INTERACTION, TRAFFICKING AND SUBCELLULAR LOCALIZATION OF MUTANT KIDNEY ANION EXCHANGER 1 (kAE1) PROTEINS IN CULTURED HUMAN EMBRYONIC KIDNEY 293 (HEK 293) CELLS

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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 974-04-7328-8

Thesis

entitled

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ACKNOWLEDGEMENT

A journey, especially of our lives, would be easier when we travel together and in accompany with a good guide. However, interdependence is certainly superior to independence. During my full five years of a Master-degree education and thesis work, I have been accompanied and supported by many people. It therefore is a great pleasure that I am now taking this opportunity to express my appreciation and gratitude to them all.

I would like to acknowledge with great appreciation to all those who gave me the opportunity to complete my Master degree and this thesis. Especially, with a deep sense of gratitude, I would like to express my sincere gratitude and appreciation to my major advisor, Prof. Dr. Pa-thai Yenchitsomanus, who mercifully offered a continuous advice, excellent guidance, and immense help in planning, and executing this thesis in time, and also flawless grammatical editing of this thesis. His immensely enthusiasm and integral view on research and his mission for providing "only high-quality work and not less", has made a deep impression on me. I owe him lots of gratitude for showing me the way to conduct a high quality research. He may not even realize how much I have learned from him.

I would also like to extend my deep and sincere gratitude to my two thesis co-advisors, Asst. Prof. Dr. Varaporn Akkarapatumwong and Lect. Dr. Sansanee Noisakran, for their invaluable suggestion, consultation and correction of this thesis. I am also grateful to Asst. Prof. Dr. Pimchai Chaiyen, the external thesis examination committee member, for her invaluable comment and correction of this thesis. I extremely thank Dr. Prida Malasit, the Head of the Division of Medical Molecular Biology (DMMB) and BIOTEC-Medical Biotechnology Unit (BMBU), for his enormous supports.

I am highly indebted to Miss Wandee Udomchaiprasertkul for her laboratorial assistance in both plasmid construction and confocal microscopic visualization, to Miss Thitima Keskanokwong for her consultation, suggestion and providing the plasmid. A special thank goes to Mr. Somchai Thiemmeca for giving me the technical assistance on flow cytometry. I sincerely thank to my lovely friends, Miss Atchara Paemanee, Miss Sita Kalayanarooj, Miss Piengpaga Ngaojanlar, and Miss Araya Jintaviwat for their helpfulness, entertainment and encouragement throughout my laboratory work. I am also thankful to all other members of DMMB and BMBU for their assistance and friendship. I also would like to thank the National Center for Genetic Engineering and Biotechnology (BIOTEC) under the National Science and Technology Development Agency (NSTDA) for awarding the scholarship and financial support.

Eventually, I am also now taking this opportunity to express the profound gratitude from my deepest heart to my beloved parents, my sister (niece), everyone in my family and Mr. Nattaphol Janeratha for their love, continuously spiritual support, understanding and patiently waiting for my graduation. This entire educational activity and thesis would not be possible without them all who are greatest to be grateful.

Nunghathai Sawasdee

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ABSTRACT

Mutations in *anion exchanger 1* (*AE1*) gene may lead to defects in kAE1 protein that result in autosomal dominant (AD) and autosomal recessive (AR) distal renal tubular acidosis (dRTA), a kidney disease characterized by a defect in acid secretion of α-intercalated cells located in the distal renal tubular region. The recessive *AE1* G701D mutation is frequently observed in Thai and Southeast Asian populations in homozygous condition (G701D/G701D), and also found in the form of compound heterozygote with Southeast Asian ovalocytosis (SAO) mutation (SAO/G701D), which was first identified in Thailand. The defects of kAE1 SAO and kAE1 G701D mutants have been shown to be different. The erythroid isoform of AE1 (eAE1) SAO shows an impaired anion transport activity in red cells and *Xenopus* oocytes, although it can be inserted into the plasma membrane. eAE1 G701D exhibits normal transport function in the red cells whereas eAE1 and kAE1 G701D lack transport activity and cell surface expression in oocytes, which can be corrected and rescued by co-expression with glycophorin A (GPA). In cultured mammalian cells, individually expressed kAE1 SAO and kAE1 G701D are retained intracellularly, showing trafficking defect. Although expression and trafficking of kAE1 SAO and G701D have been individually studied in cultured mammalian cells, no data exists on the study of co-expression between kAE1 SAO and G701D. To understand the situation that mimics compound heterozygous *AE1* SAO/G701D condition resulting in AR dRTA, this study examined the trafficking and subcellular localization of the expressed and co-expressed versions kAE1, of wild-type kAE1, kAE1 SAO and G701D, and also studied the interactions between wild-type kAE1 and kAE1 SAO, wild-type kAE1 and kAE1 G701D, and kAE1 SAO and kAE1 G701D in transiently transfected human embryonic kidney 293 (HEK 293) cells. Plasmids containing either wild-type or mutant *kAE1* genes all fused with the sequence of either six-histidine (6xHis), hemagglutinin (HA) or Myc epitope tag were constructed to be used in experiments. The wild-type and mutant kAE1 proteins fused with the epitope tags were expressed in HEK 293 cells, as detected by Western-blot method. When they were individually expressed, the wild-type kAE1 was localized at the cell surface, while the mutant kAE1 SAO and kAE1 G701D were intracellularly retained. Co-expressions of wild-type kAE1 with either kAE1 SAO or G701D demonstrated that they could interact to form heterodimers, and that the wild-type kAE1 could rescue the two mutant kAE1 to be expressed on the cell surface. The co-expression of kAE1 SAO and G701D showed that they could also interact but were intracellularly retained. This would most likely be a molecular mechanism of AR dRTA, associated with compound heterozygous *AE1* SAO/G701D mutation in the patients, especially in the Southeast Asian populations.

