envelope (NE) and mutant torsinA recruits WT protein to the NE.	(86)
Epidermolysis bullosa simplex (EBS)	Intermediate filament (IF) proteins keratin 5 (K5) and keratin 14 (K14)	Weak viscoelastic properties, and aggregation of mutant protein	(87)
Gerstmann–Straussler–Scheinker disease (GSS)	Prion protein (PrP)	Aberrant mitochondrial localization accompanied by depolarization of mitochondrial innermembrane, cytochrome c release in cytosol, DNA fragmentation, and the formation of numerous PrP-containing deposits in intracellular vacuoles resembling secondary lysosomes.	(88)
Human Tangier disease	ABCA1 transporter	Late endocytic protein trafficking defect	(89)
Huntington's disease (HD)	First exon of huntingtin	Expanded polyglutamine aggregate formation and result in down regulation of specific genes	(90, 91)
Juvenile myoclonic epilepsy (JME)	α_1-subunit of the $GABA_A$ receptor (*GABRA1*)	Severe loss-of-function of human $GABA_A$ receptor by reduced surface expression, reduced GABA-sensitivity, and accelerated deactivation.	(92)
Neurodegenerative disease retinitis pigmentosa	Rhodopsin	Generation of human rhodopsin–GFP knock in mouse for use as a visible marker of rhodopsin expression.	(93)
Sensorineural deafness	Connexins (Cx) Cx26, Cx43	Defective protein trafficking and function.	(83, 94, 95)
Wilson disease	ATP7B	Defective copper ATPase activity of ATP7B.	(96)

3.4 The outline of experimental studies in this thesis.

To examine the expression of wild-type kAE1 and mutant SAO and mutant G701D protein in the mammalian HEK293 cell line by using GFP as a molecular label, the expression vectors containing *GFP* and wild-type *kAE1* cDNA would be constructed and the mutant kAE1 SAO and G701D would be generated by site-directed mutagenesis. The plasmids containing wild-type or mutant *kAE1* cDNA fused with *GFP* would be transfected into HEK293 cells. The expression and localization of kAE1 SAO and kAE1 G701D protein would be compared with those of wild-type kAE1 protein. The wild-type kAE1 would be co-expressed with either kAE1 G701D or kAE1 SAO for studying the effect of the mutant protein on the wild-type protein and *vice versa*. To mimic the condition of compound heterozygous *AE1* G701D and SAO mutations that was found in patients, the kAE1 G701D and kAE1 SAO would be co-expressed in HEK293 cells. The protein expression, subcellular localization, and interaction would be examined by Western blotting and confocal microscopy.

CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals and reagents

The chemicals and reagents used in the experiments in this thesis are listed in the appendix.

4.1.2 Oligonucleotide primers

The oligonucleotide primers for cloning and sequencing are listed in Table 4.1.

(1) Primers for amplification of *GFP* and *kAE1* cloning

Forward and reverse primers, namely GFP *Eco*RV Fp and GFP *Not*I Rp, were designed to contain restriction sites of *Eco*RV and *Not*I, respectively. The primers GFP *Eco*RV Fp and GFP *Not*I Rp were designed and used for amplification of *GFP* from plasmid containing *GFP* gene, pDs-Green. The restriction sites of *Eco*RV and *Not*I were used for cloning the enzyme-digested *GFP* PCR product. Forward and reverse primers, kAE1 *Not*I Fp, and kAE1 *Xho*I Rp, were designed to contain restriction sites of *Not*I and *Xho*I, respectively. The restriction sites of *Not*I and *Xho*I were used for cloning of digested *kAE1* PCR product.

(2) Primers for site-directed mutagenesis

Two complementary pairs of primers, SAO Fp/SAO Rp and G701D Fp/G701D Rp, were designed and used for generating mutant *kAE1* fused with *GFP*, pcDNA3.1-*GFP*-SAO and pcDNA3.1-*GFP*-G701D, respectively from the plasmid containing *GFP* fused with wild-type *kAE1* cDNA as a template.

Table 4.1 Oligonucleotide primers for cloning and sequencing.

Primer name	Sequence (5'→ 3')	Annealing Tm (°C)
GFP *Eco*RV Fp [a]	TCTGCAGAT'ATCATGAGCAAGGGCGAGGAA	60
GFP *Not*I Rp [a]	GTGTCACGC'GGCCGCACTTGTACAGCTCGT	60
kAE1 *Not*I Fp [a]	TCGGC'GGCCGCATGGACGAAAAGAACCAGGA	55
kAE1 *Xho*I Rp [a]	TCGCTCGAGTCACACAGGCATGGCCACTTC	55
SAO Fp[b]	CCTGAGTGACATCACAGATGTCATCTTCATCTACTACTTTGC	60
SAO Rp[b]	GCAAAGTAGATGAAGATGACATCTGTGATGTCACTCAGG	60
G701D Fp[b]	GATGGTTCAAGGGCTCCGACTTCCACCTGGACCTGC	60
G701D Rp[b]	GCAGGTCCAGGTGGAAGTCGGAGCCCTTGAACCATC	60
L5 [c]	TGCTGCCTCCCACCGATGCC	55
R5 [c]	CGAAGGTGATGGCGGGTGAC	55
L9 [c]	GGTGTCCAACTCCTCAGCCC	55
R9 [c]	GGCATGGGTGACGGAACGCA	55

Notes : [a] Primers for amplifications of *GFP* or *kAE1* for cloning.

[b] Primers for site-directed mutagenesis.

[c] Primers for colony screening by PCR.

Underlined sequences are recognition sites of restriction enzymes, indicated in the primer names.

Underlined bases are the mutated bases to be generated.

(3) Primers for colony screening by colony PCR

The forward and reverse primers, L5 and R5, were used for amplification of *kAE1* fragment from the plasmid containing mutant *kAE1* SAO fused with *GFP*, and L9 and R9 for amplification of kAE1 fragment from the plasmid containing mutant *kAE1* fused with *GFP* in *E. coli* colonies.

4.1.3 Enzymes

(1) Restriction endonuclease

The resctriction endonuclease, *Dpn*I (20 U/µl), *Eco*RV (20 U/µl), *Hha*I (10 U/µl), *Not*I (10 U/µl), *Xho*I (20 U/µl), and *Bam*HI (20 U/µl) were purchased from New England Biolabs, Beverly, MA, USA. *Hpa*II (10 U/µl) was purchased from Promega, Madison, WI, USA

(2) Polymerases and other DNA modifying enzymes

Pfu DNA polymerase (3 U/µl), *Pfx* DNA polymerase (2.5 U/µl), and *Taq* DNA polymerase (5 U/µl) were purchased from Promega, Madison, WI, USA. T4 DNA Ligase (1 U/µl) was purchased from USB, USA

(3) Ribonuclease

RNaseA was purchased from USB, USA

4.1.4 DNA and protein markers

(1) DNA markers

λ-*Hin*dIII digested DNA-marker and ϕ-*Xho*I digested-marker were purchased from New England Biolabs, Beverly, MA, USA.

(2) Protein markers

SDS-PAGE molecular weight standards, Broad Range was purchased from Bio-Rad, USA.

4.1.5 Antibodies

(1) Rabbit anti HA antibody was purchased from ZYMED, USA.

(2) Rabbit anti C-terminal AE1 polyclonal antibody was a generous gift from Dr. Joseph R. Casey, Canadian Institutes of Health Research, Membrane Protein Research Group, Departments of Physiology and Biochemistry, University of Alberta, Edmonton, Alberta, Canada.

(3) Mouse anti CD147 was a gift from Dr. Watchara Kasinrerk, Department of Clinical Immunology, Faculty of Associated Medical Scinces, Chiang Mai University.

(4) Donkey anti-rabbit IgG conjugated Cy3 was purchased from Jackson Immuno Research Labolatories, Inc., West Grove, PA, USA.

(5) Alexa Fluor 488 goat anti-mouse IgG was purchased from Molecular Probes, Eugene, Oregon, USA.

4.1.6 Bacterium

Escherichia coli (*E. coli*) strain DH5α was employed as a host cell for cloning recombinant DNA plasmids.

4.1.7 Mammalian cell line

Human embryonic kidney 293 (HEK293) cells maintained in the laboratory at the Division of Medical Molecular Biology were employed as the host cells for expression of kAE1 fusion proteins. HEK293 cells were grown in Dulbecco' s Modified Eagle Medium (DMEM) supplemented with 10% calf serum and 0.5% penicillin and streptomycin (GIBCO) in 5% CO_2 at 37 °C**.**

4.1.8 Plasmid vectors and recombinant plasmids

(1) pcDNA3.1(+)

This plasmid was obtained from Invitrogen, USA. pcDNA 3.1(+) was used as a vector backbone for DNA cloning and expression vector in HEK293 cell. The structure of pcDNA3.1(+) is shown in Figure 4.1.

(2) pDs-Green

The plasmid containing *GFP* gene, pDs-Green, was constructed using pDs as a vector backbone. *GFP* gene in pDs-Green was subcloned from pGreen Lantern-1, Life Technologies, USA. pDs-Green was used as template for ampliflcation of *GFP*

gene by PCR. The structure of pGreen Lantern-1 and pDs-Green are shown in Figures 4.2 and 4.3, respectively.

(3) pcDNA 3-*kAE1*

The plasmid pcDNA3-*kAE1* was a gift from Prof. Dr. Reinhart Reithmeier, Department of Biochemistry, University of Toronto, Canada. This plasmid was used as a template for amplification of *kAE1* cDNA by PCR. The structure of pcDNA3-*kAE1* is shown in Figure 4.4.

4.1.9 Miscellaneous materials

Lipofectin was purchased from Invitrogen, USA. Qiagen Gel Extraction kit, Qiagen MiniPrep kit, and Qiagen PCR purification kit were purchased from Qiagen, Germany. Nitrocellulose transfer filter and SuperSignal West Pico Chemiluminescent Substrate were purchased from PROTRAN, USA, and PIERCE, Germany, respectively.

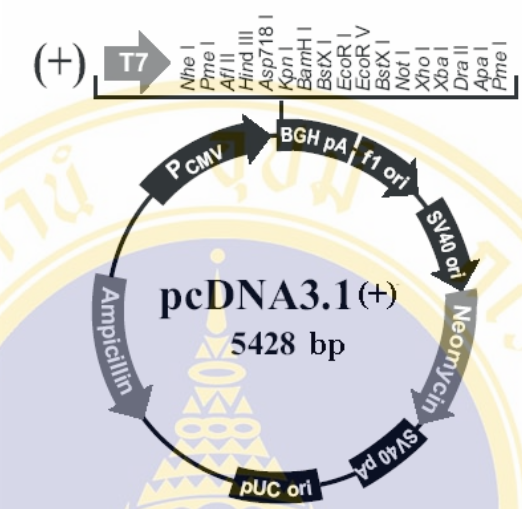


Figure 4.1 Structure of pcDNA3.1(+).

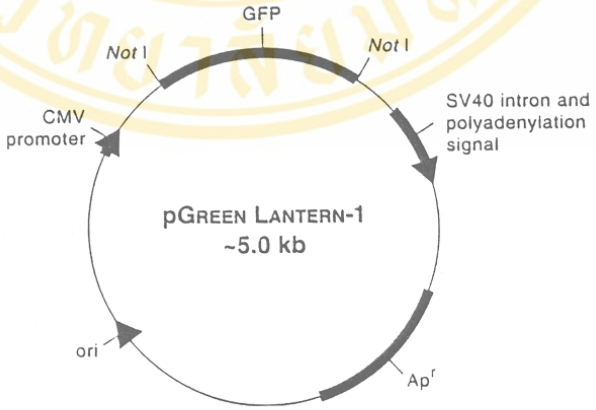


Figure 4.2 Structure of pGreen Lantern-1.

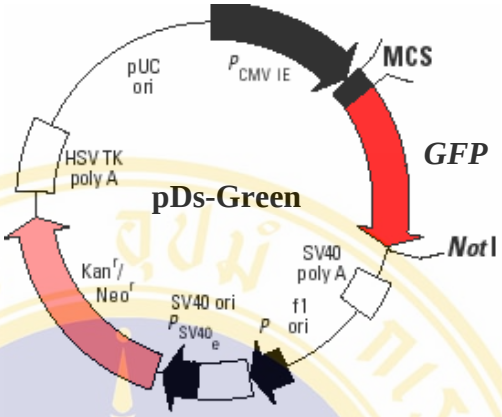


Figure 4.3 Structure of pDs-Green.

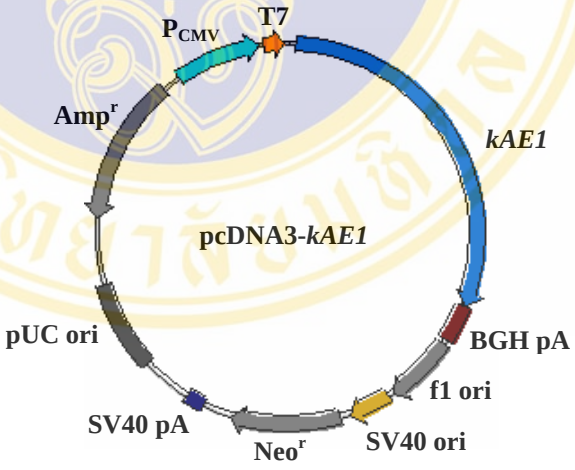


Figure 4.4 Structure of pcDNA3-*kAE1*.

4.2 Methods

4.2.1 Experimental strategy

To study the expression, subcellular localization, trafficking, and interaction of wild-type and mutant kAE1 proteins by using GFP as molecular labels, the expression vector containing *GFP* and *kAE1* cDNA fusions would be constructed. *GFP* and *kAE1* cDNA would be amplified by PCR from plasmid pDs-Green and pcDNA3-kAE1, respectively. *GFP* would be cloned into pcDNA3.1(+) followed by *kAE1* cDNA, just downstream to *GFP*, to yield pcDNA3.1(+) containing *kAE1* tagged with *GFP*, pcDNA3.1-*GFP-kAE1*. *kAE1 SAO* and *kAE1* G701D tagged with *GFP* at N-terminus would be generated by site-directed mutagenesis using wild-type kAE1 clone, pcDNA3.1-*GFP-kAE1*, as a template. Plasmids containing wild-type *kAE1*-hemagglutinin at the position 557 (HA557) and wild-type *kAE1*-HA911 would be used as templates for creation of kAE1 G701D and kAE1 SAO tagged with HA557 and HA911 by site-directed mutagenesis, respectively. The vectors containing either wild-type *kAE1* or *kE1* G701D or *kAE1* SAO fused with *GFP* would be transfected into cultured HEK293 cell lines. The plasmids containing wild-type kAE1 and mutant kAE1 tagged with HA would be used in co-transfection experiment. To examine the expression and localization wild-type kAE1 and mutant SAO and mutant G701D protein, the vectors containing wild-type or mutant *kAE1* cDNA fused with *GFP* gene would be separately transfected into HEK293 cells. To study the effect of the mutant protein on the wild-type protein and *vice versa*, the cells would be co-transfected with the plasmids containing the wild-type *kAE1* tagged with HA and *GFP-kAE1* SAO or *GFP-kAE1* G701D. To mimic the condition of compound heterozygous *AE1* G701D and SAO mutations that was found in patients, the cells would be co-transfected with the plasmids containing *GFP-kAE1* G701D and *kAE1* SAO-HA. Moreover, HEK293 cells stably expressed GFP-kAE1 and GFP-kAE1 G701D protein would be generated. HEK293 cells stably expressing GFP-kAE1 protein would be co-transfected with plasmid containing HA tagged *kAE1*or *kAE1* G701D.or *kAE1* SAO-HA. And, HEK293 cells stably expressing GFP-kAE1 G701D protein would be co-transfected with plasmid containing *kAE1*-HA or *kAE1* SAO-HA. The result from stable expression would be confirmed with transient expression. The protein expression,

subcellular localization, and interaction would be examined by Western blotting and confocal microscopy. The experimental strategy is shown in Figure 4.5.

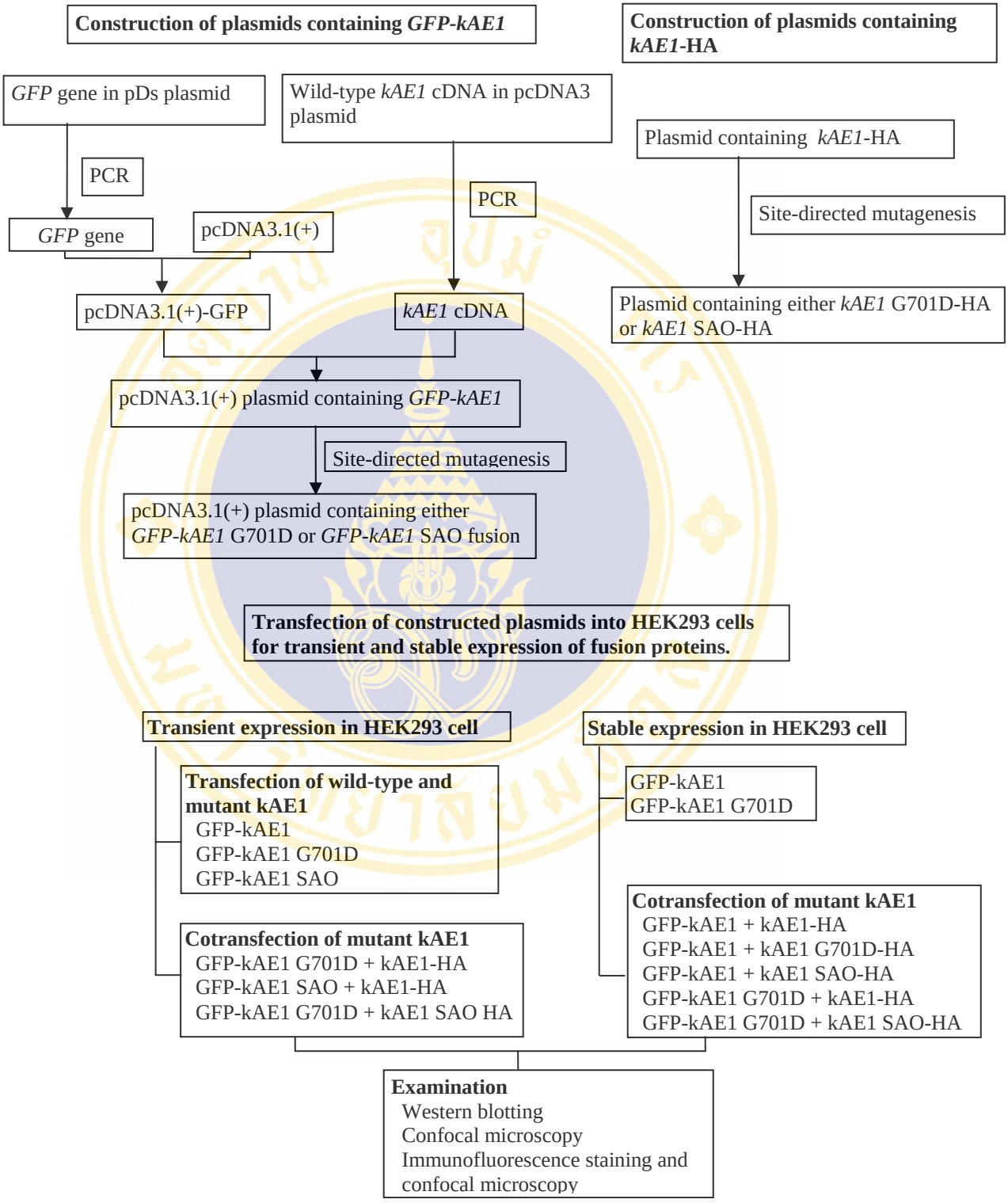


Figure 4.5 Experimental strategy.