KEY WORDS: ANION EXCHANGER 1 (AE1) / DISTAL RENAL TUBULAR ACIDOSIS (dRTA) / COMPOUND HETEROZYGOTE / SUBCELLULAR LOCALIZATION / PROTEIN TRAFFICKING

117 P. ISBN 974-04-7328-8

ปฏิสัมพันธ์การเคลื่อนย้ายและตำแหน่งในเซลล์ของโปรตีนแอนไอออนเอ็กเช็งเจอร์-วันชนิดที่พบในไตที่ผิดปกติในเซลล์ไตเพาะเลี้ยงเอ็ชอีเค 293 [INTERACTION, TRAFFICKING AND SUBCELLULAR LOCALIZATION OF MUTANT KIDNEY ANION EXCHANGER 1 (kAE1) PROTEINS IN CULTURED HUMAN EMBRYONIC KIDNEY 293 (HEK 293) CELLS]

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บทคัดย่อ

มิวเตชั่นของยีน *anion exchanger* 1 (*AE1*) สามารถทำให้เกิดความผิดปกติที่โปรตีน anion exchanger 1 ชนิดที่พบในไต (หรือ kAE1) และทำให้เกิดโรคไตผิดปกติในการขับกรด ที่มีการถ่ายทอดทั้งแบบลักษณะเด่นและแบบลักษณะด้อย โรคไตชนิดนี้เกิดจากความบกพร่องในการขับกรดของเซลล์ที่ส่วนปลายของท่อฝอยในไต มิวเตชั่นของยีน *AE1* ชนิด G701D มีการถ่ายทอดทางพันธุกรรมแบบลักษณะด้อย พบได้บ่อยในคนไทยและประชากรในเอเชียอาคเนย์ ในภาวะโฮโมซัยโกต (homozygote) และมิวเตชั่นนี้ยังพบเกิดร่วมกับมิวเตชั่นของยีน *AE1* ชนิด SAO ในภาวะคอมเปาต์เฮ็ตเทอโรซัยโกต (compound heterozygous *AE1* SAO/G701D) ซึ่งพบครั้งแรกในประเทศไทย อีกด้วย ความผิดปกติของโปรตีน AE1 ที่เกิดจากมิวเตชั่นชนิด SAO และ G701D นั้นแตกต่างกัน โปรตินผิดปกติชนิด SAO ไม่สามารถแลกเปลี่ยนไอออนได้ในเม็ดเลือดแดงและไข่กบ แม้ว่าโปรตีนนี้จะสามารถเคลื่อนที่มาที่เยื่อหุ้มเซลล์ได้ก็ตาม ส่วนโปรตีนผิดปกติชนิด G701D สามารถแลกเปลี่ยนไอออนได้ปกติในเม็ดเลือดแดง แต่ไม่สามารถแลกเปลี่ยนไอออนและไม่สามารถเคลื่อนที่มาที่เยื่อหุ้มเซลล์ได้ในไข่กบ อย่างไรก็ตาม ความผิดปกติในการแลก เปลี่ยนไอออนและการแสดงออกที่เยื่อหุ้มเซลล์ของโปรตีนในไข่กบ กลับเป็นปกติได้ เมื่อมีการแสดงออกร่วมกับ glycophorin A (GPA) ในเซลล์เพาะเลี้ยงจากสัตว์เลี้ยงลูกด้วยนม โปรตีนผิดปกติชนิด SAO และ G701D ที่มีการแสดงออกแบบเดี่ยว มีความผิดปกติเนื่องจากการคั่งค้างของโปรตีนภายในเซลล์ แสดงถึงความผิดปกติในการเคลื่อนย้ายของโปรตีน แม้ว่าการแสดงออกและการเคลื่อนที่ของโปรตีนผิดปกติชนิด SAO และ G701D จะมีการศึกษาในเซลล์เพาะเลี้ยงจากสัตว์เลี้ยงลูกด้วยนม แต่ก็ยังไม่มีข้อมูลที่ทำการศึกษาการแสดงออกของโปรตีนผิดปกติชนิด SAO และ G701D ร่วมกัน เพื่อที่จะให้เข้าใจสถานภาพจำลองของภาวะมิวเตชั่นแบบคอมเปาต์เฮ็ตเทอโรซัยโกตของ *AE1* SAO/G701D ที่ทำให้เกิดโรคไตผิดปกติในการขับกรดที่ถ่ายทอดแบบลักษณะด้อย