4.2.2 Cloning of *GFP* expression vector

(1) Amplification of *GFP* gene by PCR

GFP cDNA (containing S65T mutations) was amplified by PCR from the pDs-Green template. PCR was performed in 50 μl reaction containing 1x PCR buffer, 0.2 mM dNTPs, 12.5 pmole each of GFP *Eco*RV Fp and GFP *Not*I Rp primers, 100 ng of pDs-Green as DNA template for GFP primers and 2 units of *Pfu* DNA polymerase. After mixing and spinning of reaction mixture, PCR reaction was performed in DNA thermal cycling for 35 cycles at 94 °C for 5 min, 55 °C for 1 min and 72 °C for 1 min, followed by final extension at 72 °C for 5 min. Negative control was PCR reaction which lacking DNA template. Purification of PCR product was performed by the QIAquick PCR purification kit (QIAGEN, Germany) following manufacturer's instruction.

(2) Isolation of plasmid

Preparation of plasmid, pcDNA3.1(+), was performed by using QIAGEN plasmid mini kit (QIAGEN, Germany) according to the manufacturer's instruction. Briefly, *E.coli* containing pcDNA3.1(+) was cultured in 5 ml LB broth containing 100 μg/ml of ampicillin for 12-16 h. Bacterial cells were harvested by centrifugation at 5,000 rpm for 10 min at 4 °C and resuspended in 250 μl of buffer P1. The 250 μl of buffer P2 were added into the cell suspension and gently mixed by inverting 4-6 times and incubated at room temperature for 5 min. The 350 μl of chilled buffer P3 were added and mixed immediately by inverting 4-6 times. The cell lysis solution was clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. The clear solution was loaded onto QIAGEN mini column and centrifuged at 12,000 rpm for 30-60 sec. The flow-through was discarded and 750 μl of buffer PE was added into the column. After centrifugation at 12,000 rpm for 30-60 sec, flow-through was discarded and centrifuge for an additional 1 min to remove residual wash buffer. The column was placed on a clean 1.5 ml. microfuge tube. The DNA was eluted with 30-40 μl of buffer EB (10 mM Tris-Cl, pH 8.5), let stand for 1 min, and centrifuged for 1 min. The DNA concentration was estimated by observing the intensity of DNA fragments from the

ethidium bromide stained on agarose gel under UV transluminator comparing with the known amount of standard molecular weight DNA marker.

(3) Restriction endonuclease digestion of GFP PCR products and plasmid DNA

pcDNA3.1(+) and *GFP* PCR product [from (1) and (2)] were separately digested with restriction enzymes, *Eco*RV and *Not*I. The 20 μl reaction volume containing 2 μg of plasmid, or 200 ng of PCR product, 1x reaction buffer NEB3 and 5 units of *Eco*RV and *Not*I were incubated at 37 °C for 12-16 h. The linearized plasmid, pcDNA3.1(+), *GFP* PCR product were purified with QIAquick Gel Extraction kit (QIAGEN, Germany). The concentrations of digested vector and PCR product were determined by comparing with known concentration of standard DNA marker after running on agarose gel electrophoresis.

(4) Cloning of *GFP* gene into expression vector

GFP gene digested with *Eco*RV and *Not*I, was cloned into *Eco*RV/*Not*I cut pcDNA3.1(+) vector (Invitrogen) to generate pcDNA3.1-*GFP* at the molar ratio of 1:2. The ligation reaction was performed in a volume of 20 μl. The ligation reaction consisted of 100 ng of pcDNA3.1 (+), 24 ng of *GFP* gene, 2 units of T4 DNA ligase and 1x ligation buffer. The ligation mixture was incubated in water bath at 16 °C for 16 h. The ligation mixture was added into 100 μl of chilled *E. coli* DH5α competent cells and incubated on ice for 30 min. The mixture was heat shocked at 42 °C for 1 min and then placed on ice for 3-5 min. LB broth medium was added upto 1 ml. and incubated at 37 °C in shaking incubator for 1 h. The mixture was centrifuged at 5,000 rpm for 5 min. The supernatant was removed to leave the volume at 100 μl and the transformed cell pellet was mixed homogeneously. The cell suspension was spreaded on LB plate containing 100 μg/ml ampicillin and incubated at 37 °C for 12-16 h.

(5) Screening of colonies harboring recombinant plasmid by PCR

PCR method was performed by using *EcoR*V and GFP *Not*I primers to screen *E. coli* colonies for positive clones, pcDNA3.1-GFP. After obtaining positive clones, the colonies were selected for plasmid preparation by using QIAprep Spin Miniprep Kit (Qiagen, Germany). The recombinant plasmids were confirmed by *Bam*HI digestions. The digestion reaction was carried out in 12 μl of volume containing 300-500 ng of pcDNA3.1-*GFP*, 5 units of *Bam*HI in an appropriate buffer. The reaction mixture was incubated in waterbath at 37 °C for 3 h. *GFP* gene fragment was detected by agarose gel electrophoresis.

4.2.3 Construction of plasmid containing *GFP* fused with *kAE1* cDNA

(1) Amplification of *kAE1* cDNA by PCR

Human *kAE1* cDNA was amplified from plasmid pcDNA3-*kAE1* by PCR using a pair of oligonucleotide primers, kAE1 *Not*I Fp and kAE1 *Xho*I Rp. The reaction mixture consisted of 100 ng of pcDNA3-kAE1 as a DNA template, 1 unit of *Pfu* polymerase, 10 pmole each of primers. The reaction mixture was performed in DNA Thermal cycling profiles of DNA amplification at 94 °C for 5 min, 55°C for 1 min and 72 °C for 4 min, 35 cycles and final extended at 72 °C for 10 min. *kAE1* PCR product were purified with QIAquick Gel extraction kit (QIAGEN, Germany) following manufacturer's instruction.

(2) Restriction endonuclease digestion of *kAE1* PCR products

The purified *kAE1* cDNA was digested with restriction enzymes, *Not*I and *Xho*I. The digestion reaction was carried out in 25 μl of volume composing of 500 ng *kAE1* cDNA, 5 units of *EcoR*V and 5 units of *Xho*I, 1x buffer NEB3. The reaction mixture was incubated in waterbath at 37 °C for 12-16 h. *kAE1* cDNA was purified with QIAquick Gel Extraction kit (QIAGEN, Germany). The concentrations of digested vector and PCR product were determined by comparing with known concentration of standard DNA markers after running on agarose gel electrophoresis.

(3) Cloning of *kAE1* into *GFP* expression vector

The vector containing *GFP* was subjected to *Not*I - *Xho*I digestions. The *Not*I /*Xho*I digested *kAE1* cDNA was cloned into *Not*I and *Xho*I sites of the digested pcDNA3.1-*GFP*, downstream to *GFP*, to yield the pcDNA3.1-*GFP-kAE1* construct that would encode a GFP-kAE1 fusion protein by linking GFP to the N-terminus of kAE1. pcDNA3.1-*GFP* was ligated with *kAE1* cDNA at the molar ratio of 1:2. The ligation mixture was transformed into competent, *E. coli* DH5α. PCR method was used to screen colonies for positive clones by using kAE1 *Not*I Fp and kAE1 *Xho*I Rp primers. After obtaining positive clones, the colonies were selected for plasmid preparation by using QIAprep Spin Miniprep Kit (Qiagen, Germany). The recombinant plasmid containing *GFP-kAE1* fusion, pcDNA3.1-*GFP-kAE1*, was confirmed by *Not*I and *Xho*I digestions. The reaction was carried out in 12 μl of volume containing 300-500 ng of pcDNA3.1-*GFP kAE1*, 5 units of *Not*I and 5 units of *Xho*I in buffer NEB3. The reaction mixture was incubated in waterbath at 37 °C for 3 h. *kAE1* gene fragment was detected by agarose gel electrophoresis. The sequence of *GFP-kAE1* construct was determined by direct sequencing. This wild-type construct, pcDNA3.1-*GFP-kAE1*, was used as a template for site-directed mutagenesis to generate *GFP*-mutant *kAE1* constructs.

4.2.4 Construction of plasmid containing *GFP* fused with mutant *kAE1* cDNA by site-directed mutagenesis.

Plasmid containing either *kAE1* G701D or *kAE1* SAO tagged at the N-terminus with *GFP* was generated by using pcDNA3.1-*GFP-kAE1* as template, according to the protocol of the QuickChange TM site-directed mutagenesis kit from Stratagene (Invitrogen, USA). pcDNA3.1-*GFP-kAE1* G701D and pcDNA3.1-*GFP-kAE1* SAO were generated by using the pairs of G701D Fp/G701D Rp and SAO Fp/SAO Rp primers, respectively. PCR was performed in 50 μl reaction volume containing 5 μl of 10x *Pfx* DNA polymerase buffer, 0.2 mM dNTPs, 20 pmole each of primers, 1 μl of 2.5 U of *Pfx* DNA polymerase, and distilled water up to 50 μl. PCR profile was 95 °C for 30 s for the first round of the reaction, followed by 18 cycles of 95 °C for 30 s,

55 °C for 1 min and 68 °C for 18 min. The PCR product was digested with 1 μl of *Dpn*I (2U/μl, NEB) at 37°C for 3 h to digest methylated parental DNA template. The 15 μl of digested PCR reaction was transformed into 200 μl of competent DH5α cell. PCR using L5/R5 primers or L9/R9 primers was performed to screen colonies for the putative positive clones. The *kAE1* PCR product from *GFP-kAE1* SAO plasmid was determined by 2% agarose gel electrophoresis, whereas *kAE1* PCR products from *GFP-kAE1* G701D plasmid was detected by digestion with *Hpa*II restriction enzyme. The digested product was detected by 2% agarose gel electrophoresis. Wild-type *kAE1* PCR product could be digested with *Hpa*II while *kAE1* G701D PCR product could not. The positive colonies were grown and plasmids were purified by QIAprep Spin Miniprep Kit (Qiagen, Germany). The mutations were confirmed by sequencing performed by a commercial company, Macrogen (Korea).

4.2.5 Construction of plasmid containing wild-type *kAE1*-HA and mutant *kAE1*-HA

Plasmid containing wild-type *kAE1*-HA557 was a gift from Prof. Dr. Reinhart Reithmeier, Department of Biochemistry, University of Toronto, Canada. The *kAE1* HA557 containing an extracelllular heamagglutinin (HA) epitope inserted at the position 557 was constructed by inserting the nucleotides encoding HA, TAC CCA TAC GAT GTT CCA GAT TAC GCT, at *Xba*I and *Hind*III sites of *kAE1*. Plasmid containing wild-type *kAE1*-HA911 was a gift from Miss. Thitima Keskanokwong, Institute of Molecular Biology and Genetics, Mahidol University (Salaya Campus). HA epitope was inserted after codon 911 of *kAE1*.

Plasmid containing either *kAE1* G701D or *kAE1* SAO tagged with either HA557 or HA911were generated by using pcDNA3-*kAE1*-HA557 and pcDNA3-*kAE1*-HA911 as template, respectively, according to the protocol of the QuickChange TM site-directed mutagenesis kit from Stratagene. pcDNA3-*kAE1* G701D-HA and pcDNA3-*kAE1* SAO-HA, tagged with either HA557 or HA911, were generated by using pairs of G701D Fp/G701D Rp and SAO Fp/SAO Rp primers, respectively. The PCR condition and screening of colonies for positive clones were performed by PCR, following the method that was described for the construction of plasmid containing *GFP* gene fused with mutant *kAE1* cDNA by site-directed mutagenesis.

4.2.6 Agarose-gel electrophoresis

One percent agarose-gel was prepared by completely dissolving agarose powder upon heating in 1x TAE buffer. Each DNA sample was mixed with 1/6 volume of loading dye and loaded into a gel slot under 1x TAE buffer. Electroporesis was performed at 100 volts for appropriate time depend on size of DNA fragment. The gel was stained with ethidium bromide solution for 5 min and destained with distilled water. DNA patterns were visualized and photographed during UV light exposure.

4.2.7 Automated DNA sequencing

The sequencing reaction was performed by using ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Kit and analyzed by ABI PRISM™ 377 DNA sequencer. The reaction mixture was performed in 20 μl of volume containing 5 μl of Big Dye premix (terminator ready mix), 0.32 pmole of a sequencing primer and 500 ng of the reacombinant plasmid. The sequencing reaction was amplified for 25 cycles consisting of 96 °C for 10 sec. 50 °C for 5 sec. and 60°C for 4 min. The sequencing product was precipitated by adding 2 μl of 3 M sodium acetate (NaOAc) pH 4.8 and 50 μl of cold absolute ethanol, and kept on ice for 10 min. The pellet was collected by centrifugation at 12,000 rpm for 15 min and washed twice with 70% ethanol. After centrifugation, the supernatant was discarded and the pellet was dried at room temperature. The pellet was resuspended in 25 μl of template suppression reagent (TSR), heated at 95 °C for min to denature the sequencing product, followed by chilling on ice and analyzed by automated DNA sequencer ABI PRISM™ 377. The DNA sequencing was performed by Macrogen (Korea).

4.2.8 Transient expression of fusion proteins in cultured HEK 293 cell line

(1) Cell culture

HEK293 cells were grown in Dulbecco's modified Eagle' medium (DMEM) supplemented with 10 % fetal bovine serum (PERBIO), 100 U/ml penicillin and 100 ug/ml streptomycin (complete medium). The cell culture was maintained in 25-cm^2 flask at 37 ºC with 5% CO_2. Cells were subcultured twice per week following standard trypsinization protocol. Briefly, 100% confluent cells in flask was washed with 5 ml of PBS and then treated with 2 ml of 1%Trypsin in 2.5 mM EDTA/PBS solution for 3-5 min at 37 ºC or until the cells were detached. Pre-warmed complete medium at the volume of 5 ml was added into the flask. The cells were split by pipetting up-down twice. For routine subculture, 0.3-0.5 ml of cell suspension was placed into 5 ml fresh complete medium in a 25-cm^2 flask. The flask of cells was incubated normally at 37 ºC with 5% CO_2.

To maintain long term stocks of cell lines, they must be kept frozen in liquid nitrogen. Cells were subcultured as previously describe and 5 ml of cell suspension was transferred to 50 ml centrifuge tube and spin at 800 rpm for 5 min. After medium was removed, the cell pellet was resuspended with 1 ml ice-cold cell freezing medium (40% FBS and 10% DMSO in DMEM) and then transferred to cryovial. This cryovial was placed in pre-chilled isopropanol containing rack at -80 ºC for 16-24 h and transferred to keep in liquid nitrogen tank. Whenever the stock of cell line kept in liquid nitrogen was needed, it was recovered by removing the vial from the liquid nitrogen tank and placing immediately in 37 ºC water bath. When the cells were thawed, outside of the vial was sprayed with 70% ethanol, wiped off, and then the content was pipetted into a new flask containing fresh medium. The cells were cultured in 37 ºC incubator with 5% CO_2 until confluent and passaging or subculturing process were carried out again.

(2) Transfection of constructed vectors into cell lines

The day before transfection, the cells were collected by trypsinization and seeded in 6-well plates. DEAE-Dextran or Lipofectin method was used to transiently transfect the cells.

(2.1) DEAE – Dextran transfection

HEK293 cells were grown in 6-well plate for a day. 50% confluent cells were transfected with 1 μg of a recombinant plasmid. The transfection mixture consisted of 1 μg of recombinant plasmid, 10 μl of 10mM chloroquine, 50 μl of 10 mg/ml DEAE-Dextran, and serum-free DMEM in a total volume of 1 ml. The mixture was overlaid onto cells and incubated for 3 h. After incubation, cells were shocked with 10% DMSO in serum-free DMEM for 1 min, and then washed with PBS. After that transfected cells were cultured in complete medium with 5% CO_2 at 37 ºC for 48 h. The expression of protein in transfected cells was determined by Western blot.

(2.2) Lipofectin transfection

HEK293 cells were grown in 6-well plate containing round cover glass class 1 (Menzel-Giaser; diameter 12 mm) for a day. 40-60% confluent cells were transfected by using Lipofectin (Invitrogen, USA) according to the manufacturer's protocol for transient transfection of adherent cells. For each transfection, 2-5 μg of recombinant plasmid were diluted into 250 μl of serum-free medium and 5 μl of 1 mg/ml Lipofectin reagent were diluted into 100 μl serum-free medium, and thus were allowed to stand at room temperature for 30-45 min. The solution of plasmid was combined with solution of Lipofectin reagent, mixed gently, and incubated at room temperature for 10-15 min. Thereafter 650 μl of serum-free medium were added into each tube containing the Lipofectin reagent-DNA complexes, mixed gently and the complex was overlaid onto the cells that had been washed once with 2 ml of serum-free medium. After incubation for 5-24 h at 37 °C in a CO_2 incubator, DNA-containing medium were replaced with 2 ml of complete medium in 5% CO_2 at 37 ºC for 48-72 h. The localization of fusion protein in transfected cells were determined by using immunofluorescence method at 48-72 h post transfection.

4.2.9 Stable expression of GFP- kAE1 fusion protein in HEK 293 cell

HEK293 cells were transfected with either pcDNA3.1-*GFP-kAE1* or pcDNA3.1-*GFP-kAE1* G701D using Lipofectin reagent. Two days later, the transfected cells were subcultured into complete medium containing 1 mg/ml of

geneticin (G418, Sigma, St. Louis, MO) and maintained in this medium for 14 days. To select clones stably expressing GFP fused with kAE1, single colony of geneticin-resistant transfectants were then picked and subcultured into 24-well plates and maintained in the presence of 1 mg/ml of geneticin for 7 days. The stable clones were identified by confocal laser scanning microscopy. The cells were maintained and subcultured in complete medium containing 1 mg/ml of geneticin according to the protocol previously described.

4.2.10 Determination of fusion protein expression in cell line by Western blot analysis

Two days after transfection, the transiently transfected HEK293 cells in 6-well plate were washed once with 1 ml of PBS. After that the cells were resuspended with 1 ml of PBS and transferred to 1.5 ml microcentrifuge tube and centrifuged at 2,000 rpm for 5 min. The cells were lysed with 200 µl of PBS containing 1% $C_{12}E_8$ and protease inhibitors (100 µM PMSF, 1 µM aprotinin, 1 µM leupeptin, and 1 µM pepstatin) on ice for 30 min. After centrifugation at 3,000 rpm for 5 min, protein samples were subjected to electrophoresis on 8% SDS-PAGE, and transferred to nitrocellulose membranes. Membrane were blocked for 1 h in 5% skim milk in TBST (TBS with 0.1% Tween 20) and then incubated with rabbit anti-Ct AE1 antibody at 1: 5,000 dilution in TBST containing 0.5% skim milk for 2 h. After washing 3 times for 5 min each in TBST, the membranes were incubated with 1: 1,000 goat anti-rabbit antibody conjugated to horseradise peroxidase for 1 h. After washing 3 times in TBST, specific proteins were detected by ECL plus Western Blotting Detection System for 5 min according to manufacturer's instruction. To detect a chemiluminescence, the membrane was exposed with X-ray film in cassette for 1 min.