ดังนั้นการศึกษานี้จึงได้ตรวจหาการเคลื่อนย้ายและตำแหน่งที่อยู่ภายในเซลล์ที่มีการแสดงออกแบบเดี่ยวของโปรตีนผิดปกติชนิด SAO และ G701D และยังศึกษาปฏิสัมพันธ์กันระหว่างโปรตีนปกติกับโปรตีนผิดปกติชนิด SAO หรือ G701D โดยเฉพาะปฏิสัมพันธ์ระหว่างโปรตีนผิดปกติชนิด SAO กับ G701D ในเซลล์ไตเพาะเลี้ยงชนิดเอ็ชอีเค 293 อีกด้วย พลาสมิดที่มียีน *AE1* แบบปกติหรือแบบผิดปกติซึ่งติดอยู่กับลำดับนิวคลีโอไทด์ของ six-histidine (6xHis), hemagglutinin (HA) or Myc ถูกสร้างขึ้นเพื่อใช้ในการทดลอง โปรตีนชนิดปกติและผิดปกติที่ถูกทำให้มีการแสดงออกในเซลล์ไตเพาะเลี้ยงเอ็ชอีเค 293 สามารถตรวจหาโปรตีนด้วยวิธี Western blot ได้ เมื่อทำให้มีการแสดงออกของโปรตีนแต่ละชนิดแบบเดี่ยว พบว่า โปรตีนปกติอยู่บนเยื่อหุ้มเซลล์ ส่วนโปรตีนผิดปกติชนิด SAO และ G701D ค้างอยู่ภายในเซลล์ เมื่อทำให้มีการแสดงออกร่วมกันของโปรตีนปกติกับโปรตีนผิดปกติชนิด SAO หรือ G701D พบว่าโปรตีนเหล่านี้สามารถมีปฏิสัมพันธ์กันและสามารถปรากฏที่เยื่อหุ้มเซลล์ แสดงว่าโปรตีนปกติช่วยทำให้โปรตีนที่ผิดปกติทั้งสองชนิดเคลื่อนย้ายไปยังเยื่อหุ้มเซลล์ ส่วนโปรตีนผิดปกติชนิด SAO และ G701D ที่ทำให้มีการแสดงออกพร้อมกันภายในเซลล์ยังคงมีปฏิสัมพันธ์กัน แต่ค้างอยู่ด้วยกันภายในเซลล์ ซึ่งน่าจะเป็นคำอธิบายถึงกลไกที่ทำให้เกิดโรคไตผิดปกติในการขับกรดที่มีการถ่ายทอดแบบลักษณะด้อย จากมิวเตชั่นแบบคอมแปาต์เฮ็ตเทอโรซัยโกตของ *AE1* SAO/G701D ที่พบในประชากรแถบเอเชียอาคเนย์

117 หน้า. ISBN 974-04-7328-8

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LIST OF ABBREVIATIONS

AD	=	autosomal dominant
AE1	=	anion exchanger 1
AR	=	autosomal recessive
bp	=	base pair
BSA	=	bovine serum albumin
°C	=	degree Celsius
CAII	=	carbonic anhydrase II
cDNA	=	complementary DNA
Cl^-	=	chloride
C-terminal	=	carboxy terminal
C-terminus	=	carboxy terminus
Cy3	=	cyanine 3
dATP	=	deoxyadenosine-5'-triphosphate
dCTP	=	deoxycytidine-5'-triphosphate
dGTP	=	dexoyguanosine-5'triphosphate
dTTP	=	deoxythymidine-5'-triphosphate
dNTP	=	dATP, dCTP, dGTP, dTTP
del	=	deletion
DIDS	=	4,4'-diisothiocyanostilbene-2,2'-disulfonate
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	dimethysulfoxide
DNA	=	deoxyribonucleic acid
dRTA	=	distal renal tubular acidosis
eAE1	=	erythroid anion exchanger 1
EDTA	=	ethylenediaminetetraacetic acid
ER	=	endoplasmic reticulum

LIST OF ABBREVIATIONS (cont.)