4.2.11 Examination of GFP fusion protein in the transfected cells

To detect the expression of GFP-tagged kAE1 in transfected cells, cover glasses were washed once with PBS and fixed with 4% paraformaldehyde for 1 h at room temperature. After washing 3 times with PBS, cover glasses were mounted with Antifade and detected by a Zeiss LSM510 META confocal microscope. Green fluorescence was visualized by excitation at 488 nm and emission was monitored at 520 nm. Percentage of cell surface expression of GFP-kAE1 fusion protein comparing to its total expression was estimated from numbers of green color pixels by using histogram function of software controlling the confocal microscope.

4.2.12 Examination of HA fusion protein in the transfected cell by immunofluorescence

To detect the expression of HA-tagged kAE1 in transfected cells, the transiently transfected HEK293 cells were washed with DMEM serum-free medium. The cells were fixed with 4% paraformadehyde in PBS at room temperature for 1 h. After washing with 100 mM glycine in PBS for 5 min, the cells were permeabilized in 0.2% Triton X-100 for 15 min. The cells were washed 3 times with PBS for 5 min each. Nonspecific binding was blocked with 1 % BSA in PBS for 30 min, followed by further washing with PBS. The cells were incubated with primary antibody (1: 1,000 dilution of a rabbit anti-HA antibody or with a 1:50 dilution of mouse anti-CD147 antibody) in PBS containing 1% BSA for 1 h at room temperature. After washing 3 times with PBS, the cells were incubated with secondary antibody (1: 8,000 dilution of Cy3-conjugated donkey anti-rabbit IgG or 1:250 dilution of goat anti-mouse Alexa 488-conjugated IgG) for 30 min at room temperature. The cells were washed 3 times with PBS and mounted with Antifade, the expression of protein was examined by Zeiss LSM510 META confocal microscope. Green fluorescence was visualized by excitation at 488 nm and emission was monitored at 520 nm, while red fluorescence was visualized by excitation at 543 nm and emission was monitored at 560 nm. Percentage of cell surface expression of GFP- and HA-tagged kAE1 proteins could similarly be estimated from numbers of their color pixels as described above.

CHAPTER V
RESULTS

5.1 Cloning of *GFP* into pcDNA3.1 (+) expression vector

GFP gene was amplified from the plasmid containing *GFP* gene, pDs-Green, by PCR using oligonucleotide primers (GFP *Eco*RV Fp and GFP *Not*I Rp) to introduce recognition sites for restriction enzymes, *Eco*RV and *Not*I, to the 5' and 3' ends of the PCR protduct, respectively. The *GFP* PCR product with the size of 714 bp (Figure 5.1) was digested with a combination of *Eco*RV and *Not*I to generate the fragment comprising 701 bp. This fragment was purified and then ligated into *Eco*RV/*Not*I digested pcDNA3.1(+) vector to generate pcDNA3.1-*GFP*, 6.1 kb. The recombinant clones were screened by PCR method using GFP *Eco*RV Fp and GFP *Not*I Rp primers from the transformant colonies. The recombinant clones that showed the PCR product with the size of 714 bp, corresponding to the expected size, were selected (Figure 5.2). Ten clones containing pcDNA3.1-*GFP* were obtained and subjected to plasmid preparation for confirmation by *Bam*HI digestions; *Bam*HI could digest at one position in the vector and another position in *GFP* generating two fragments with the sizes of 5.5 kb and 600 bp. After the digestions, all ten clones gave two DNA fragments with the sizes of 5.5 kb and 600 bp (Figure 5.3), indicating the presence of *GFP* gene in the pcDNA3.1(+) vector. Three out of ten positive clones containing pcDNA3.1-*GFP* plasmid were selected for plasmid preparation and use for cloning wild-type kAE1, downstream and fused to *GFP*.

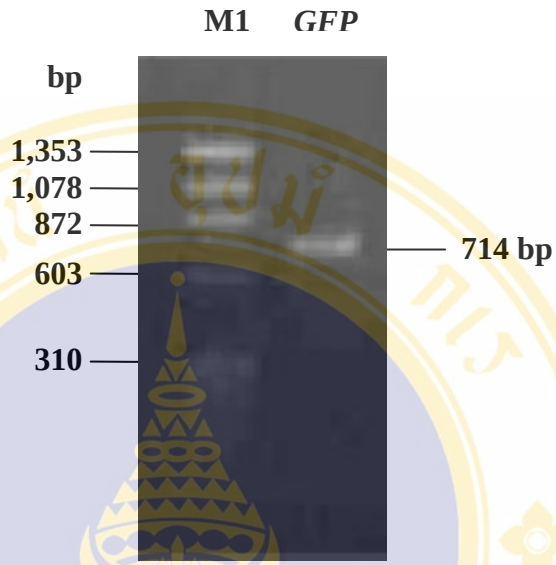


Figure 5.1 **Amplification of *GFP* gene.**
GFP PCR product, 714 bp, was generated using pDs-Green as template and GFP *Eco*RV Fp and GFP *Not*I Rp as amplifying primers. Lane M was *Hae*III-digested ϕX174 DNA markers.

Figure 5.2 **Screening of pcDNA3.1-*GFP* plasmid from transformant colonies by PCR.**

PCR screening of colonies containing pcDNA3.1-*GFP* using GFP *Eco*RV Fp and GFP *Not*I Rp primers to amplify *GFP* fragment. Lane M is *Hae*III-digest ϕX174 DNA markers. Lanes 1-10 show the *PCR* product of *GFP* with the size of 714 bp amplified from pcDNA3.1-*GFP* transformant colonies, numbers 5-15. Lane 11 is a positive control of *GFP* fragment amplified from pDsGreen.

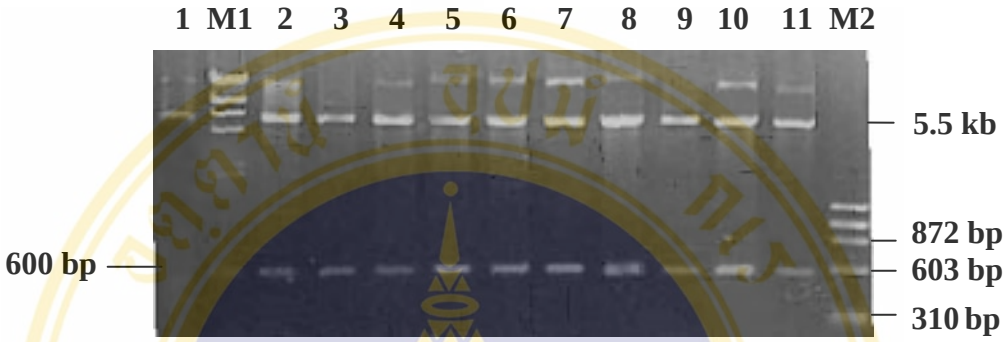


Figure 5.3 Screening of pcDNA3.1-*GFP* by restriction endonuclease digestion. The colonies positive for pcDNA3.1-*GFP* screening by PCR were subjected to plasmid preparation to confirm by *Bam*HI digestions. Lane 1 is an empty pcDNA3.1(+) vector. Lane M1 is *Hind*III-digested λ DNA markers. Lanes 2-11 were pcDNA3.1-*GFP* from clone numbers 5-15. The pcDNA3.1-*GFP* plasmids were digested by *Bam*HI into two fragments, 5.5 kb (upper band) and 600 bp (lower band). Lane M2 is *Hae*III-digest ϕX174 DNA markers.

5.2 Construction of plasmid containing *GFP* fused with *kAE1* cDNA

Wild-type *kAE1* cDNA was amplified from pcDNA3-*kAE1* (a gift from Prof. Dr. Reinhart Reithmeier, Department of Biochemistry, University of Toronto, Canada) by PCR using kAE1 *Not*I Fp and kAE1 *Xho*I Rp primers. *kAE1* PCR product, 2.5 kb, (Figure 5.4) were digested with *Not*I and *Xho*I and was purified by Qiagen PCR purification kit. The *kAE1* fragment was cloned into pcDNA3.1-*GFP* to generate the plasmid containing *GFP* fused with *kAE1*, pcDNA3.1-*GFP-kAE1* (Figure 5.5). After transformation, PCR method was used as a preliminary screening the transformant colonies. Five clones containing the recombinant plasmid that could be amplified for *kAE1* with the PCR products of 2.5 kb (Figure 5.6) were selected for further screening. pcDNA3.1-*GFP-kAE1* plasmids were then extracted from these five clones and analyzed by restriction endonuclease digestions with either *Not*I and *Xho*I or *Eco*RV and *Not*I. When digested with *Not*I and *Xho*I, all five clones gave two DNA fragments, 6.1 kb and 2.5 kb, for the plasmid vector and *kAE1* fragment. These five recombinant plasmids were digested with *Bam*HI, which could digest at one position each in the plasmid, *GF*, and *kAE1* cDNA, generating three fragments with the sizes of 7.49 kb, 560 bp, and 550 bp, respectively. All five clones contained the recombinant plasmids that could be digested into the three fragments (7.49 kb, 560 bp, and 550 bp), indicating the presence of *GFP* and *kAE1* cDNA in the recombinant plasmids. Figure 5.7 shows the result of restriction endonuclease digestions of pcDNA3.1-*GFP-kAE1* from clone number 22. This recombinant plasmid was used as a template for site-directed mutagenesis to generate the plasmid containing *GFP* fused with mutant *kAE1*.

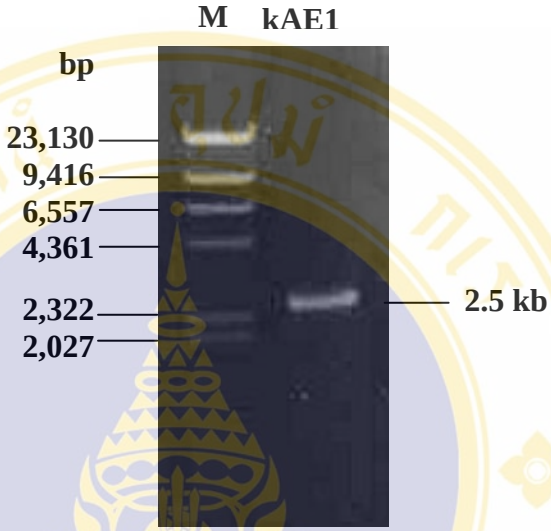


Figure 5.4 Amplification of human *kAE1* cDNA.

Human *kAE1* cDNA was amplified from plasmid pcDNA3-*kAE1* with kAE1 *Not*I (Fp) and kAE1 *Xho*I (Rp) primers. Lane M is *Hind*III-digested λ DNA markers. *kAE1* is amplified to produce the size of 2.5 kb.

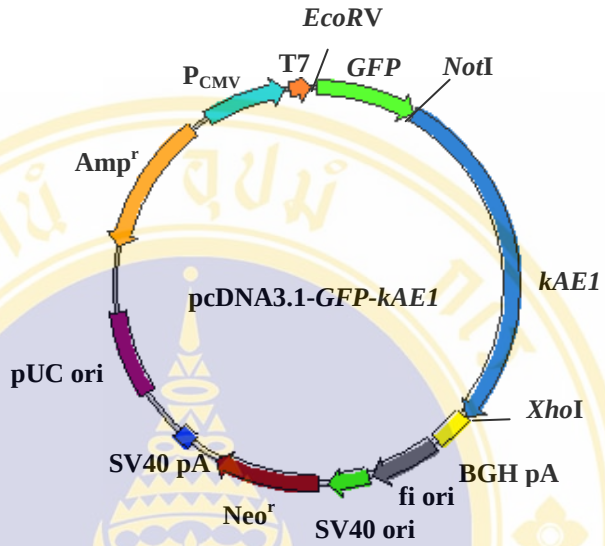


Figure 5.5 **Physical map of the constructed plasmid, pcDNA3.1-*GFP-kAE1*, for expression of kAE1 tagged with GFP in HEK 293 cells.**

Plasmid pcDNA3.1(+) was inserted with *GFP* and *kAE1* cDNA.

The recombinant protein generated from this plasmid will have *GFP* fused to *kAE1* at the N-terminus.

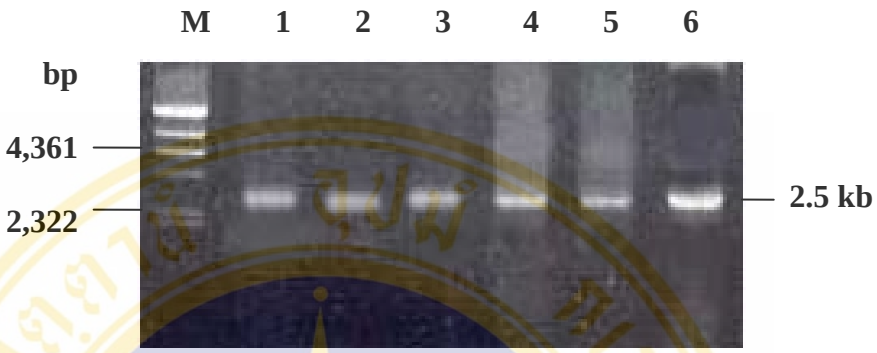


Figure 5.6 Screening of colonies containing pcDNA3.1-*GFP-kAE1* plasmid by PCR method.

PCR reactions were performed by using the lysed transformant colonies and kAE1 *Not*I Fp/kAE1 *Xho*I Rp primers. Lane M is *Hind*III-digested λ DNA markers. Lanes 1-5 are PCR products from clone numbers 22-26. Lane 6 is a positive control using pcDNA3-*kAE1* as template for PCR.

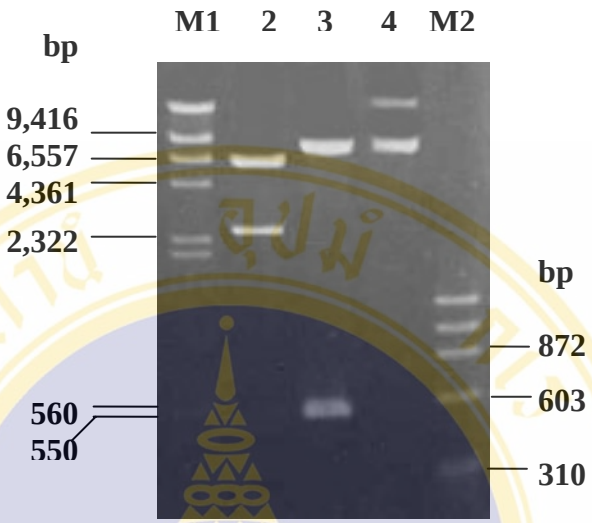


Figure 5.7 **Restriction endonuclease digestion of pcDNA3.1-*GFP-kAE1*.** Purified pcDNA3.1-*GFP-kAE1* plasmid was digested with either *Not*I and *Xho*I (lane 2), or *Bam*HI (lane 3). *Not*I and *Xho*I digested the recombinant plasmid into two fragments, 6.1 kb and 2.5 kb, for the vector and *kAE1* fragment, respectively. *Bam*HI digested at one position each in the vector, *GFP*, and *kAE1* cDNA, generating three fragments with the sizes of 7.49 kb, 560 bp, and 550 bp, respectively, indicating the presence of *GFP* and *kAE1* cDNA in the recombinant plasmids. The fragments with the sizes of 560 and 550 bp located close together and were seen as a single band. Lane 4 is uncut pcDNA3.1-*GFP-kAE1*. Lane M1 is *Hind*III-digested λ DNA markers. And, lane M2 is *Hae*III-digested ϕX174 DNA markers.

5.3 Construction of plasmid containing *GFP* fused with mutant *kAE1* cDNA by site-directed mutagenesis

Plasmids containing *GFP-kAE1* G701D and *GFP-kAE1* SAO fusions were generated by using pcDNA3.1-*GFP-kAE1* as template, according to the protocol of the QuickChange TM site-directed mutagenesis kit from Stratagene. After transformation, the PCR method using L9/R9 and L5/R5 primers were performed for screening the positive clones containing *kAE1* G701D and *kAE1* SAO, respectively. To differentiate the wild-type *kAE1* and *kAE1* G701D amplified by using L9/R9 primer pair, the PCR products with the size of 290 bp were digested with *Hpa*II. *Hpa*II could digest the PCR product from the wild-type *kAE1* but could not digest the PCR product from *kAE1* G701D at the mutated site (it could digest at one site outside the mutated site, generating fragments with the sizes of 270 and 20 bp). The PCR product from wild-type *kAE1* was digested with *Hpa*II at two positions giving three fragments with the sizes of 164, 106, and 20 bp (Figure 5.8). However, sometimes the digestion of the PCR product from the wild-type *kAE1* might be partially incomplete, generating the fragment of 270 bp. Figure 5.8 shows an example of *Hpa*II digestion of the PCR product from pcDNA3.1-*GFP-kAE1* G701D obtained from clone number 19. For screening of the clones containing *GFP-kAE1* SAO by PCR using L5/R5 primer pair, the PCR products from the wild-type *kAE1* (318 bp) and *kAE1* SAO with a 27 bp-deletion (291 bp) could be differentiated by agarose gel electrophoresis. The PCR product with a shorter fragment of *kAE1* SAO moved faster on the electrophoresis. Figure 5.9 shows different mobilities of the PCR products amplified from pcDNA3.1-*GFP-kAE1* SAO obtained from clone numbers 12 and 19, comparing to that from pcDNA3.1-*GFP-kAE1*. From these screenings, four positive clones containing pcDNA3.1-*GFP-kAE1* G701D and ten positive clones containing pcDNA3.1-*GFP-kAE1* SAO were obtained.

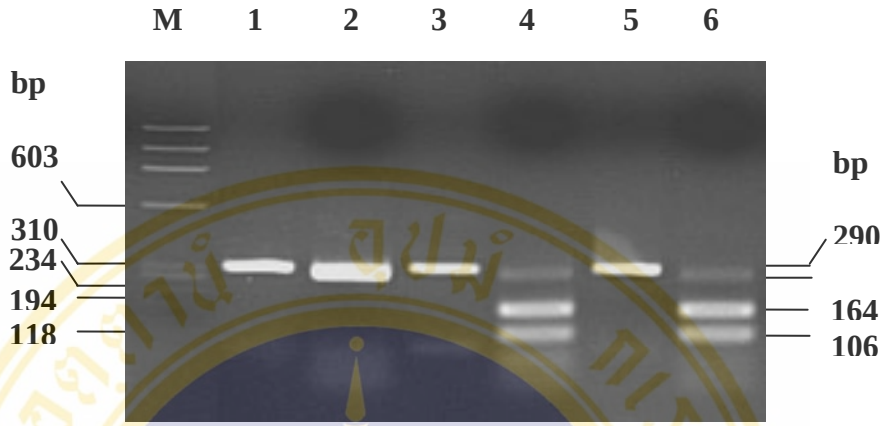


Figure 5.8 Agarose gel electrophoresis of *Hpa*II digested k*AE1* amplified by PCR from pcDNA3.1-*GFP-kAE1* G701D generated by site-directed mutagenesis.

kAE1 was amplified by PCR using L9/R9 primers and digested with *Hpa*II. The undigested PCR was 290 bp. The PCR product of *kAE1* G701D could not be digested with *Hpa*II at the mutated site, but be digested at one site, generating the fragments with sizes of 270 and 20 bp (the latter is undetectable). The PCR product from the wild-type kAE1 could be digested with *Hpa*II at two positions giving product sizes of 164, 106, and 20 bp (the latter is undetectable). A partially incomplete *Hpa*II digestion of wild-type kAE1 product might occur and generate a fragment with the size of 270 bp. Lane M is *Hae*II digested ϕX174 DNA markers. Lanes 1 and 2 are undigested and digested PCR products amplified from pcDNA3.1-*GFP-kAE1* G701D obtained from clone number 19 generated by the site-directed mutagenesis. Lanes 3 and 4 are undigested and digested PCR products amplified from the original plasmid, pcDNA3.1-*GFP-kAE1*. Lanes 5 and 6 are undigested and digested PCR products amplified from the positive control plasmid, pcDNA3-*kAE1*.

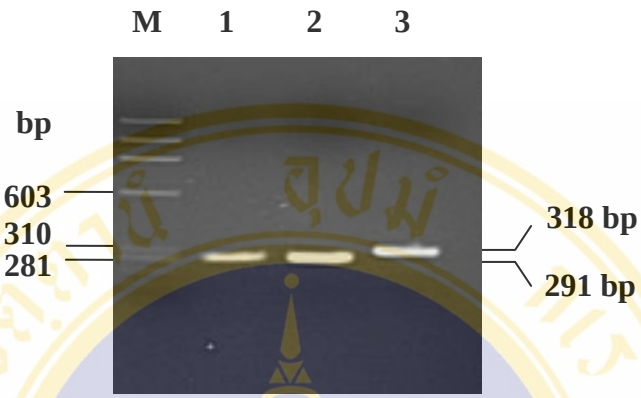
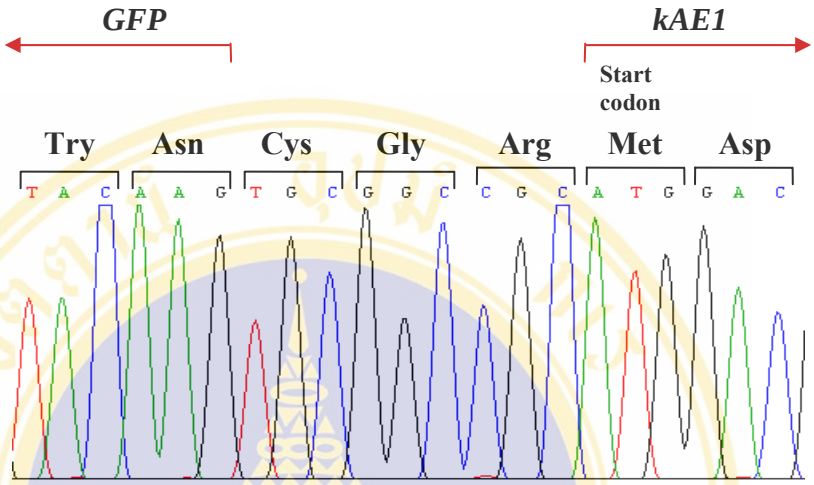


Figure 5.9 **Agarose gel electrophoresis of PCR products of k*AE1* amplified by PCR from pcDNA3.1-*GFP-kAE1* SAO, generated by site-directed mutagenesis.**

kAE1 was amplified by PCR using L5/R5 primers and the PCR products were examined on electrophoresis using 2% agarose gel. Lane M is *Hae*III-digested ϕX174 DNA markers. Lanes 1 and 2 are PCR products of *kAE1* SAO amplified from pcDNA3.1-*GFP-kAE1* SAO obtained from clone numbers 12 and 19, generated by site-directed mutagenesis. Lane 3 is the PCR product of *kAE1* of a positive control amplified from pcDNA3-*kAE1*.

5.4 Sequence analyses of *GFP-kAE1* and *GFP-kAE1* mutants in the recombinant plasmids

To examine the nucleotide sequence of *GFP-kAE1* in the recombinant plasmid, the entire region of *GFP-kAE1* in pcDNA3.1-*GFP-kAE1* obtained from clone number 22 was analyzed by DNA sequencing. The result showed that the nucleotide sequence of the entire region of *GFP-kAE1* was all correct. Figure 5.10 shows a chromatogram of DNA sequence in the *GFP-kAE1* fusion region. The pcDNA3.1-*GFP-kAE1* plasmid was used as a template for site-directed mutagenesis to generate pcDNA3.1-*GFP-kAE1* G701D and pcDNA3.1-*GFP-kAE1* SAO mutant clones. The entire *kAE1* region of *kAE1* G701D (from clone number 19) and *kAE1* SAO (from clone number 19) were then analyzed by DNA sequencing. Both recombinant plasmids harbored the *kAE1* G701D and *kAE1* SAO mutations as expected. Figure 5.11 shows chromatograms of DNA sequences in the mutated regions, generated by site-directed mutagenesis, in comparison with that in the corresponding regions of wild-type *kAE1*. pcDNA3.1-*GFP-kAE1* contains a CGG at codon 701 for glycine (Figure 5.11 A) and pcDNA3.1-*GFP-kAE1* G701D comprises a CAG at codon 701 for aspartic acid as designed (Figure 5.11 B). While the chromatogram of the wild-type *kAE1* from pcDNA3.1-*GFP-kAE1* (Figure 5.11 C) maintains DNA sequence in the region of 27 nucleotides encoding for nine amino acids residues 400-408, pcDNA3.1-*GFP-kAE1* SAO harbors a deletion of 27 nucleotides encoding for nine amino acids (residues 400-408) (Figure 5.11 D) as designed.



pcDNA3.1-*GFP-kAE1*

Figure 5.10 Partial sequencing profile showing nucleotide sequence in the junction region of *GFP-kAE1* in pcDNA3.1-*GFP-kAE1* plasmid.

The entire regions of *GFP* and *kAE1* were analyzed by DNA sequencing and found to be all correct. The chromatogram in this figure shows the junction region between *GFP* and *kAE1*. The 3' terminus of *GFP* is fused to the 5' terminus of *kAE1*, separated by 9 nucleotides (encoding 3 amino acid residues). The partial sequences of *GFP* and *kAE1* are indicated. The sequences of *GFP* and *kAE1* are in the right reading frame.

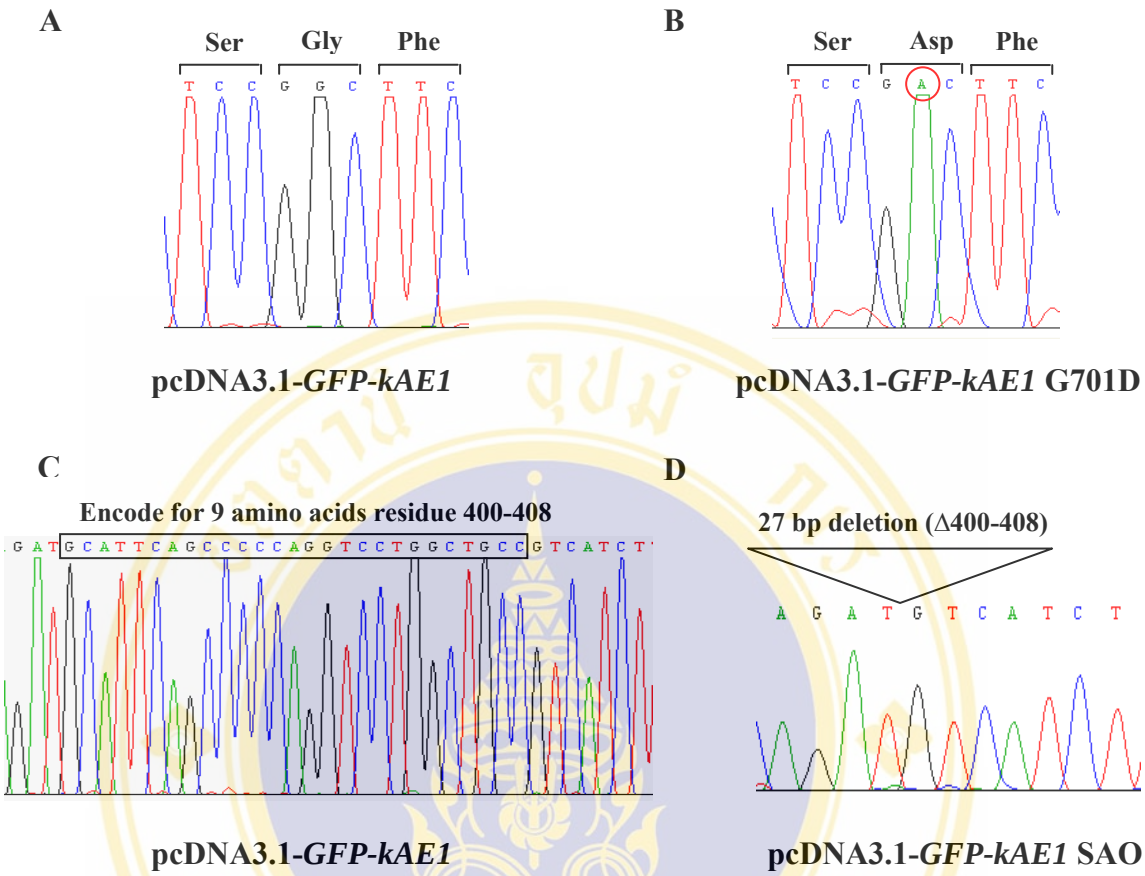


Figure 5.11 Partial sequencing profiles in the mutated regions of pcDNA3.1-*GFP kAE1* G701D and pcDNA3.1-*GFP-kAE1* SAO.

A. The nucleotide sequence of pcDNA3.1-*GFP-kAE1* in *kAE1* region corresponding to the mutated region in B. The codon 701 is GGC encoding for glycine.

B. The nucleotide sequence of pcDNA3.1-*GFP-kAE1* G701D in the mutated region of *kAE1*. The codon 701 is changed to GAC encoding for aspartic acid.

C. The nucleotide sequence of pcDNA3.1-*GFP-kAE1* in *kAE1* region corresponding to the mutated region in D. The sequence in the region of codons 400-408 is maintained.

D. The nucleotide sequence of pcDNA3.1-*GFP-kAE1* SAO in the mutated region of *kAE1*. The sequence in the region of codons 400-408 is deleted.

5.5 Determination of GFP-kAE1 fusion protein transiently expressed in HEK293 cells by Western blot analysis

To examine the expression of GFP-kAE1 fusion protein in HEK293 cells, 5 μg each of pcDNA3-*kAE1*, pcDNA3.1-*GFP-kAE1*, pcDNA3.1-*GFP-kAE1* G701D and pcDNA3.1-*GFP-kAE1* SAO were individually transfected in HEK293 cells by using DEAE-Dextran transfection protocol. The protein expression were examined by Western blot analysis using anti-C-terminal AE1 (anti-Ct AE1) antibody. The HEK293 with mock transfection was used as control and it was found that there was no endogenous kAE1 expression in HEK293 cells. The cells transfected with pcDNA3-*kAE1* expressed kAE1 protein with the molecular weight (MW) of ~82 kDa. The calculated MW of GFP-kAE1 and GFP-kAE1 G701D fusion proteins is ~109 kDa, and that of GFP-kAE1 SAO is ~106 kDa. The cells transfected with pcDNA3.1-*GFP-kAE1*, pcDNA3.1-*GFP-kAE1* G701D, and pcDNA3.1-*GFP* kAE1 SAO expressed the fusion proteins with MW of ~110 kDa, close to the calculated MWs (Figure 5.12).

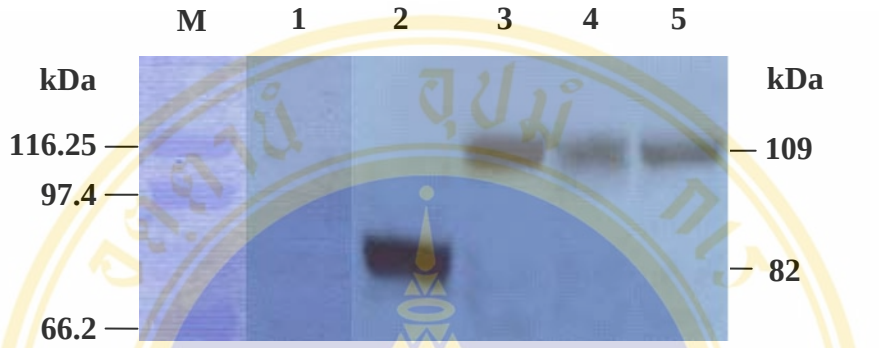


Figure 5.12 Western blot analysis of kAE1, GFP-kAE1, GFP-kAE1 G701D, and GFP-kAE1 SAO proteins expressed in HEK 293 cells.

The kAE1 and GFP-kAE1 fusion proteins expressed in transfected HEK293 cells were analyzed by Western blot method using anti-Ct AE1 antibody. Lane M is the broad range protein molecular weight standard. Lane 1 is the lysate from HEK293 with mock transfection. Lane 2 is kAE1 expressed from the cells transfected with pcDNA3-*kAE1*. Lane 3 is GFP-kAE1 fusion protein expressed from the cells transfected with pcDNA3.1-*GFP-kAE1*. Lane 4 is GFP-kAE1 G701D fusion protein expressed from the cells transfected with pcDNA3.1-*GFP-kAE1* G701D. And, lane 5 is GFP-kAE1 SAO fusion protein expressed from the cells transfected with pcDNA3.1-*GFP-kAE1* SAO.

5.6 Examination of expression and cellular localization of GFP-kAE1 fusion protein transiently expressed in HEK293 cells

To examine expression and cellular localization of GFP fused with the wild-type kAE1 (GFP-kAE1) and mutant kAE1 (GFP-kAE1 G701D and GFP-kAE1 SAO) in HEK293 cells, 5 μg each of pcDNA3.1-*GFP-kAE1*, pcDNA3.1-*GFP-kAE1* G701D, and pcDNA3.1-*GFP-kAE1* SAO were individually transfected into HEK293 cells by using Lipofectin transfection protocol. After two days of transfection, the cells were examined under Zeiss LSM 510 META confocal microscope. The expression and cellular localization of the three fusion proteins could directly be examined for green fluorescent signal generated from GFP fused to the wild-type and mutant kAE1 proteins by confocal microscope at the excitation wave length 488 nm and emission wave length at 520 nm. To also locate the surface of HEK293 cells, a cell surface marker-CD147 (β-subunit of Na^+/K^+-ATPase) was stained by the immunofluorescence method using mouse anti-CD147 antibody, followed by goat anti-mouse IgG antibody conjugated with Cy3, showing red fluorescent signal at the excitation wave length 543 nm and emission wave length at 560 nm. As shown in Figure 5.13, HEK293 cells transfected with either pcDNA3.1-*GFP-kAE1* G701D or pcDNA3.1-*GFP-kAE1* SAO demonstrated markedly different expression and cellular localization patterns of the fusion proteins from that transfected with pcDNA3.1-*GFP-kAE1*. In addition to cytoplasmic expression, the GFP-kAE1 fusion protein was predominantly expressed at the cell surface which was co-localized with the surface marker - CD147 (Figure 5.13 A-C). In contrast, HEK293 cells transfected with either pcDNA3.1-*GFP-kAE1* G701D or pcDNA3.1-*GFP-kAE1* SAO showed predominantly intracellular localization without the intense cell-surface expression, as demonstrated for the surface marker-CD147 (Figure 5.13 D-F and 5.13 G-I). The cell surface expression of GFP-kAE1, GFP-kAE1 G701D, and GFP-kAE1 SAO proteins as estimated from green color pixels on the cell surface comparing to total green color pixels were approximately 53.5±2.5 %, 4.5±1.5%, 6.9±7.6%, respectively (Table 5.1).

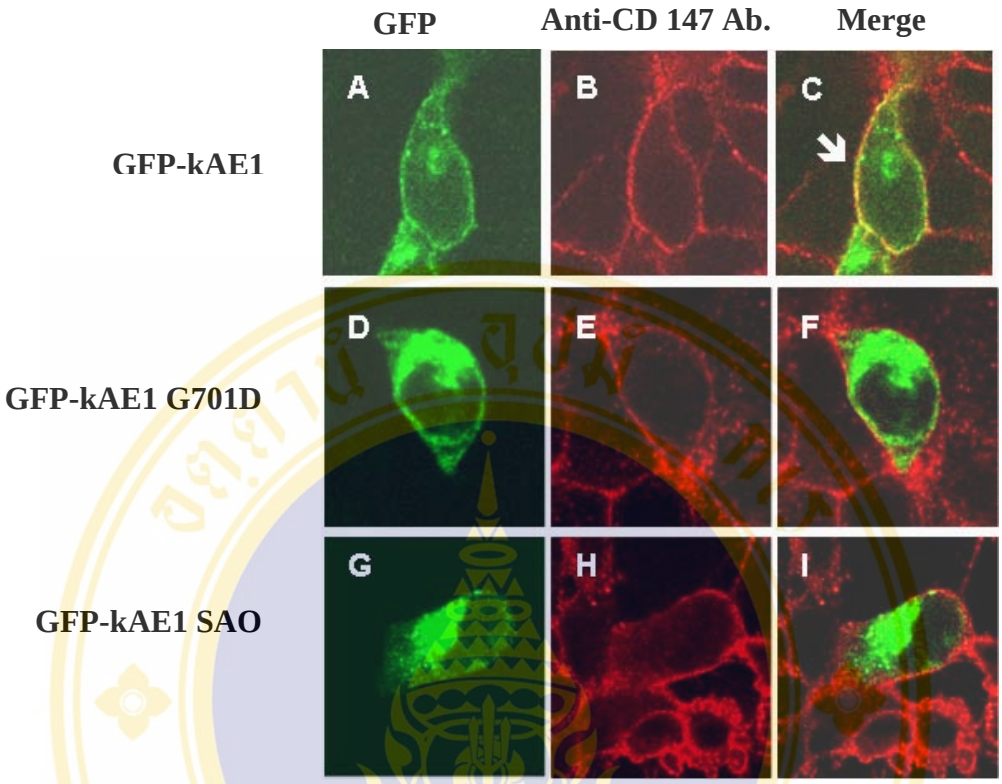


Figure 5.13 Expression and cellular localization of GFP-kAE1, GFP-kAE1 G701D, and GFP-kAE1 SAO fusion proteins in HEK293 cells.

Transiently transfected HEK293 cells expressing GFP-kAE1, GFP-kAE1 G701D, GFP-kAE1 SAO fusion proteins were examined for green fluorescent signal from GFP and stained for cell surface marker - CD147 by the immunofluorescence method using mouse anti-CD147 antibody, followed by goat anti-IgG antibody conjugated with Cy3, showing red fluorescent signal. Panels A-C show expression and cellular localization of GFP-kAE1. The fusion protein is expressed in cytoplasm and also co-localized with the surface marker. Panels D-F and G-I show expression and cellular localization of GFP-kAE1 G701D and GFP-kAE1 SAO, respectively. The fusion proteins are predominantly expressed and localized intracellularlly, without cell surface localization. *Left column* is confocal images visualizing GFP fusion proteins. *Middle column* is confocal images visualizing CD147. *Right column* is merged images.

5.7 Examination of expression and cellular localization of kAE1 tagged with hemagglutinin epitope (kAE1-HA) transiently expressed in HEK293 cells by immunofluorescence method

Since the wild-type and mutant kAE1 tagged with hemagglutinin (HA) epitope would be used to co-express with kAE1-GFP fusion proteins to study the protein-protein interaction to mimic the heterozygous and compound heterozygous conditions, the expression and cellular localization of wild-type and mutant kAE1-HA were also studied. HEK293 cells were individually transfected with 2 μg each of pcDNA3-*kAE1*-HA912, pcDNA3-*kAE1* G701D-HA912, and pcDNA3-*kAE1* SAO-HA912 by using Lipofectin transfection protocol. After two days of transfection, the cells were stained by immunofluorescence method by using rabbit anti-HA antibody, followed by donkey anti-rabbit IgG antibody conjugated with Cy3, and the cells were then examined by the confocal microscope. Figure 5.14 shows the results of expression and cellular localization of wild-type and mutant kAE1-HA in HEK293 cells. The cells transfected with pcDNA3-*kAE1*-HA peripherally expressed kAE1-HA at the cell surface (Figure 5.14A). In contrast, the cells transfected with either pcDNA3-*kAE1* G701D-HA or pcDNA3-*kAE1* SAO-HA predominantly expressed the mutant kAE1-HA proteins in the cytoplasm (Figures 5.14C and E).