FACS	=	fluorescence activated cell sorting
FBS	=	fetal bovine serum
FITC	=	fluoresceine isothiocyanate
g	=	gram (s)
x g	=	gravity centrifugal force
GPA	=	glycophorin A
h	=	hour
H^+	=	acid or hydrogen ion
HA	=	hemagglutinin
HCO_3^-	=	bicarbonate ion
H_2DIDS	=	4,4'-diidothiocyanodihydrostilbene-2,2'-disulfonate
HEK	=	human embryonic kidney
His	=	histidine
H^+-ATPase	=	hydrogen adenosine triphosphatase
H^+/K^+-ATPase	=	hydrogen/potassium adenosine triphosphatase
Ig(s)	=	immunoglobulins
kAE1	=	kidney anion exchanger 1
kb	=	kilobase
kDa	=	kiloDalton
LB	=	Luria-Bertani broth
M	=	Molar
MDCK	=	Madin-Darby canine kidney
mg	=	milligram (s)
min	=	minute
ml	=	milliliter (s)
mM	=	millimolar
mmol	=	millimole

LIST OF ABBREVIATIONS (cont.)

MT	=	mutant
MW	=	molecular weight
µg	=	microgram (s)
µl	=	microliter (s)
µM	=	micromolar
NaOAc	=	sodium acetate
ng	=	nanogram
nmol	=	nanomole
NBC	=	sodium bicarbonate co-transporter
nt	=	nucleotide (s)
N-terminal	=	amino terminal
N-terminus	=	amino terminus
OD	=	optical density
PBS	=	phosphate-buffered saline
PCR	=	polymerase chain reaction
PDZ	=	PSD-95/Dlg/ZO-1 domain
pH	=	power of hydrogen
pmol	=	picomole
RBCs	=	red blood cells
RNase	=	ribonuclease
Sec	=	second (s)
SAO	=	Southeast Asian ovalocytosis
SDS	=	sodium dodecyl sulfate
SDS-PAGE	=	SDS-polyacrylamide gel electrophoresis
SLC	=	solute carrier family
TAE	=	tris-acetate-EDTA
Taq	=	*Thermus aquaticus*
TBST	=	Tris-Buffered Saline Tween-20
TEMED	=	N,N,N',N'-tetramethylethylenediamine

LIST OF ABBREVIATIONS (cont.)

TM	=	transmembrane
TMs	=	transmembrane segments
U	=	unit (s)
UV	=	ultraviolet
WT	=	wild-type

CHAPTER I

INTRODUCTION

Hereditary distal renal tubular acidosis (dRTA) is a renal disease characterized by defective acid secretion of α-intercalated cells located at the epithelial lining of distal tubular region of nephron, resulting in systemic metabolic acidosis and other accompanying clinical manifestations including hypokalemia, muscle weakness, impaired growth and development, rickets, and nephrocalcinosis (1-4). This disease can be caused by defect of H^+-ATPase responsible for acid or proton (H^+) secretion at apical membrane or by abnormality of kidney anion exchanger 1 (kAE1) that exchanges chloride/bicarbonate (Cl^-/HCO_3^-) at basolateral membrane of the α-intercalated cells (5, 6). The defect of kAE1 will lead not only to failure of basolateral bicarbonate extrusion and reabsorption into circulation but also to failure of apical acid secretion by the α-intercalated cells. kAE1 is a truncated isoform of erythroid AE1 (eAE1 or band 3) protein that interacts with cytoskeleton proteins to maintain the flexible biconcave disc-shape of the erythrocytes (7) and mediates Cl^-/HCO_3^- exchange in red blood cells (8). Both eAE1 and kAE1 are encoded by *SLC4A1* or *AE1* gene located on chromosome 17q21-q22 (9-12). Mutations of this gene may result in abnormal red blood cells and/or dRTA. The *AE1* mutations associated with dRTA may be inherited in either autosomal dominant (AD) or autosomal recessive (AR) fashion (13, 14).

The reported *AE1* mutations associated with AD dRTA are R589H, R589S, R589C, G609R, S613F, A888L+889X and R901X, which were normally observed in heterozygous conditions, while those associated with AR dRTA consisting of G701D, V858M and ΔV850, which were found in homozygous conditions (14). Since Southeast Asian ovalocytosis (SAO) is a common *AE1* mutation in the Southeast Asian populations, several genotypes of AR dRTA were compound heterozygous conditions between SAO and other mutations including SAO/G701D, SAO/ΔV850,

SAO/A858D, and SAO/R602H. Moreover, other compound heterozygous genotypes, G701D/S773P and ΔV850/V858D, were also reported to cause AR dRTA (14).

Several AE1 mutant proteins have been examined for anion transport function and protein trafficking in red blood cells, *Xenopus* oocytes, and cultured mammalian cells. The variants of R589 showed mild to moderate reduction in sulfate transport in red blood cells (15, 16), Cl^-/Cl^- transport in *Xenopus* oocytes (16), and exhibited trafficking defect and intracellular retention in human embryonic kidney 293 (HEK 293) cells (17, 18). AE1 901X exhibited normal sulfate activity in red blood cells but retained in an intracellular compartment in HEK 293 cells (19) and also mis-targeted to the apical membrane in polarized Mardin-Darby canine kidney (MDCK) cells (20). Co-expression of wild-type AE1 with either AE1 R589H or R901X mutant protein resulted in retention of the wild-type protein in intracellular compartment (17, 18). These results suggest that AD dRTA associated with *AE1* mutations is not attributed by the reduction of anion transport activity but by impaired trafficking of AE1 mutant proteins to the basolateral membrane of the cells or mis-targeting of the mutant protein to the apical membrane (21). In addition, hetero-oligomerization of wild-type and mutant protein leads to intracellular retention of both wild-type and mutant protein, to be known as 'dominant negative effect', is likely responsible for the AD dRTA phenotype (17-19, 21).