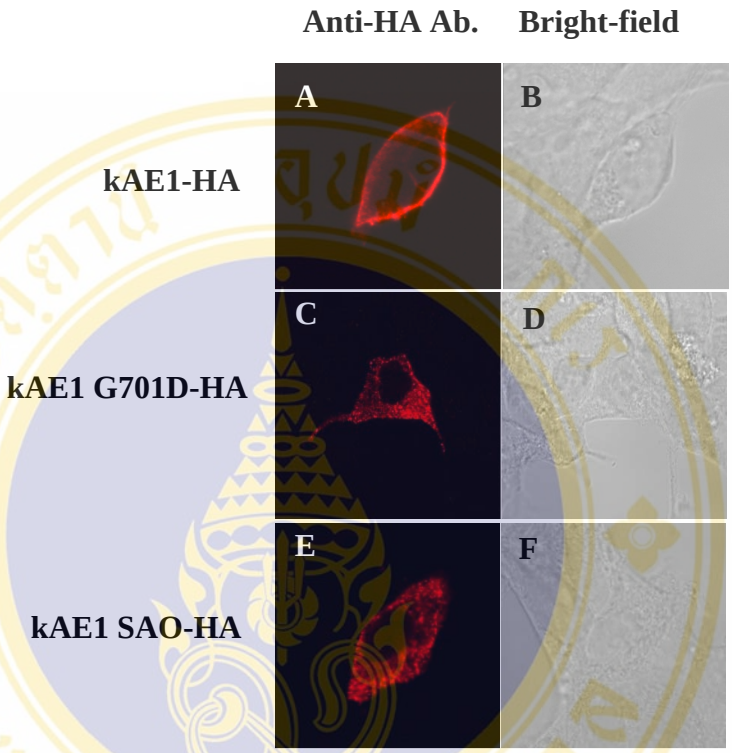


Figure 5.14 Expression and cellular localization of kAE1-HA, kAE1 G701D-HA, and kAE1 SAO-HA in HEK293 cells examined by immunofluorescence method.

HEK293 cells were transfected with either pcDNA3-*kAE1*-HA, pcDNA3-*kAE1* G701D-HA, or pcDNA3-*kAE1*-SAO-HA and stained by immunofluoresence method. Panels A-B, C-D, and E-F showed the transfected cells expressing kAE1-HA, kAE1 G701D-HA, and kAE1 SAO-HA, respectively. The kAE1-HA is predominantly expressed at the cell surface while the kAE1 G701D-HA and kAE1 SAO-HA are predominantly expressed in the cytoplasm. *Left column* is confocal images visualizing HA-tagged proteins. *Right column* is bright-field images.

5.8 Co-expression and localization of wild-type and mutant kAE1 proteins in HEK293 cells

To study the effect of mutant kAE1 on the wild-type kAE1 and *vice versa*, and the effect on each other of the two mutant kAE1 (kAE1 G701D and kAE1 SAO), HEK293 cells were co-transfected with four pairs of plasmids including pcDNA3.1-*GFP-kAE1*/pcDNA3-*kAE1*-HA, pcDNA3.1-*GFP-kAE1* G701D/pcDNA3-*kAE1*-HA, pcDNA3.1-*GFP-kAE1* SAO/pcDNA3-*kAE1*-HA, pcDNA3.1-*GFP-kAE1* G701D /pcDNA3-*kAE1* SAO-HA. After two days of transfection, the expression and cellular localization of GFP fusion proteins could directly be observed for green fluorescent signal by the confocal microscope whereas the HA-tagged kAE1 proteins were visualized by immunofluorescence staining method and confocal microscopy as previously described. As illustrated in Figures 5.15A-C, the HEK293 cells co-transfected with pcDNA3.1-*GFP-kAE1*/pcDNA3-*kAE1*-HA clearly co-expressed GFP-kAE1 and kAE1-HA on the plasma membrane (surface expression ~38.8±3.2%, Table 5.1) and the remaining in cytoplasm. The similar findings were observed when the HEK293 cells were co-transfected with either pcDNA3.1-*GFP-kAE1* G701D/pcDNA3-*kAE1*-HA (Figures 5.15 D-F) or pcDNA3.1-*GFP-kAE1* SAO/pcDNA3-*kAE1*-HA (Figures 5.15 G-I), which mimic the heterozygous conditions. The transfected cells predominantly co-expressed GFP-kAE1 G701D/kAE1-HA or GFP-kAE1 SAO/kAE1-HA on the plasma membrane (surface expressions ~50.0±13.7% and 49.9±1.9%, respectively) but the fusion proteins were spread out with less intensity in the cytoplasm. To mimic the compound heterozygous *AE1* G701D and SAO mutations reported in the patients with dRTA and SAO, the HEK293 cells were co-transfected with pcDNA3.1-*GFP-kAE1* G701D/pcDNA3-*kAE1* SAO-HA. In contrast to the findings observed in the previously co-transfected cells, the HEK293 cells co-transfected with pcDNA3.1-*GFP-kAE1* G701D/pcDNA3-*kAE1* SAO-HA largely co-expressed GFP-kAE1 G701D and kAE1 SAO-HA in the cytoplasm; the two mutant proteins were not or rarely localized on the plasma membrane (surface expression ~4.5±0.9%) (Figure 5.15 J-L and Table 5.1). The cellular localization of GFP-kAE1 in the transiently expressed HEK293 cells were concluded and shown in Table 5.2.

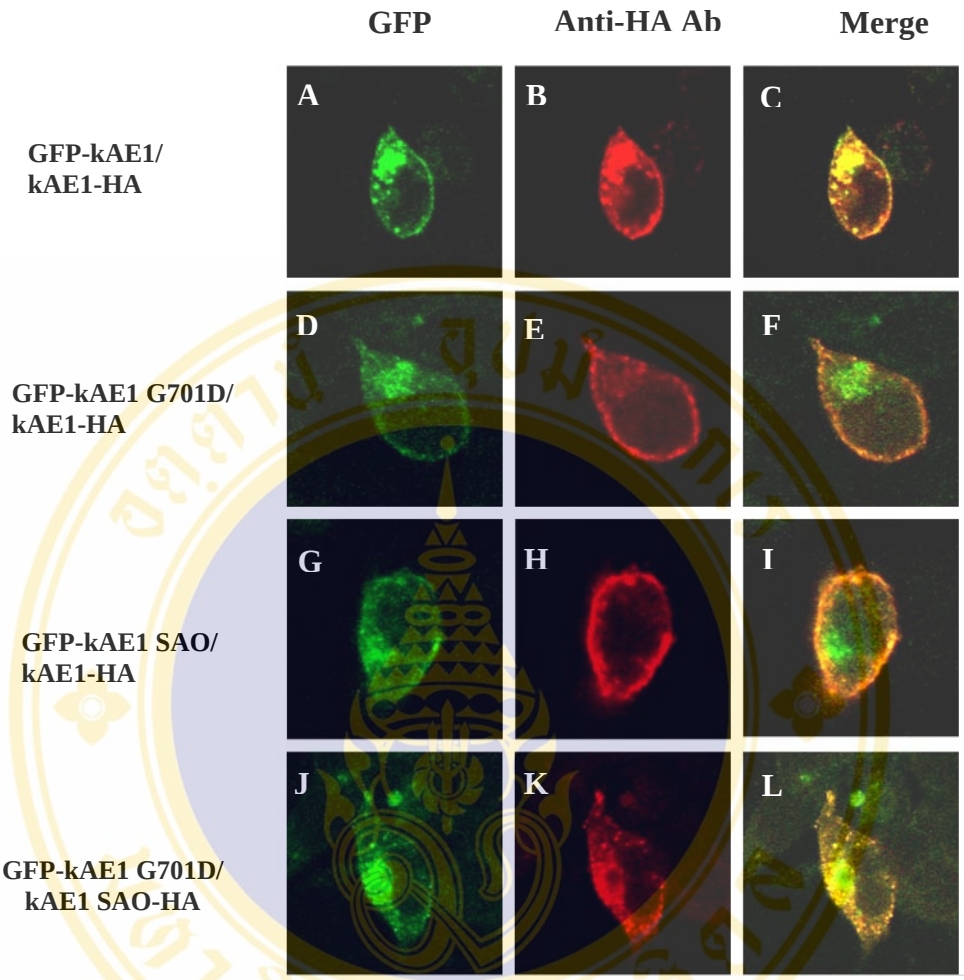


Figure 5.15 Co-expression and localization of wild-type and mutant kAE1 proteins in HEK293 cells.

HEK293 cells were co-transfected with four pairs of plasmids. Panels A-C are the cells co-transfected with pcDNA3.1-*GFP-kAE1*/pcDNA3-*kAE1*-HA. The proteins are clearly co-expressed on plasma membrane and partly in cytoplasm. Panels D-F and G-I are the cells co-transfected with pcDNA3.1-*GFP-kAE1* G701D/pcDNA3-*kAE1*-HA and pcDNA3.1-*GFP-kAE1* SAO/pcDNA3-*kAE1*-HA, respectively. In both transfected cells, the proteins are obviously co-expressed on the plasma membrane and partly in the cytoplasm. Panels J-L are the cells co-transfected with pcDNA3.1-*GFP-kAE1* G701D/pcDNA3-*kAE1* SAO-HA. The proteins are largely co-expressed in the cytoplasm but not or rarely localized at the plasma membrane. *Left column* is confocal images visualizing GFP fusion proteins. *Middle column* is confocal images visualizing HA-tagged proteins. *Right column* is merged images.

Table 5.1 Estimated percentages of kAE1 cell-surface expression from numbers of kAE1 pixels located on the cell surface against total kAE1 pixels in transiently expressed HEK293 cells.

kAE1 fusion protein	Number of kAE1 pixel*		Cell-surface expression (%)	Average cell-surface expression (mean ± SD) (%)
	Total	Surface		
GFP-kAE1 /CD147	3969	2143	54	53.5 ± 2.5
	7629	4255	55.8	
	5691	2890	50.8	
GFP-kAE1 G701D /CD147	12482	653	5.2	4.5 ± 1.5
	10769	306	2.8	
	12497	697	5.6	
GFP-kAE1 SAO /CD147	13422	2079	15.5	6.9 ± 7.6
	5938	62	1.0	
	14279	590	4.1	
GFP-kAE1 /kAE1-HA	13657	4830	35.4	38.8 ± 3.2
	16617	6513	39.2	
	15075	6299	41.8	
GFP-kAE1 G701D /kAE1-HA	20209	9406	46.5	50.0 ± 13.7
	10666	6333	59.4	
	20111	6445	32.0	
GFP-kAE1 SAO /kAE1-HA	11678	5980	51.2	49.9 ± 1.9
	20734	10051	48.5	
GFP-kAE1 G701D /kAE1 SAO-HA	21529	912	4.2	4.5 ± 0.9
	18269	1027	5.6	
	20998	807	3.8	

*The numbers of pixels were acquired from three cells (except for one).

Table 5.2 Cellular localization of kAE1 in transiently expressed HEK293 cells

kAE1	Cellular localization
GFP-kAE1	cell surface
GFP-kAE1 G701D	intracellular retention
GFP-kAE1 SAO	intracellular retention
GFP-kAE1/kAE1-HA	cell surface
kAE1-HA/GFP-kAE1 G701D	cell surface
kAE1-HA/GFP-kAE1 SAO	cell surface
GFP-kAE1G701D/kAE1 SAO-HA	intracellular retention

5.9 Examination of expression and cellular localization of GFP-kAE1 fusion proteins stably expressed in HEK293 cells

The HEK293 cells stably expressing GFP-kAE1 fusion proteins were generated for co-expression and localization studies by transfection with another plasmid construct. The HEK293 cells stably expressing either GFP-kAE1 or GFP-kAE1 G701D was successfully generated but the HEK293 cells stably expressing GFP-kAE1 SAO was not successfully produced in three independent experiments. The expressed GFP-kAE1 and GFP-kAE1 G701D proteins were determined by Western blot analysis using rabbit anti C-terminal AE1 polyclonal antibodies (Figure 5.16). The results showed that the HEK293 cells stably expressing either GFP-kAE1 or GFP-kAE1 G701D produced the fusion proteins with MW of ~109 kDa as expected. When the HEK293 cells stably expressing either GFP-kAE1 or GFP-kAE1 G701D were examined for cellular localization of the proteins, comparing to the surface marker - CD147, it was found that, in addition to cytoplasmic localization, GFP-kAE1 was also co-expressed with the surface marker on the cell membrane (surface expression ~34.2%, Table 5.3) whereas GFP-kAE1 G701D was mainly retained intracellularly and had less cell-surface localization (surface expression ~7.6%, Table 5.3) (Figure 5.17).

To study the effect of mutant kAE1 on wild-type kAE1 and *vice versa*, and the effect between kAE1 G701D and kAE1 SAO, HEK293 cells stably expressing GFP-kAE1 was transfected with pcDNA3-*kAE1*-HA or pcDNA3-*kAE1* G701D-HA or pcDNA3-*kAE1* SAO-HA. And, HEK293 cells stably expressing GFP-kAE1 G701D was co-transfected with pcDNA3-*kAE1*-HA or pcDNA3-*kAE1* SAO-HA. After two days of transfection, the expression and cellular localization of GFP fusion proteins and HA-tagged kAE1 proteins were visualized by immunofluorescence staining method and confocal microscopy as previously described. As shown in Figures 5.18 A-C, in the transfection of HEK293 cells stably expressing GFP-kAE1 with pcDNA3-*kAE1*-HA, the transfected cells co-expressed GFP-kAE1 and kAE1-HA on the plasma membrane (~65%) with less amounts in the cytoplasm. The similar findings were observed when the HEK293 cells stably expressing GFP-kAE1 was transfected with either pcDNA3-*kAE1* G701D-HA (Figures 5.18 D-F) or pcDNA3-*kAE1* SAO-HA (Figures 5.18 G-I), and the HEK293 cells stably expressing GFP-kAE1 G701D

transfected with pcDNA3-*kAE1*-HA (Figures 5.18 J-L). The transfected cells predominantly co-expressed GFP-kAE1/kAE1 G701D-HA or GFP-kAE1/kAE1 SAO-HA or GFP-kAE1 G701D/kAE1-HA on the plasma membrane (Figures 5.18 D-L and Table 5.3) with the percentages of cell-surface expression of about 43.9±4.2%, 34.2±9.1%, and 37.5±2.2%, respectively. These findings are consistent with those found in the transiently expressed HEK293 cells in the previous experiments but with lower percentages of cell-surface expression. To again mimic the compound heterozygous *AE1* G701D and SAO mutations reported in the patients with dRTA and SAO, the HEK293 cells stably expressing GFP-kAE1 G701D was transfected with pcDNA3-*kAE1* SAO-HA. As same as the result of transiently transfected HEK293 cells previously described, the transfected cells largely co-expressed GFP-kAE1 G701D and kAE1 SAO-HA in the cytoplasm with the cell-surface expression of about 5.8±4.0% (Figure 5.18 M-O, Table 5.3). The cellular localization of GFP-kAE1 in the stably expressed HEK293 cells were concluded and showed in Table 5.4.

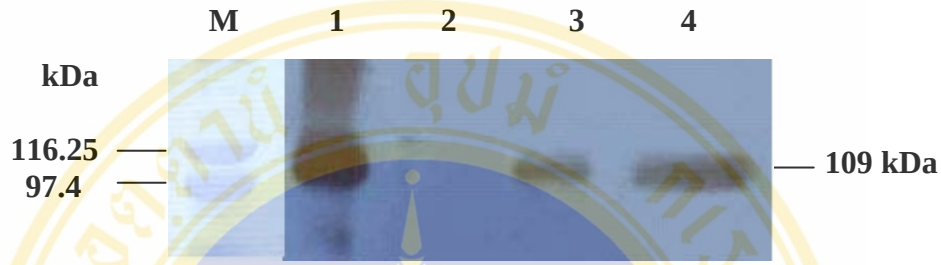


Figure 5.16 Western blot analysis of GFP-kAE1 fusion proteins stably expressed in HEK293 cells.

The GFP-kAE1 and GFP-kAE1 G701D fusion proteins stably expressed in HEK293 cells were analyzed by Western blot method using anti-Ct AE1 antibody. Lane M is the broad range protein molecular weight standard. Lane 1 is red cell membrane proteins containing eAE1 loaded as a positive control. Lane 2 is the lysate from untransfected HEK293 cells served as a negative control. Lane 3 is the lysate from HEK293 cells stably expressing GFP-kAE1. Lane 4 is the lysate from the cells stably expressing GFP-kAE1 G701D. The two fusion proteins have MW of about 109 kDa.

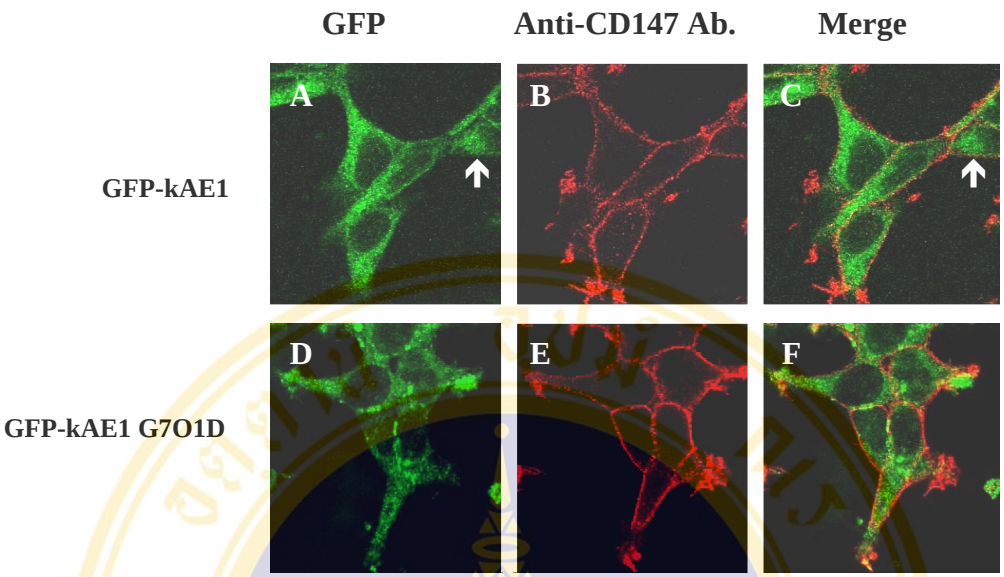


Figure 5.17 Cellular localization of GFP-kAE1 fusion proteins stably expressed in HEK293 cells.