Homozygous *AE1* G701D mutation associated with AD dRTA was first reported in two Thai sibs (22) and also observed in other Thai pediatric patients (23). eAE1 G701D exhibits normal anion transport function in the red blood cells while eAE1 and kAE1 G701D lacked anion transport function and cell surface expression in *Xenopus* oocytes. However, this defective anion transport function and cell surface expression could be corrected and rescued by the co-expression with glycophorin A (GPA), an erythroid integral membrane and eAE1 chaperone protein, indicating that eAE1 and kAE1 G701D requires GPA for trafficking to the cell surface (22). The defect in the alpha-intercalated cells may be similar as they have no detectable GPA. As a result, kAE1 G701D may not be able to accumulate at the cell surface of these cells although its function is still normal and thus impaired trafficking is likely to be molecular mechanism associated with this mutation.

In the study of a novel compound heterozygote of G701D/S773P reported in Thai patient with AR dRTA (24), it was found that while kAE1 S773P showed a low level of protein expression and a decreasing half-life compared with wild-type AE1 and it was also retained in endoplasmic reticulum (ER), kAE1 G701D exhibited defective trafficking to the plasma membrane and was retained in the Golgi in non-polarized HEK 293 and LLC-PK1 cells and polarized MDCK cells (25, 26). kAE1 G701D and S773P could form either homodimer or heterodimer with wild-type or with each other. Hetero-oligomers of wild-type kAE1 with kAE1 G701D or S773P, in contrast to the dominant kAE1 R589H mutant, could be delivered to the plasma membrane. These results indicated that the wild-type kAE1 could rescue kAE1 G701D and S773P trafficking or the wild-type kAE1 shows a 'dominant-positive effect' in relation to the kAE1 mutant proteins (14).

The cases of dRTA with ovalocytosis were originally reported in Thai patients who carried compound heterozygous SAO/G701D mutations (27), which were also found later by other research group (Bruce 2000). Southeast Asian ovalocytosis (SAO), an in-frame 9-amino-acid deletion at the boundary between cytoplasmic and membrane domains of AE1, is occurred from a mutation causing a deletion of 27 base-pairs in codons 400-408 of *AE1* exon 11. The heterozygous *AE1* SAO mutation is not sufficient to cause dRTA (27, 28). The transport function of AE1 SAO was found to be impaired in red blood cells and oocytes (28, 29) although the protein could be inserted into the plasma membrane (30). The effect of the SAO deletion on stability and trafficking of AE1 and kAE1 was examined in transfected HEK 293 and MDCK cells (31). In HEK 293 cells, expression levels and stabilities of SAO proteins were significantly reduced, and the mutant protein was retained intracellularly with its absence at the cell surface, suggesting that erythroid-specific factors missing in HEK cells may be required for cell-surface expression. Although misfolded, SAO proteins could form heterodimers with the normal proteins, as well as homodimers. In MDCK cells, kAE1 SAO was also retained intracellularly, while kAE1 was localized to the cell surface or the basolateral membrane after polarization. When kAE1 SAO was co-expressed with kAE1 in MDCK cells, kAE1 SAO was retained intracellularly; however, it also co-localized with kAE1 at the cell surface. Thus, it was proposed that in the kidney of heterozygous SAO individuals, dimers of

kAE1 and heterodimers of kAE1 SAO and kAE1 traffic to the basolateral membrane of the α-intercalated cells, while homodimers of kAE1 SAO are retained in the endoplasmic reticulum and rapidly degraded. This would result in sufficient expression of kAE1 to maintain adequate bicarbonate reabsorption and proton secretion without dRTA.

Although the expression and trafficking of kAE1 SAO and G701D were individually studied in cultured mammalian cells, no data exists on the study of co-expression between kAE1 SAO and G701D in the cultured mammalian cells to examine the situation that mimics compound heterozygous *AE1* SAO/G701D condition resulting in AR dRTA. In order to examine expression and co-expression of the wild-type kAE1, kAE1 SAO, and kAE1 G701D, the mammalian expression plasmids containing the wild-type and mutant *kAE1* fused with sequences of epitope tags including hemagglutinin (HA), six-histidine (6xHis), and Myc were constructed for transfection and co-transfection into HEK 293 cells. The wild-type and mutant kAE1 fusion proteins would be then examined for their expression, interaction, trafficking and subcellular localization by Western blotting, co-immunoprecipitation, His-tagged protein co-purification, fluorescence activated cell sorting (FACS) analysis, and indirect immunofluorescence techniques. The findings from this study would provide a better understanding of the molecular mechanism of AR dRTA caused by compound heterozygous *AE1* SAO/G701D mutations.

CHAPTER II

OBJECTIVES

The objectives of this study are:

1. To examine expression, trafficking, and subcellular localization of individually expressed wild-type kAE1, kAE1 SAO, or kAE1 G701D in human embryonic kidney 293 (HEK 293) cells,
2. To examine interaction, trafficking, and subcellular localization of co-expressed wild-type kAE1 and kAE1 SAO or kAE1 G701D in HEK 293 cells,
3. To examine interaction, trafficking, and subcellular localization of co-expressed kAE1 SAO and kAE1 G701D in HEK 293 cells to mimic the compound heterozygous *AE1* SAO/G701D condition.

CHAPTER III

LITERATURE REVIEW

3.1 Hereditary distal renal tubular acidosis (dRTA) and anion exchanger 1 (AE1)

Hereditary distal renal tubular acidosis (dRTA) is a kidney disease caused by a defect in the secretion of hydrogen (H^+) ions in the distal renal tubule (the late portion of the kidney tubule). This causes an accumulation of acid and a reduction of bicarbonate in the bloodstream. The acidic condition of the body causes calcium to dissolve from the bones. The high level of calcium in the bloodstream and the excessive serum calcium is excreted by the kidneys, causing a loss of total body calcium and resulting in rickets or osteomalacia, impaired growth of children, skeletal deformities, and muscle weakness. The incidence of kidney stones and nephrocalcinosis is increased as the result of excessive excretion of calcium and phosphate through the kidneys. Hereditary dRTA can be caused by the defect of H^+-ATPase that regulates H^+ ion excretion at the apical membrane of the epithelial alpha-intercalated cells of distal tubule of nephron or by the defect of kidney anion exchanger 1 (kAE1) that mediates chloride and bicarbonate (Cl^-/HCO_3^-) exchange at the basolateral membrane of the alpha-intercalated cells (Figure 3.1). The defects of kAE1 may occur from mutations in human solute carrier family 4, anion exchanger, member 1 *(SLC4A1)* or anion exchanger 1 *(AE1)* gene, which encodes both erythroid (eAE1, band 3) and kidney (kAE1) isoforms of the AE1 protein. As *SLC4A1* is expressed in two different tissues, its mutations show pleiotrophic effect, resulting in two distinct and seemingly unrelated disorders, red cell abnormalities (e.g. hereditary spherocytosis and ovalocytosis) and dRTA.

The abnormality of kAE1 that mediates anion (Cl^-/HCO_3^-) exchange at the basolateral membrane of the α-intercalated cells can lead to a defect in HCO_3^- extrusion across this membrane resulting in its intracellular accumulation. To maintain acid-base and electroneutral balances in the cells, H^+ ions would be reserved

instead of secreting through the apical membrane. Consequently, the intracellular accumulation of both HCO_3^- and H^+ ions would inhibit the dissociation of carbonic acid (H_2CO_3) (Figure 3.1). The failure of H^+ ion secretion through the apical membrane into the tubular lumen due to kAE1 defect will eventually result in dRTA.

3.2 Molecular biology of AE1

3.2.1 *AE1* gene

The human *SLC4A1* or *AE1* gene is located on chromosome17q21-q22 and is a member of the *anion exchanger (AE)* family encoding the anion exchanger 1 (AE1 or band 3) protein (9). It spans approximately 20 kb and consists of 20 exons separated by 19 introns. The gene contains no TATA or CCAAT boxes in its upstream region (32-34) but consists of transcription factor consensus binding sites, activator protein 1 (AP1), activator protein 2 (AP2), CACCC boxes, GATA (erythroid factor 1), and E-boxes in this region. The TATA and CCAAT boxes are also found in intron 3. The human *AE1* gene is transcribed by two different promoters and contains alternative splicing: the transcript from the putative upstream promoter produces erythroid band 3 or anion exchanger 1 (eAE1), whereas that from the internal promoter in intron 3 produces kidney band 3 or anion exchanger 1 (kAE1) (35). eAE1 mRNA is transcribed from all exons comprising 4,906 nucleotides (nt), excluding its poly (A) tail(32-34). While kAE1 mRNA lacks the sequences of exons 1-3 of the eAE1 transcript and contains a part of intron 3 (designated as exon K1) in its 5'untranslated region (5' UTR) that results from alternative splicing (10-12, 33, 36). kAE1 mRNA contains complete exon 4-20 sequences with the translation initiation (ATG) site for methionine (Met) at codon 66 (10, 11). The RNA cleavage site or poly A addition site is defined at the downstream end of exon 20. A consensus poly A signal (AATAAA) is located 20 nucleotides upstream of the RNA cleavage site. The diagram of human *AE1* gene is shown in Figure 3.2.