HEK293 cells stably expressing GFP-kAE1 and GFP-kAE1 G701D were examined for green fluorescent signal from GFP and stained for cell surface marker-CD147 by the immunofluorescence method. Panels A-C show expression and cellular localization of GFP-kAE1 in the stably expressing HEK293 cells. The fusion protein is expressed in cytoplasm and also co-localized with the surface marker. Panels D-F show expression and cellular localization of GFP-kAE1 G701D in the stably expressing HEK293 cells. The fusion protein is predominantly expressed and localized in cytoplasm. *Left column* is confocal images visualizing GFP fusion proteins. *Middle column* is confocal images visualizing CD147. *Right column* is merged images.

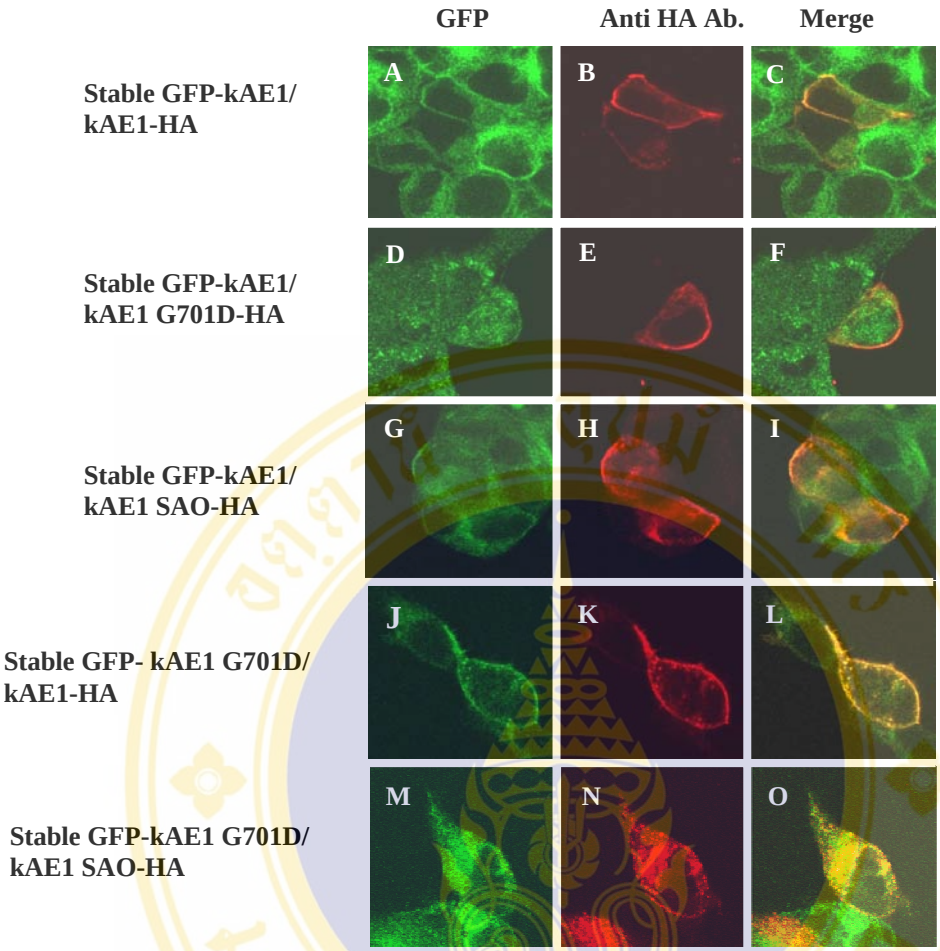


Figure 5.18 Co-expression and localization of wild-type and mutant kAE1 proteins stably expressed in HEK293 cells.

HEK293 cells stably expressing either GFP-kAE1 or GFP-kAE1 G701D were co-transfected with the second plasmid construct. Panels A-C are the HEK293 cells stably expressing GFP-kAE1 transfected with pcDNA3-*kAE1*-HA. The proteins are co-expressed on plasma membrane in the doubly transfected cells. Panels D-F and G-I are the HEK293 cells stably expressing GFP-kAE1 transfected with either pcDNA3-*kAE1* G701D-HA or pcDNA3-*kAE1* SAO-HA, respectively. In both transfected cells, the proteins are co-expressed on the plasma membrane and also in the cytoplasm. Panels J-L and M-O are the HEK293 cells stably expressing GFP-kAE1 G701D transfected with either pcDNA3-*kAE1*-HA or pcDNA3-*kAE1* SAO-HA, respectively. The GFP-kAE1 G701D and kAE1-HA proteins are largely co-expressed at the cell surface and less in the cytoplasm, whereas the GFP-kAE1 G701D and kAE1 SAO-HA proteins are predominantly co-expressed in the cytoplasm. *Left column* is confocal images visualizing GFP fusion proteins. *Middle column* is confocal images visualizing HA-tagged proteins. *Right column* is merged images.

Table 5.3 Estimated percentages of kAE1 cell-surface expression from numbers of kAE1 pixels located on the cell surface against total kAE1 pixels in stably expressed HEK293 cells.

kAE1 fusion protein	Number of kAE1 pixel*		Cell-surface expression (%)	Average cell-surface expression (mean ± SD) (%)
	Total	Surface		
ST GFP-kAE1/CD147	1244	426	34.2	34.2
ST GFP-kAE1 G701D /CD147	5637	426	7.6	7.6
ST GFP-kAE1 /kAE1-HA	14897	9170	61.6	64.8±4.5
	17011	11555	67.9	
ST GFP-kAE1 /kAE1 G701D-HA	9243	4330	46.8	43.9±4.2
	15391	6291	40.9	
ST GFP-kAE1 /kAE1 SAO-HA	6300	2555	40.6	34.2±9.1
	13931	3854	27.7	
ST GFP-kAE1 G701D /kAE1-HA	33120	11893	35.9	37.5±2.2
	30122	11760	39.0	
ST GFP-kAE1 G701D /kAE1 SAO-HA	20742	605	2.9	5.8±4.0
	22014	1900	8.6	

*The numbers of pixels were acquired from two cells (except for the first two fusion proteins).

Table 5.4 Cellular localization of GFP-kAE1 in the stably expressed HEK293 cells

Expression		Cellular localization
Stable expression	Co-expression	
GFP-kAE1	-	cell surface
	kAE1-HA	cell surface
	kAE1 G701D-HA	cell surface
	kAE1 SAO-HA	cell surface
GFP-kAE1 G701D	-	intracellular retention
	kAE1-HA	cell surface
	kAE1 SAO-HA	intracellular retention

CHAPTER VI
DISCUSSION

The mutations of *SLC4A1* (or *AE1*) encoding kAE1 have been found to cause either AD or AR dRTA. It is of great interest that majority of these *AE1* mutations do not produce defect of anion transport function but often result in aberrant trafficking of kAE1, leading to intracellular retention or mis-targeting of the mutant proteins (10, 12). AR dRTA associated with *AE1* mutations, which are frequently observed in Southeast Asia, may result from either homozygous or compound heterozygous conditions (11, 12, 16, 27, 97). Since *AE1* SAO mutation is prevalent in Southeast Asia, compound heterozygosities between SAO and other recessive mutations are usually present. Two patients with dRTA and SAO, caused by compound heterozygous G701D/SAO mutations, were firstly reported in two Thai families from southern Thailand (16) and later three patients with the same genotype were found in two Malaysian families (5). Recently, a case with this genotype was also reported in another Malaysian family from Sarawak (98). Additionally, the research group in the Division of Medical Molecular Biology identified a few more patients with the G701D/SAO mutations in Thai families (unpublished data).

The *AE1* G701D mutation *per se* is particularly interesting because while eAE1 G701D contains normal anion transport activity and RBC surface expression, kAE1 G701D seems to contain a defective function in the kidney as evident by the presence of dRTA phenotype in the patients with homozygous G701D mutation. This can be explained by the presence of glycophorin A (GPA) in RBC but absence of this protein in the kidney α–intercalated cells (11). This explanation was supported by the result that anion transport activity and surface expression of eAE1 and kAE1 G701D could be rescued by GPA in *Xenopus* oocyts (11) (Table 6.1). The recent studies have also showed impaired anion transport and intracellular retention of kAE1 G701D in HEK293 and non-polarized LLC-PK1 (14), and polarized MDCK cells (15) (Table 6.1). However, the impaired kAE1 G701D trafficking in non-polarized LLC-PK1 and

Table 6.1 The studies of transport activity and cellular localization of kAE1 mutant proteins resulted from *AE1* G701D and SAO mutations.

Mutation	Expression	Reference
G701D	Normal anion transport activity and cell surface expression in RBC from patients with homozygous condition. Reduced anion transport activity and cell surface expression in *Xenopus* oocytes but these could be rescued by co-expression with glycophorin A (GPA).	Tanphaichitr *et al*, 1998 (11)
	Intracellular retention in HEK293 cells	**Present work**
	No anion transport activity in HEK293 cells, and intracellular retention in HEK293 and non-polarized LLC-PK1 cells.	Kittanakom *et al*, 2004 (14)
	Intracellular and Golgi retention in non-polarized and polarized MDCK cells.	Cordat *et al*, 2006 (15)
SAO	Reduced anion transport activity in RBC from individuals with heterozygous SAO mutation.	Schofield *et al*, 1992 (17)
	Presence of cell surface expression but no anion transport activity in *Xenopus* oocytes.	Grove *et al*, 1993 (18)
	Intracellular retention in HEK293 cells	**Present work**
	Intracellular and ER retention in HEK293 cells, non-polarized and polarized MDCK cells.	Cheung *et al*, 2005 (19)
G701D/SAO	Reduced anion transport activity in RBC from patients with compound heterozygosity.	Vasuvattakul *et al*, 1999 (16)
	Reduced anion transport in RBC from patients with compound heterozygosity and in oocytes.	Bruce *et al*, 2000 (5)
	Intracellular retention in HEK293 cells	**Present work**

polarized MDCK cells could be corrected by the co-expression with wild-type kAE1 (14, 15). Although eAE1 SAO is able to traffic to the surface of RBC, this mutant protein is inactive, resulting in decreased RBC anion transport activity (~50%) in individuals with heterozygous SAO mutation (17). This was also evident by the lack of anion transport activity although it is present on the surface of oocytes (18). The later expression studies in HEK293 cells and in non-polarized and polarlized MDCK cells demonstrated that kAE1 SAO was intracellularly retained (19), indicating different trafficking mechanisms in HEK293 and MDCK cells from that in RBC and oocytes. Similar to kAE1 G701D, the impaired kAE1 SAO trafficking could also be corrected by the co-expression with wild-type kAE1 (19).

In RBC from the patients with compound heterozygous *AE1* G701D/SAO mutation, the anion transport activity is reduced to be comparable to that in RBC from individuals with heterozygous SAO (5, 16) (Table 6.1), again indicating the normal anion transport activity of kAE1 G701D in RBC as mentioned above. The loss of anion transport activity of the co-expressed kAE1 G701D and SAO in *Xenopus* oocyte could partially be rescued by addition of GPA (5), which would be contributed from kAE1 G701D. Although the patients with the compound heterozygous *AE1* G701D/SAO mutation are frequently observed in Thailand and other Southeast Asian countries, the molecular defect associated with this compound heterozygous condition is still unclear. As reviewed above, the defects of kAE1 G701D and SAO in the cultured mammalian cells have individually been studied and only the co-expression of kAE1 G701D and SAO in oocytes was investigated. However, the co-expression of kAE1 G701D and SAO in cultured mammalian cells, which mimics the compound heterozygous *AE1* G701D/SAO condition in kidney cells, to observe their interaction and effect to each other have not been examined. Thus, the experimental studies in this thesis aimed to investigate the expression and co-expression of kAE1 SAO and G701D in HEK293 cells by using green fluorescent protein (GFP) fusion and hemagglutinin A (HA) tagging to follow trafficking of the two mutant proteins when co-expressed with the wild-type kAE1 and with each other. GFP has been used to fuse with eAE1 and kAE1 to study their trafficking and localization in human K562 cells (20). eAE1 and kAE1 fused with GFP at their N-termini could predominantly be expressed at the surface of K562 cells and the chloride transport activity of the

proteins was found to be normal. These results indicated that the N-terminal tagging of eAE1 and kAE1 with GFP does not affect the targeting and anion transport properties of eAE1 and kAE1. Therefore, GFP fusion at the N-terminus of kAE1 should be able to use for study the expression, trafficking, and localization of wild-type and mutant kAE1 proteins in mammalian cell lines.

Initially, both GFP and red fluorescent protein (RFP) were used to fuse with wild-type kAE1 and mutant kAE1 proteins in order to produce different fluorescence signals for the subsequent studies. However, unlike GFP-kAE1, the RFP-kAE1 fusion resulted in obviously cytoplasmic aggregation of the protein producing red fluorescent signal in the transfected HEK293 cells. This result may occur from the interfering of RFP to the folding and structure of wild-type kAE1 and it would also affect the interpretation of result if RFP would be used to fuse with the mutant kAE1 proteins in the following experiments. Thus, only GFP was chosen and hemagglutinin A (HA) was then used as an epitope tag instead of RFP to fuse with the wild-type and mutant kAE1.

In the studies of this thesis, either GFP or HA were individually fused to wild-type kAE1, kAE1 G701D, and kAE1 SAO. While GFP was fused at the N-termini, HA was tagged to the C-termini of wild-type and mutant kAE1 proteins. HA can be tagged at the N-terminus (6) or at the extracellular loop 3 after Val557 (HA557) of eAE1 and kAE1 (14, 34) for expression in MDCK and HEK293 cells without any effect on AE1 trafficking. In this work, HA was tagged to kAE1 at the C-terminus after the last amino acid - Val911 (kAE1-HA911). This kAE1-HA911 was preliminarily tested for expression in HEK293 cells, comparing to kAE1-HA557. The recombinant plasmids, pcDNA3-*kAE1*-HA911 and pcDNA3-*kAE1*-HA557, for transfection study were generated by others and available in our laboratory. It was found that kAE1-HA911 was predominantly detected at the surface of HEK 293 cells. The cell surface expression of kAE1-HA911 was as good as or better than that of kAE1-HA557. Thus, wild-type and mutant kAE1-HA911 were used in the following experimental studies.

After the construction of recombinant plasmid containing *GFP* gene fused with wild-type *kAE1* cDNA (pcDNA3.1-*GFP-kAE1*) (Figures 5.5-5.7), the recombinant plasmids containing *GFP* fused with mutant *kAE1* (pcDNA3.1-*GFP-kAE1* G701D and

pcDNA3.1-*GFP-kAE1* SAO) were generated by site-directed mutagenesis and the mutations were confirmed by RFLP and gel electrophoresis (Figures 5.8 and 5.9) as well as nucleotide sequencing (Figures 5.10 and 5.11). The plasmid construct containing either *GFP-kAE1*, *GFP-kAE1* G701D or *GFP-kAE1* SAO was transfected into HEK293 cells and the expression of GFP-kAE1 fusion proteins was examined by Western blot analysis using anti-Ct AE1 antibody (Figure 5.12). The result showed that both the GFP fused with wild-type and mutant kAE1 were expressed as the proteins with the size of about 109 kDa, larger than that of wild-type kAE1 without GFP fusion (~82 kDa), as expected. The calculated MW for GFP-kAE1 and GFP-kAE1 G701D fusion proteins is ~109 kDa, and that for GFP-kAE1 SAO is ~106 kDa but they were unable to separate from each other. The result of Western blot analysis indicated that the three plasmid constructs could express the GFP-kAE1 fusion proteins to be used for the next experiments.

To determine the expression and localization of GFP-kAE1, GFP-kAE1 G701D and GFP-kAE1 SAO fusion proteins in HEK293 cells, the cells were individually transfected with each plasmid construct, after two days of transfection the cells were stained for cell surface marker, CD147, using anti-CD147 antibody and anti-IgG antibody conjugated with Cy3. The GFP fusion protein directly generated green fluorescent signal while the stained cell surface marker-CD147 produced red fluorescent signal (Figure 5.13). While GFP-kAE1 was predominantly co-expressed with CD147 at the surface of HEK293 cells, GFP-kAE1 G701D and GFP-kAE1 SAO were predominantly present in the cytoplasm and not co-localized with the CD147 surface marker (Figure 5.13 and Table 5.1). In addition, the aggregation of both fusion proteins was prominent, suggesting the effect of mutations on structure of GFP-kAE1 fusion proteins. This preliminary result indicates normal trafficking and localization of GFP-kAE1 but abnormal trafficking and localization of GFP-kAE1 G701D and GFP-kAE1 SAO, which was firstly demonstrated using GFP fusion. When kAE1-HA, kAE1 G7O1D-HA or kAE1 SAO-HA was individually expressed in HEK293 cells and stained by anti-HA antibody and anti-IgG antidody conjugated with Cy3 (Figure 5.14), similar results of normal trafficking and localization of kAE1-HA but abnormal trafficking and localization of kAE1 G7O1D-HA or kAE1 SAO-HA

were detected. The latter result confirms the finding that was previously reported (14, 15).

When GFP-kAE1 G701D or GFP-kAE1 SAO was co-expressed with wild-type kAE1-HA in HEK293 cells, the results showed that although GFP-kAE1 G701D and GFP-kAE1 SAO were partly expressed and localized in cytoplasm, the two mutant fusion proteins could also be co-expressed with kAE1-HA on the cell surface (Figure 5.15 and Table 5.1). This indicates that the wild-type kAE1 can rescue the defective trafficking of the mutant kAE1 G701D and kAE1 SAO proteins, the so-called 'dominant positive effect' of the wild-type kAE1 on the mutant kAE1 proteins (12). Thus, these findings confirmed the results that have recently been reported (14, 15, 19). It should be pointed out that the expressions of kAE1 G701D and kAE1 SAO were distinct in different cell types. kAE1 G701D is able to express at RBC surface but unable to express at the surface of oocytes and mammalian (HEK293, LLC-PK1, and MDCK) cells (15) that lack GPA. Interestingly, kAE1 SAO, which is inactive in anion transport, is able to express at the surface of both RBC and oocytes but unable to express on the surface of mammalian (HEK293 and MDCK) cells (19). However, the impaired trafficking of kAE1 G701D and kAE1 SAO in the mammalian cells can be corrected by the co-expression of wild-type kAE1, explaining the absence of dRTA phenotype in individuals with heterozygous *AE1* G701D or SAO mutation.

When GFP-kAE1 G701D and kAE1 SAO-HA were co-expressed in HEK293 cells to mimic the compound heterozygous *AE1* G701D/SAO condition detected in many patients with dRTA and SAO, in contrast to the results that described above, the two mutant proteins were intracellularly retained, without (or rarely if any) presence at the cell surface (~4.5%) (Figure 5.15 and Table 5.1). Although the cell surface marker was not stained in this co-expression study, cytoplasmic localization of the two mutant proteins was predominant (~95.5%) and clear, as compared to the results of co-expressions between the mutant and wild-type kAE1 proteins. Other methods such as investigation of cell surface expression of the mutant proteins by using HA or myc epitope tag at the 3rd extracellular loop of the mutant kAE1 for detection by fluorescence-activated cell sorting (FACS) in the co-expression in HEK293 or other cells may be used to confirm the absence of cell surface expression of these two mutant proteins. Since both mutant proteins were retained in cytoplasm, one mutant

(e.g. GFP-kAE1 G701D) could not rescue the other (e.g. kAE1 SAO-HA) or *vice versa*. The co-expression result is also agreeable with that of individual expression of kAE1 G701D and kAE1 SAO fused with either GFP or HA (Figures 5.13 and 5.14, and Table 5.1). This finding of co-expression between kAE1 G701D and kAE1 SAO in HEK293 cells was first demonstrated in the present work, which explains the molecular pathogenesis of dRTA causing by this compound heterozygous condition.

To confirm the results of transiently expressed HEK293 cells as described above, the stably expressed HEK293 cells were also generated for the co-expression experiments. The HEK293 cells stably expressed wild-type and mutant GFP-kAE1, the cells were created by transfections of the cells with the plasmids containing either *GFP-kAE1*, *GFP-kAE1* G701D, or *GFP-kAE1* SAO and cultured in the media with the presence of selective antibiotic-geneticin to select the tranfected cells with the plasmid containing neomycin resistant (Neo^r) gene. After two weeks, the surviving clones were transferred for continuing culture in the media containing geneticin for co-transfection experiment. From the transfections with the three plasmids, only two clones stably expressing either GFP-kAE1 or GFP-kAE1 G701D could be produced. The production of clone stably expressing GFP-kAE1 SAO was not successful although the experiments were repeated three times. This may result from the unknown adverse effect of kAE1 SAO on the stably transfected cells. Western blot analysis using anti-Ct AE1 antibody showed that the HEK293 cells stably expressing either GFP-kAE1 or GFP-kAE1 G701D could produce the fusion proteins with MW of ~109 kDa as expected (Figure 5.16). To examine cellular localization of the fusion protein, the cells were stained for the surface marker - CD147 and subjected to visualization by confocal microscope. While GFP-kAE1 was more predominantly co-expressed with CD147 at the surface of HEK293 cells, GFP-kAE1 G701D was mainly retained intracellularly and showed less cell-surface localization (Figure 5.17 and Table 5.3). This finding confirmed the result of normal trafficking and localization of GFP-kAE1 but abnormal trafficking and localization of GFP-kAE1 G701D that had been observed in transiently transfected HEK293 cells.

When the HEK293 cells stably expressing GFP-kAE1 was co-expressed with either kAE1 G701D-HA or kAE1 SAO-HA, and the HEK293 cells stably expressing GFP-kAE1 G701D co-expressed with kAE1-HA (Figures 5.18), similar findings that

the co-transfected cells predominantly co-expressed the wild-type and mutant proteins on the cell surface as well as in the cytoplasm were observed. To again mimic the compound heterozygous *AE1* G701D/SAO conditions, the HEK293 cells stably expressing GFP-kAE1 G701D was co-expressed with kAE1 SAO-HA. The result showed that the co-transfected cells predominantly co-expressed the mutant proteins in the cytoplasm with very little or no cell surface localization (Figure 5.18 and Table 5.3). This result is consistent with that of the HEK293 cells transiently co-expressing the two mutant kAE1 proteins (Figure 5.15).

The results of co-expressions between wild-type and mutant kAE1 that showed predominant cell surface expression in HEK293 cells in both transient and stable expression experiments indicated that wild-type and mutant kAE1 could interact to each other and both kAE1 G701D and kAE1 SAO could be rescued by wild-type kAE1 as previously reported. The predominant intracellular retention of the co-expressed kAE1 G701D and kAE1 SAO in HEK293 cells in both transient and stable expression experiments indicated their impaired trafficking and the failure to rescue each other. However, it is possible that the two mutant proteins may or may not physically interact to each other although they were located at the same place and the two fluorescent colors (green and red) were superimposed to generate new color (yellow) in the cytoplasm. The studies by co-immunoprecipitation and his-tag co-purification methods by Miss Nunghathai Sawasdee in our laboratory demonstrated that kAE1 G701D and kAE1 SAO could form heterodimer in HEK293 cells. Thus, both kAE1 G701D and kAE1 SAO homodimers, and their heterodimer present trafficking defect and intracellular retention in HEK293 cells. This would explain the molecular pathogenesis of the compound heterozygous G701D/SAO condition as it will cause the lack of a functional kAE1 at the basolateral membrane of kidney α-intercalated cells resulting in inadequate anion exchange activity in the patients with dRTA.

CHAPTER VII
CONCLUSION

To gain a better understanding on the molecular mechanism of AR dRTA caused by a compound heterozygous *AE1* G701D/SAO mutation, the expression and localization of kAE1 SAO and G701D were studied by using GFP fusion and HA tagging to visualize these mutant proteins by a confocal microscopy when co-expressed in cultured HEK293 cells. All three fusion proteins including GFP-kAE1, GFP-kAE1 G701D, and GFP-kAE1 SAO could transiently be expressed but two, GFP-kAE1 and GFP-kAE1 G701D, were stably expressed in HEK293 cells, all of which could be detected by Western blot analysis. When individually expressed, GFP-kAE1 was co-localized with a surface marker-CD147 whereas GFP-kAE1 G701D and GFP-kAE1 SAO were intracellularly retained. However, these two GFP-kAE1 fusion mutant proteins could be rescued to predominantly locate at the cell surface when they were co-expressed with wild-type kAE1-HA. Co-expression of GFP-kAE1 G701D with kAE1 SAO-HA resulted in intracellular retention with little or no cell surface expression in both transiently and stably expressing HEK293 cells, indicating their severely trafficking impairment. This would likely be the explanation in term of molecular mechanism of AR dRTA associated with the compound heterozygous *AE1* G701D/SAO condition because the trafficking impairment and intracellular retention of the two kAE1 mutant proteins will lead to the absence or inadequacy of functional kAE1 at the basolateral membrane of kidney α-intercalated cells in the patients with dRTA.

REFERENCES

1. Karet FE, Gainza FJ, Gyory AZ, Unwin RJ, Wrong O, Tanner MJ, et al. Mutations in the chloride-bicarbonate exchanger gene AE1 cause autosomal dominant but not autosomal recessive distal renal tubular acidosis. Proc Natl Acad Sci U S A 1998;95(11):6337-42.
2. Toye AM, Bruce LJ, Unwin RJ, Wrong O, Tanner MJ. Band 3 Walton, a C terminal deletion associated with distal renal tubular acidosis, is expressed in the red cell membrane but retained internally in kidney cells. Blood 2002;99:342-347.
3. Bruce LJ, Cope DL, Jones GK, Schofield AE, Burley M, Povey S, et al. Familial distal renal tubular acidosis is associated with mutations in the red cell anion exchanger (Band 3, AE1) gene. J Clin Invest 1997;100(7):1693-707.
4. Jarolim P, Shayakul C, Prabakaran D, Jiang L, Stuart-Tilley A, Rubin HL, et al. Autosomal dominant distal renal tubular acidosis is associated in three families with heterozygosity for the R589H mutation in the AE1 (band 3) Cl-/HCO3- exchanger. J Biol Chem. 1998;273(11):6380-6388.
5. Bruce LJ, Wrong O, Toye AM, Young MT, Ogle G, Ismail Z, et al. Band 3 mutations, renal tubular acidosis and South-East Asian ovalocytosis in Malasia and Papua New Guinea : loss of up to 95% band 3 transport in red cells. Biochem. J. 2000;350:41-51.
6. Rungroj N, Devonald MA, Cuthbert AW, Reimann F, Akkarapatumwong V, Yenchitsomanus P, et al. A novel missense mutation in AE1 causing autosomal dominant distal renal tubular acidosis retains normal transport function but is mistargeted in polarized epithelial cells. The Journal of Biological Chemistry 2004;279(14):13833-13838.
7. Quilty JA, Cordat E, Reithmeier RA. Impaired trafficking of human kidney anion exchanger (kAE1) caused by hetero-oligomer formation with a truncated mutant associated with distal renal tubular acidosis. Biochem J 2002;368(Pt 3):895-903

8. Quilty JA, Li J, Reithmeier RA. Impaired trafficking of distal renal tubular acidosis mutants of the human kidney anion exchanger kAE1. Am J Physiol Renal Physiol 2002;282(5):F810-20.

9. Devonald MA, Smith AN, Poon JP, Ihrke G, Karet FE. Non-polarized targeting of AE1 causes autosomal dominant distal renal tubular acidosis. Nat Genet. 2003;33(2):125-127.

10. Toye AM. Defective kidney anion exchanger 1 (AE1, Band 3) trafficking in dominant distal renal tubular acidosis (dRTA). Biochemical Society Symposium 2005;72:47-63.

11. Tanphaichitr VS, Sumboonnanonda A, Ideguchi H, Shayakul C, Brugnara C, Takao M, et al. Novel AE1 mutations in recessive distal renal tubular acidosis. Loss-of-function is rescued by glycophorin A. J Clin Invest 1998;102(12):2173-9.

12. Yenchitsomanus P, Kittanakom S, Rungroj N, Cordat E, Reithmeier RA. Molecular mechanisms of autosomal dominant and recessive distal renal tubular acidosis caused by SLC4A1 (AE1) mutations. Journal of Molecular and Genetic Medicine 2005;1(2):00-00.

13. Sritippayawan S, Sumboonnanonda A, Vasuvattakul S, Keskanokwong T, Sawasdee N, Paemanee A, et al. Novel compound heterozygous SLC4A1 mutations in Thai patients with autosomal recessive distal renal tubular acidosis. Am J Kidney Dis. 2004;44(1):64-70.

14. Kittanakom S, Cordat E, Akkarapatumwong V, Yenchitsomanus P, Reithmeier RA. Trafficking Defects of a Novel Autosomal Recessive Distal Renal Tubular Acidosis Mutant (S773P) of the Human Kidney Anion Exchanger (kAE1). The Journal of Biological Chemistry 2004;279:40960-40971.

15. Cordat E, Kittanakom S, Yenchitsomanus P, Li J, Du K, Lukacs G, et al. Dominant and Recessive Distal Renal Tubular Acidosis Mutations of Kidney Anion Exchanger 1 Induce Distinct Trafficking Defects in MDCK Cells. Traffic 2006 2006;7:1-12.

16. Vasuvattakul S, Yenchitsomanus P, Vachuanichsanong P, Thuwajit P, Kaitwatcharachai C, Laosombat V, et al. Autosomal recessive distal renal tubular acidosis associated with Southeast Asian ovalocytosis. Kidney

Int 1999;56:1674–1682.

17. Schofield AE, Reardon DM, Tanner MJ. Defective anion transport activity of the abnormal band 3 in hereditary ovalocytic red blood cells. Nature 1992;355:836-868.
18. Groves JD, Ring SM, Schofield AE, Tanner MJ. The expression of the abnormal human red cell anion transporter from South-East Asian ovalocytes (band 3 SAO) in Xenopus oocytes. FEBS Lett 1993;330(2):186-90.
19. Cheung JC, Cordat E, Reithmeier RA. Trafficking defects of the Southeast Asian ovalocytosis deletion mutant of anion exchanger 1 membrane proteins. Biochemical society 2005;1(1):1.
20. Beckmann R, Toye AM, Smythe JS, Anstee DJ, Tanner MJ. An N-terminal GFP tag does not alter the functional expression to the plasma membrane of red cell and kidney anion exchanger (AE1) in mammalian cells. Molecular Membrane Biology 2002;19:187-200.
21. Alper SL. Genetic diseases of acid-base transporters. Annu Rev Physiol 2002;64:899-923.
22. Showe LC, Ballantine M, Huebner K. Localization of the gene for the erythroid anion exchange protein, band 3 (EMPB3), to human chromosome 17. Genomics 1987;1(1):71-6.
23. Tanner MJ. The structure and function of band 3 (AE1): recent developments (review). Mol Membr Biol 1997;14(4):155-65.
24. Reithmeier RA, Landolt-Marticorena C, Casey JR, Sarabia VE, Wang J. Molecular characterization of the erythrocyte chloride-bicarbonate exchanger. Soc Gen Physiol Ser 1993;48:161-8.
25. Wagner S, Vogel R, Lietzke R, Koob R, Drenckhahn D. Immunochemical characterization of a band 3-like anion exchanger in collecting duct of human kidney. Am J Physiol 1987;253(2 Pt 2):F213-21.
26. Alper SL. The band 3-related anion exchanger (AE) gene family. Annu Rev Physiol 1991;53:549-64.
27. Yenchitsomanus PT, Sawasdee N, Paemanee A, Keskanokwong T, Vasuvattakul

S, Bejrachandra S, et al. Anion exchanger 1 mutations associated with distal renal tubular acidosis in the Thai population. J Hum Genet 2003;48(9):451-6.

28. Zhang D, Kiyatkin A, Bolin JT, Low PS. Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. Blood 2000;96(9):2925-33.
29. Reithmeier RA. A membrane metabolon linking carbonic anhydrase with chloride/bicarbonate anion exchangers. Blood Cells Mol Dis 2001;27(1):85-9.
30. Zhu Q, Lee DW, Casey JR. Novel topology in C-terminal region of the human plasma membrane anion exchanger, AE1. J Biol Chem 2003;278(5):3112-20.
31. Muth TR, Caplan MJ. Transport protein trafficking in polarized cells. Annu Rev Cell Dev Biol 2003;19:333-66.
32. Toye AM, Banting G, Tanner MJ. Regions of human kidney anion exchanger 1 (kAE1) required for basolateral targeting of kAE1 in polarised kidney cells: mis-targeting explains dominant renal tubular acidosis (dRTA). J Cell Sci 2004;117(Pt 8):1399-410.
33. Shayakul C, Alper SL. Defects in processing and trafficking of the AE1 Cl^-/HCO_3^- exchanger associated with inherited distal renal tubular acidosis. Clin Exp Nephrol 2004;8:1-11.
34. Cordat E, Li, J. and Reithmeier, R. A. F. Carboxyl-terminal truncations of human anion exhanger impair its trafficking to the plasma membrane. Traffic 2003;4:642-651.
35. Sheng M, Sala C. PDZ domains and the organization of supramolecular complexes. Annu Rev Neurosci 2001;24:1-29.
36. Bruce LJ, Tanner MJ. Erythroid band 3 variants and disease. Baillieres Best Pract Res Clin Haematol 1999;12(4):637-54.
37. Iolascon A, Miraglia del Giudice E, Perrotta S, Alloisio N, Morle L, Delaunay J. Hereditary spherocytosis: from clinical to molecular defects. Haematologica 1998;83(3):240-257.
38. Rybicki AC, Qiu JJ, Musto S, Rosen NL, Nagel RL, Schwartz RS. Human

erythrocyte protein 4.2 deficiency associated with hemolytic anemia and a homozygous 40glutamic acid-->lysine substitution in the cytoplasmic domain of band 3 (band 3Montefiore). Blood 1993;81(8):2155-2165.

39. Inoue T, Kanzaki A, Kaku M, Yawata A, Takezono M, Okamoto N, et al. Homozygous missense mutation (band 3 Fukuoka: G130R): a mild form of hereditary spherocytosis with near-normal band 3 content and minimal changes of membrane ultrastructure despite moderate protein 4.2 deficiency. Br J Haematol. 1998;102(4):932-939.
40. Jarolim P, Palek J, Rubin HL, Prchal JT, Korsgren C, Cohen CM. Band 3 Tuscaloosa: Pro327----Arg327 substitution in the cytoplasmic domain of erythrocyte band 3 protein associated with spherocytic hemolytic anemia and partial deficiency of protein 4.2. Blood 1992;80(2):523-529.
41. Jarolim P, Murray JL, Rubin HL, Taylor WM, Prchal JT, Ballas SK, et al. Characterization of 13 novel band 3 gene defects in hereditary spherocytosis with band 3 deficiency. Blood 1996;88(11):4366-4374.
42. Quilty JA, Reithmeier RA. Trafficking and folding defects in hereditary spherocytosis mutants of the human red cell anion exchange. Traffic 2000;12:987-998.
43. Cheidde L, Vieira TC, Lima PR, Saad ST, Heilberg IP. A novel mutation in the anion exchanger 1 gene is associated with familial distal renal tubular acidosis and nephrocalcinosis. Pediatrics 2003;112(6 Pt 1):1361-7.
44. Rysava R, Tesar V, Jirsa MJ, Brabec V, Jarolim P. Incomplete distal renal tubular acidosis coinherited with a mutation in the band 3 (AE1) gene. Nephrol. Dial. Transplant 1997;12:1869-1873.
45. Sritippayawan S, Kirdpon S, Vasuvattakul S, Wasanawatana S, Susaengrat W, Waiyawuth W, et al. A de novo R589C mutation of anion exchanger 1 causing distal renal tubular acidosis. Pediatr Nephrol 2003.
46. Liu SC, Zhai S, Palek J, Golan DE, Amato D, Hassan K, et al. Molecular defect of the band 3 protein in Southeast Asian ovalocytosis. New Eng. J. Med 1990;323:1530-1538.
47. Ribeiro ML, Alloisio N, Almeida H, Gomes C, Texier P, Lemos C, et al. Severe hereditary spherocytosis and distal renal tubular acidosis associated with

the total absence of band 3. Blood 2000;96(4):1602-4.

48. Yenchitsomanus P, Sawasdee N, Paemanee A, Keskanokwong T, Vasuvattakul S, Bejrachandra S, et al. Novel AE1 mutations in recessive distal renal tubular acidosis: loss-of-function is rescued by glycophorin A. J. Clin. Invest. 1998;102:2173-2179.
49. Yenchitsomanus PT, Vasuvattakul S, Kirdpon S, Wasanawatana S, Susaengrat W, Srethiphayawan S, et al. Autosomal recessive distal renal tubular acidosis caused by G701D mutation of anion exchanger 1 gene. Am J Kidney Dis 2002;40(1):21-9.
50. Garcia AM, Lodish HF. Lysine 539 of human band 3 is not essential for ion transport or inhibition by stilbene disulfonates. J Biol Chem 1989;264(33):19607-13.
51. Bartel D, Lepke S, Layh-Schmitt G, Legrum B, Passow H. Anion transport in oocytes of Xenopus laevis induced by expression of mouse erythroid band3 protein-encoding cRNA and of a cRNA derivative obtained by site-directed mutagenesis at the stilbene disulfonate binding site. Embo J 1989;8(12):3601-9.
52. Jonathan WJ, Cheryl AT. Epitope tagging. Annu. Rev. Genet. 1998;32:601-618.
53. Higgins SJ. Protein Expression: A Practical Approach: Oxford University Press.
54. Sambrook JR. Molecular Cloning: a laboratory manual; 2001.
55. Lippincott-Schwartz J, Smith C. Insights into secretory and endocytic membrane traffic using green fluorescent protein chimeras. Curr Opin Neurobiol 1997;7(5):631-639.
56. Yu Y, Oberg K, Wang G, Szaley AA. Visualization of molecular and cellular events with green fluorescent proteins in developing embryos : a reveiw. Luminescence 2003;18(1):1-18.
57. Sahly I EH, Khoutorsky A, Shapira E, Spira ME. Effective expression of the green fluorescent fusion proteins in cultured Aplysia neurons. J Neurosci Methods 2003;126(2):111-117.
58. Sullivan KF, Kay SA. Green Fluorescent Proteins. Methods in Cell Biology.; 1999.
59. Cody CW, Prasher DC, Westler WM, Prendergast FG, Ward WW. Chemical

structure of the hexapeptide chromophore of the Aequorea green-fluorescent protein. Biochemistry 1993;32(5):1212-1218.