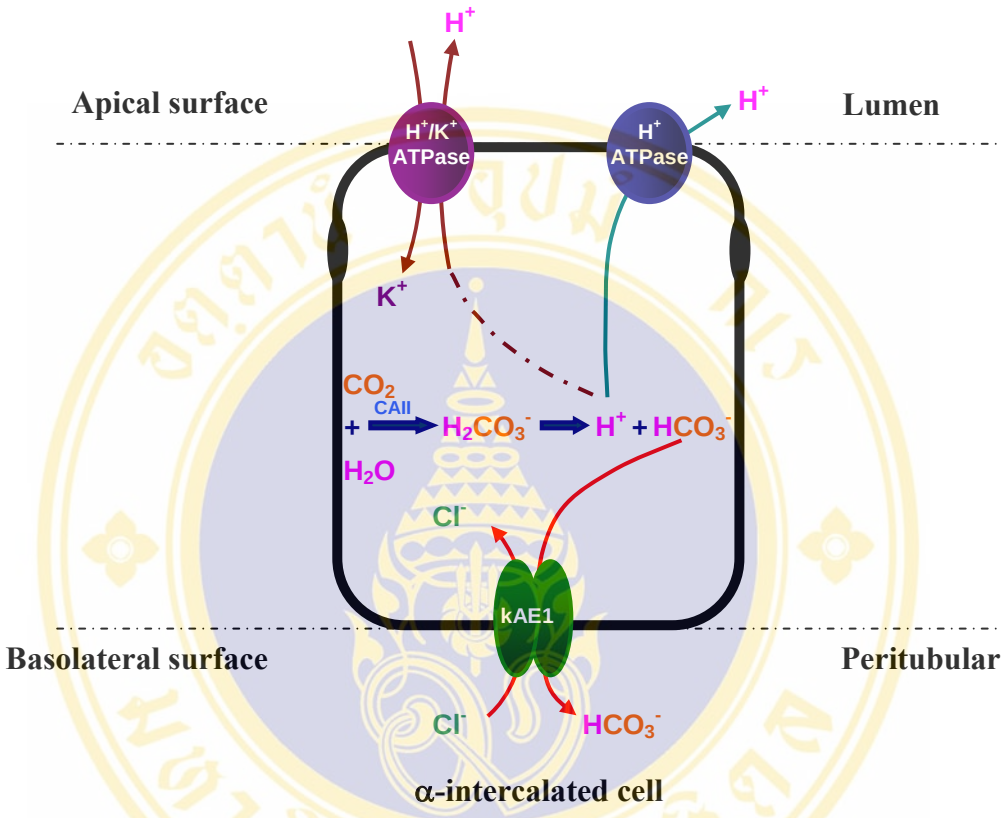


Figure 3.1 **Schematic diagram of the α-intercalated cell in the collecting tubule and ion transporters involving in acid-base balance.**

H^+-ATPase and H^+/K^+-ATPase are located at the apical membrane while kidney anion (Cl^-/HCO_3^-) exchanger 1 (kAE1) at the basolateral membrane of the α-intercalated cell. H^+ and HCO_3^- are dissociated from $H_2CO_3^-$. While H^+ is secreted through the apical membrane by H^+-ATPase and H^+/K^+-ATPase, HCO_3^- is exchanged with Cl^- to exist through the basolateral membrane by kAE1.

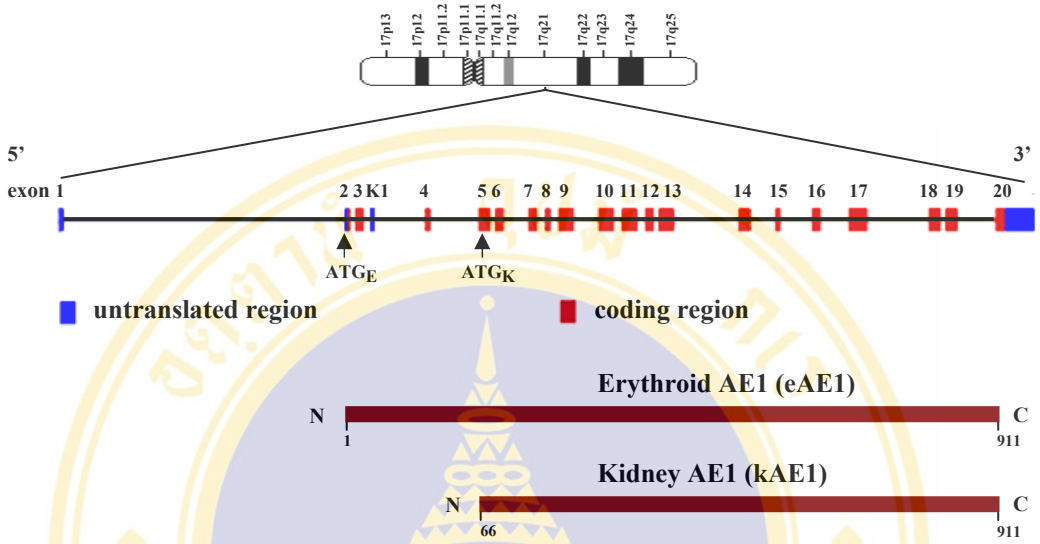


Figure 3.2 **Schematic diagram of human anion exchanger 1 (AE1) gene (modified form Yenchitsomanus *et al*. 2003)** (13)**.**