60. Ormo M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ. Crystal structure of the Aequorea victoria green fluorescent protein. Science 1996;273(5280):1392-1395.
61. Roessel PV, Brand AH. Imaging into the future: visualizing gene expression and protein interactions with fluorescent proteins. Nature cell biology 2002;4.
62. Yang F, Moss LG, Phillips GNJ. The molecular structure of green fluorescent protein. Nat Biotechnol 1996;14(10):1246-1251.
63. Spergel DJ, Kruth U, Shimshek DR, Sprengel R, Seeburg PH. Using reporter genes to label selected neuronal populations in transgenic mice for gene promoter, anatomical, and physiological studies. Progress in Neurobiology 2001;63:673-686.
64. Reddi PP, al. e. Transcriptional regulation of spermiogenesis: insights from the study of the gene encoding the acrosomal protein SP-10. J. Reprod. Immunol 2002;53(1-2):25-36.
65. Doubrovin M, Ponomarev V, Beresten T, Balatoni J, Bornmann W, Finn R, et al. Imaging transcriptional regulation of p53-dependent genes with positron emission tomography in vivo. PNAS 2001;98:9300–9305.
66. Melin P, Thoreau V, Norez C, Bilan F, Kitzis A, Becq F. The cystic fibrosis mutation G1349D within the signature motif LSHG of NBD2 abolishes the activation of CFTR chloride channels by genistein. Biochemical Pharmacology 2004;67:2187-2196.
67. Bartholomew A, Patil S, Mackay A, Nelson M, Buyaner D, Hardy W, et al. Baboon mesenchymal stem cells can be genetically modified to secrete human erythropoietin in vivo. Hum. Gene Ther 2001;12(12):1527-1541.
68. Miller CL, Imren S, Antonchuk J, Kalberer C, Fabry ME, Nagel RL, et al. Feasibility of Using Autologous Transplantation to Evaluate Hematopoietic Stem Cell-Based Gene Therapy Strategies in Transgenic Mouse Models of Human Disease. Molecular Therapy 2002;6(3):422-428.
69. Priller J, Persons DA, Klett FF, Kempermann G, Kreutzberg GW, Dirnagl U.

Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells in vivo. J. Cell Biology 2001;155(5):733-738.

70. Priller J, Flugel A, Wehner T, Boentert M, Haas CA, Prinz M, et al. Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment. Nat Med. 2001;12:1356-1361.
71. Hakamata Y, Tahara K, Uchida H, Sakuma Y, Nakamura M, Kume A, et al. Green fluorescent protein-transgenic rat: a tool for organ-transplantation research. Biochem. Biophys. Res. Commun 2001;286(4):779-785.
72. Schmidt A, Bockmann M, Stoll A, Racek T, Putzer BM. Analysis of adenovirus gene transfer into adult neural stem cells. Virus Research 2005;114(1-2):45-53.
73. Roh S, Shim H, Hwang WS, Yoon JT. In vitro development of green fluorescent protein (GFP) transgenic bovine embryos after nuclear transfer using different cell cycles and passages of fetal fibroblasts. Reprod. Fertil. Dev 2000;12(1-2):1-6.
74. Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M. A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). FEBS Lett 1995;375(1-2):125-128.
75. Takada T, Iida K, Awaji T, Itoh K, Takahashi R, Shibui A, et al. Selective production of transgenic mice using green fluorescent protein as a marker. Nature Biotechnology 1997;15:458-461.
76. Chishima T, Yang M, Miyagi Y, Li L, Tan Y, Baranov E, et al. Governing step of metastasis visualized in vitro. Proc.Natl. Acad. Sci. USA 1997;94(21):11573-11576.
77. Contag CH, Jenkins D, Contag PR, Negrin RS. Use of reporter genes for optical measurements of neoplastic disease in vivo. Neoplasia 2000;2(1-2):41-52.
78. Hoffman RM. Visualization of GFP-expressing tumors and metastasis in vivo. Biotechniques 2001;30(5):1016-1026.
79. Lippincott-Schwartz J A-BN, Patterson GH. Photobleaching and photoactivation: following protein dynamics in living cells. Nat Cell Biol. 2003:Suppl: S7-S14.

80. Pollok BA, Heim R. Using GFP in FRET-based applications. Trends in cell biology 1999;9:57-60.

81. Simpson AM, Schwartz GJ. Distal renal tubular acidosis with severe hypokalaemia, probably caused by colonic H(+)-K(+)-ATPase deficiency. Arch Dis Child 2001;84(6):504-7.

82. Miesenbock G, De Angelis DA, Rothman JE. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 1998;394(6689):192-195.

83. Thomas T, Telford D, Laird DW. Functional Domain Mapping and Selective Trans-dominant Effects Exhibited by Cx26 Disease-causing Mutations. The journal of biological chemistry 2004;279:19157-19168.

84. Ivana De Domenico DMW, Elizabeta Nemeth, Michael B. Vaughn, Giovanni Musci, Tomas Ganz,, Kaplan. aJ. The molecular basis of ferroportinlinked hemochromatosis. PNAS 2005;102:8955-8960.

85. Yamashita F, Horie M, Kubota T, Yoshida H, Yumoto Y, Kobori A, et al. Characterization and Subcellular Localization of KCNQ1 with a Heterozygous Mutation in the C Terminus. J Mol Cell Cardiol 2001;33:197-207.

86. Goodchild RE, Dauer WT. Mislocalization to the nuclear envelope: An effect of the dystonia-causing torsinA mutation. PNAS 2004;101:847-852.

87. Gu LH, Coulombe PA. Defining the Properties of the Nonhelical Tail Domain in TypeII Keratin 5: Insight from a Bullous Disease-causing Mutation. Molecular Biology of the Cell 2005;16:1427-1438.

88. Hachiya NS, Watanabe K, Kawabata MY, Jozuka A, Kozuka Y, Sakasegawa Y, et al. Prion protein with Y145STOP mutation induces mitochondria-mediated apoptosis and PrP-containing deposits in vitro. Biochemical and Biophysical Research Communications. 2005;327:894-899.

89. Edward BN, John AS, Stephen JDJ, Catherine LK, Christian AC, Adele C, et al. The ABCA1 Transporter Modulates Late Endocytic Trafficking insights from the correction of the genetic defect in tangier disease. The journal of biological chemitry 2004;279:15571-15578.

90. Novoselova TV, Margulis BA, Novoselov SS, Sapozhnikov AM, van der Spuy J,

Cheetham ME, et al. Treatment with extracellular HSP70/HSC70 protein can reduce polyglutamine toxicity and aggregation. Journal of Neurochemistry 2005;94:597-606.

91. Kotliarova S, Jana NR, Sakamoto N, Kurosawa M, Miyazaki H, Nekooki M, et al. Decreased expression of hypothalamic neuropeptides in Huntington disease transgenic mice with expanded polyglutamine-EGFP fluorescent aggregates. Journal of Neurochemistry 2005;93:641-653.
92. Krampfl K, Maljevic S, Cossette P, Ziegler E, Rouleau GA, Lerche H, et al. Molecular analysis of the A322D mutation in the $GABA_A$ receptor α_1-subunit causing juvenile myoclonic epilepsy. European Journal of Neuroscience 2005;22:10-20.
93. Fung C, Allan B, Theodore GW, John HW. Knock-in human rhodopsin–GFP fusions as mouse models for human disease and targets for gene therapy. PNAS 2004;101:9109-9114.
94. Thomas T, Jordan K, Simek J, Shao Q, Jedeszko C, Walton P, et al. Mechanisms of Cx43 and Cx26 transport to the plasma membrane and gap junction regeneration. Journal of Cell Science. 2005;118:4451-4462.
95. Thomas T AT, Hodgins M, Laird DW. Transport and function of cx26 mutants involved in skin and deafness disorders. Cell Commun Adhes 2003;10(4-6):353-358.
96. Harada M, Kawaguchi T, Kumemura H, Terada K, Ninomiya H, Taniguchi E, et al. The Wilson Disease Protein ATP7B Resides in the Late Endosomes with Rab7 and the Niemann-Pick C1 Protein. American Journal of Pathology 2005;166:499-510.
97. Wrong O, Bruce LJ, Unwin RJ, Toye AM, Tanner MJ. Band 3 mutations, distal renal tubular acidosis, and Southeast Asian ovalocytosis. Kidney Int 2002;62(1):10-19.
98. Choo KE, Nicoli TK, Bruce LJ, Tanner MJ, Ruiz-Linares A, Wrong OM. Recessive distal renal tubular acidosis in Sarawak caused by AE1 mutations. Pediatr Nephrol 2005.

APPENDIX

Appendix

Chemicals, solution and buffers

1. Chemicals

Chemicals	Company
Absolute ethanol (C_2H_5OH)	BHD
Acrylamide (C_3H_5NO)	Sigma
Agarose SeaKem GTG $(C_{12}H_{18}O_9)_n$	BMA
Ammonium persulfate $(NH_4)_2S_2O_8$	USB
Ampicillin sodium	T.P. Laboratories
2'-Deoxyadenosine 5'-triphosphate or dATP ($C_{10}H_{12}N_5O_{12}P_3Na_4$)	Promega
2'-Deoxycytidine 5'-triphosphate or dCTP ($C_9H_{12}N_3O_{13}P_3Na_4$)	Promega
2'-Deoxyguanosine 5'-triphosphate or dGTP ($C_{10}H_{12}N_5O_{13}P_3Na_4$)	Promega
2'-Deoxythymidine 5'-triphosphate or dTTP ($C_{10}H_{13}N_2O_{14}P_3Na_4$)	Promega
Dimethyl Sulfoxide (DMSO)	Sigma
Ethidium bromide	Bio-Rad
Ethylenediamine tetraacetic acid or EDTA ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	USB
FBS	PERBIO
Glycerol ($C_3H_8O_3$)	BHD
Hydrochloric acid (HCl)	Merck
Isopropanol ($CH_3CHOHCH_3$)	BHD
β-Mercaptoethanol ($HSCH_2CH_2OH$)	Fluka chemika
N, N'-methylene-bis-acrylamide ($C_7H_{10}N_2O_2$)	Sigma

N, N, N', N'-tetrametyl-ethylenediamine	Bio-Rad
Or TEMED ($C_{14}H_{11}NO_3$)	
Phenylmethylsulfonyl fluoride (PMSF)	Sigma
Sodium chloride (NaCl)	Merck
Sodium hydroxide (NaOH)	Merck
di-sodium hydrogen phosphate anhydrous (Na_2HPO_4)	Merck
Sodium dodecyl sulfate or SDS ($C_{12}H_{25}O_4SNa$)	Sigma
Tris (Hydroxymethyl aminomethane)	Sigma
Tryptone (Pancreatic Digest of casein)	Difco
Yeast extract	Difco

2. Reagents for PCR reaction and agarose gel electrophoresis

2.1 0.5 M EDTA (pH 8.0)

A 186.1 g of EDTA.Na_2.2H_2O was added into 800 ml of deionized water. The solution was stirred on a magnetic stirrer and adjusted pH to 8.0 with NaOH. The solution was adjusted the final volume to 1 liter with deionized water.

2.2 50x concentrated stock solution of Tris-acetate-EDTA (TAE) electrophoresis buffer

To 800 ml of distilled water, add:

Tris base	242.0	g
Glacial acetic acid	57.1	ml
0.5 M EDTA (pH 8.0)	100	ml

The mixture was shaken until the solutes dissolved and adjusted the volume of solution to 1 liter with distilled water. It was diluted with deionized water just before usage.

2.3 1% Agarose gel in 1 X TAE buffer

Agarose gel	1.0	g
1X TAE buffer	100	ml

The mixture was heated in microwave oven until agarose gel was completely dissolved, poured it into gel tray. Comb was placed to create well.

2.4 6X loading dye

Glycerol	3	ml
Xylene cyanol FF	0.025	g
Bromophenol blue	0.025	g

These chemicals were mixed and dissolved in 10 ml of distilled water.

3. Solution and buffers for SDS-PAGE and Western blotting

3.1 30.8% (w/v) Acrylamide-Bisacrylamide

To 80 ml of distilled water, add:

Acrylamide	30.0	g
Bis-acrylamide	0.8	g

The mixture was stirred on magnetic stirrer until dissolved and adjusted to the final volume to 100 ml. The solution was filtrated through 125 mm diameter-filtered paper (Whatman No. 1)

3.2 Resolving gel buffer pH 8.8: 3 M Tris-HCl

Tris	36.3	g
1 M HCl	48	ml

Tris was dissolved in 1 M HCl and adjusted pH to 8.8. The solution was adjusted the final volume to 100 ml with distilled water.

3.3 10% (w/v) Sodium dodecyl sulphate

Sodium dodecyl sulphate (SDS) 10 g was dissolved in 100 ml distilled water and stored at room temperature.

3.4 10% (w/v) Ammonium persulfate

Ammonium persulfate 1.0 g was dissolved in distilled water and adjusted the final volume to 10 ml.

3.5 Stacking gel buffer pH 6.8: 0.5 M Tris-HCl

Tris	6.0	g
1 M HCl	48	ml

Tris was dissolved in 1 M HCl and adjusted pH to 6.8 with 1 M HCl.

The mixture was adjusted the final volume tto 100 ml with deionized water.

3.6 10% Resolving gel of SDS-PAGE

30.8% (w/v) Acrylamide-bisacrylamide	1.67	ml
3.0 M Tris-HCl (pH 8.8)	0.630	ml
10% SDS	0.050	ml
10% Ammonium persulfate	0.038	ml
Distilled water	2.63	ml
TEMED	0.0025	ml

These solutions were mixed, loaded into a protein gel electrophoresis set and poured distilled water to on surface of gel to protect gel oxidation. Water was poured off when gel was completely set for 30-45 minutes.

3.7 3.85% Stacking gel of SDS-PAGE

30.8% (w/v) Acrylamide-bisacrylamide	0.250	ml
0.5 M Tris-HCl (pH 6.8)	0.500	ml
10% SDS	0.020	ml
10% Ammonium persulfate	0.015	ml
Distilled water	1.22	ml
TEMED	0.0015	ml

These solutions were mixed and loaded into a protein gel electrophoresis set. Gel was completely set for 30-45 minutes.

3.8 2X Leammli sample buffer

Per 10 ml:

β-mercaptoethanol	1	ml
10% SDS	6	ml
80% glycerol	2.5	ml
Stacking buffer	100	µl
5% bromophenol blue	100	µl
Distilled water	0.3	ml

These chemicals were homogeneously mixed and stored at 4°C.

3.9 100 mM PMSF

0.09 of PMSF was dissolved in 5 ml of absolute ethanol.

3.10 10X Running buffer pH 8.3 (0.25 M Tris-HCl, 1.92 M Glycine, 1% (w/v) SDS)

To 900 ml of distilled water, add:

Tris	30.3	g
Glycine	144.0	g
SDS	10.0	g

These chemicals were dissolved and adjusted the final volume to 1 liter with deionized water. The solution was diluted to 1X with deionized water just before usage.

3.11 Towbin buffer (25 Mm Tris, 192 mM Glycine, 20 %(v/v) Methanol, 0.1%SDS, pH 8.3)

To 600 ml of distilled water, add:

Tris	3	g
Glycine	14.4	g
SDS	1	g

The mixture was dissolved. A 200 ml of methanol was added, adjusted the final volume to 1 liter with distilled water and stored at 4°C.

3.12 Blocking buffer

5 g of skimmed milk was dissolved in 100 ml of 1X PBS.

3.13 Phophate Buffered-Saline (PBS), pH 7.4

To 900 ml of the distilled water, add:

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_4PO_4	0.2	g

These chemicals were mixed, dissolved in distilled water, and adjusted the final

volume to 1 liter.

3.14 Destaining solution

Per liter:

Methanol	50	ml
Glacial acetic acid	75	ml

Theses solutions were mixed and adjusted the final volume to 1 liter.

4. Reagents for mammalian cell line transfection

4.1 DEAE-Dextran

10 mg/ml DEAE Dextran in PBS 10 ml

DEAE Dextran	0.1	g
PBS	10	ml

Stir until the reagents are completely dissolved. Filter through a 0.22 µm membrane and make aliquots (500 µl/tube)

4.2 Chloroquine

10 mM Chloroquine in PBS 20 ml

Chloroquine	0.1	g
PBS	20	ml

Stir until the reagents are completely dissolved. Filter through a 0.22 µm membrane and make aliquots (500 µl/tube)**.**

5. Reagents for DNA extraction from *E. coli*

5.1 Resuspended buffer (P1 buffer)

Per 100 ml:

Tris	0.606	g
EDTA	0.372	g

The mixture was dissolved in distilled water and adjusted pH to 8.0. After the solution was dissolved, volume was adjusted the final volume to 100 ml.

5.2 Lysis buffer (P2 buffer)

Per 100 ml:

1N NaOH	20	ml
20% SDS	5	ml

Theses solution were mixed and adjusted the final volume to 100 ml.

5.3 Neutralizing buffer (P3 buffer)

Per 100 ml:

Potassium acetate	29.45	g
Glacial acetic acid	11	ml

Theses chemicals were dissolved in 80 ml distilled water and adjusted pH to 5.5. Then it was adjusted to the final volume to 100 ml.

6. Growth media

6.1. Bacterial media

6.1.1. Low salt Luria-Bertani plate (LB plate)

Per liter:

To 950 ml of distilled water, add:

Peptone	10	g
Yeast extract	5	g
NaCl	10	g
Agar	15	g

The mixture was shaken until the solutes dissolved and adjusted the final volume of solution to 1 liter with distilled water. This media was sterilized by autoclaving for 20 minutes.

6.1.2 Low salt Luria-Bertani broth (LB broth)

LB broth medium was made the same way as LB plate, leaving out the agar.

6.1.3 Luria-Bertani media with ampicillin

When the medium cool down to approximately 50°C, 100 mg/ml ampicillin was added to LB medium to a final concentration of 50 μg/ml.

6.2 Mammalian cell line media

6.2.1 Stock DMEM

Per liter:

Dulbecco's Modified Eagle Medium	13.4	g
$NaHCO_3$	3.7	g

Stir until the solution was completely dissolved. Filter through a 0.22 µm membrane and 0.5 µm membrane by using sterile media filter set

6.2.2 Complete DMEM

Per liter:

Stock DMEM	900	ml
FBS	100	ml
60.79 U/ml of penicillin G/ 60 ug/ml of streptomycin	12	ml

6.3 Cell dissociation solution

6.3.1 2.5 mM EDTA in PBS

Per 500 ml:

EDTA.NA2.2H_2O	0.4653	g

This chemical was dissolved in PBS, pH 7.4, stirred, and adjusted at a final volume to 500 ml in volumetric flask. The reagent was sterilized by autoclaved at 121 °C for 15 min.

6.3.2 Trypsin solution (10% Trypsin in 2.5 mM EDTA/PBS)

Per 20 ml:

Trypsin	2	g

This chemical was dissolved in 20 ml of 2.5 mM EDTA/PBS, stirred until completely dissolved, and sterilized by filtrated through 0.2 µM cellulose acetate filter membrane. The reagent was diluted to the desire concentration with sterile 2.5 mM EDTA/PBS before use.

BIOGRAPHY

NAME	Miss. Wandee Udomchaiprasertkul
DATE OF BIRTH	15 MAY 1980
PLACE OF BIRTH	Chonburi, Thailand
INSTITUTION ATTENDED	Burapha University, 1998-2002 Bachelor of Science (Microbiology) Mahidol University, 2002-2006 Master of science (Immunology)
RESEARCH GRANT	Graduate Followship Programme, National Science and technology Development Agent (NSTDA), Thailand Siriraj Research Grant for Graduate Studies, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand
POSTER PRESENTATION	A part of this project will be presented as a poster entitled “Molecular Defect of Distal Renal Tubular Acidosis Caused by Compound Heterozygous Anion Exchanger 1 Mutations” in Siriraj-Rama Conference 2006
HOME ADDRESS	3/10 Success Soi, Sriracha, Chonburi, Thailand. Email: wandeee15@yahoo.com