The gene is located on chromosome 17 at the region 17q1-22. It spans 20 kb and consists of 20 exons and 19 introns. The untranslated and coding regions are indicted by blue and red boxes, respectively. The same gene encodes both erythroid AE1 (eAE1) and kidney AE1 (kAE1) isoforms of AE1 by using alternative promoters and different start codon (ATG_E and ATG_K), resulting in different lengths of polypeptides. The eAE1 contains 911 amino acids while kAE1 has a truncated 65 amino acids at the N-terminus, 846 amino acids.

3.2.2 Expression and function of AE1

The human AE1 (anion exchanger 1, band 3) protein is expressed in erythrocytes and in the α-intercalated cells of the kidney distal collecting tubule. eAE1 or band 3 is a multifunctional polytopic membrane protein containing 911 amino acids (32, 34) with molecular weight (MW) of approximately 95 kDa carrying a single glycosylation site at Asn642. It is a major abundant glycoprotein of red cell membrane and expressed at a high copy number of approximately 1.2×10^6 molecules per cell. It is arranged as dimers in the membrane which can associate via the cytosolic domain to form tetramers required for bind to a cytoskeletal protein, ankyrin. This binding is critical for maintenance of the biconcave disc-shape of red blood cells (37, 38). It was found that eAE1 is present mainly as dimers in ankyrin-deficient cells (37). eAE1 serves as the critical attachment site of the peripheral membrane skeleton. It also mediates exchange of Cl^- and HCO_3^- across the phospholipid bilayer and plays a central role in respiration of carbon dioxide (CO_2) in peripheral tissues (39).

kAE1, a truncated form of eAE1 with missing the first 65 amino acids, is expressed in the basolateral membrane of acid secreting α-intercalated cells of the kidney. It provides a major exit route for HCO_3^- in exchange for Cl^- across the basolateral membrane of the α -intercalated cells during H^+ secretion through the apical membrane into the tubular lumen (11, 40). kAE1 does not bind to ankyrin (41). The carboxyl-terminal tails of eAE1 and kAE1 bind to carbonic anhydrase (CA) II, the enzyme that catalyzes the inter-conversion of H_2O and CO_2 to H_2CO_3 and its dissociation to H^+ and HCO_3^-. This interaction is required for optimal HCO_3^- transport (42, 43).

3.2.3 Protein structure of AE1

The AE1 protein comprises two major domains that are cytoplasmic (structurally) and membrane (functionally) distinct (Figure 3.3) (44). The 40-kDa amino-terminal cytosolic domain of eAE1 (M1-K360) is responsible for the binding of a number of cytosolic proteins including components of the red cell cytoskeleton (e.g. ankyrin, band 4.1, and band 4.2). This domain also binds to the glycolytic enzyme complex (45). The carboxyl-terminal membrane domain (G361-E882) of both eAE1 and kAE1 spans the membrane 12-13 times and is responsible for the anion exchange function (46, 47). Both eAE1 and kAE1 has a short acidic cytoplasmic carboxyl-terminal tail (residues L883-V911), containing binding sites for CA II (48, 49) and for protein complexes involving in the targeting of kAE1 to the basolateral membrane of kidney α-intercalated cells (20, 50). The proposed topology model of the membrane domain of human AE1 is shown in Figure 3.4 (51, 52). The structure of AE1, like most other polytopic membrane proteins with a high helical content, is thought to be based on a tightly packed helical bundle with the loops that connect the helical spans extending outside the membrane (53).

Three-dimensional structure of AE1 has been proposed (54). It is a dimer with dimensions of ~60x110 Å and a thickness of 80 Å (Figure 3.5), the U-shaped structure with an opening on the top and sides like a canyon. The basal domain is 40 Å thick and probably spans the lipid bilayer. The protrusions form the sides of converges into a depression at the center of the dimmer on the top of the basal domain. This depression may represent the opening to a transport channel located at the dimmer interface. Based on the available biochemical data, it is believed that the protrusions on the upper side of the basal domain is the cytosolic side of the membrane and the smooth surface on the lower side is extracytosolic (54).

The structure and function relationship of residues at the dimeric interface of AE1 has been studied by cysteine-directed cross-linking (55). The proposed dimeric interface relates with tentative positional constraints of TMs 1-4 and 6. The loop-loop contacts across the dimeric interface are not primarily responsible for allosteric interactions between monomers within the functional dimeric protein complex.

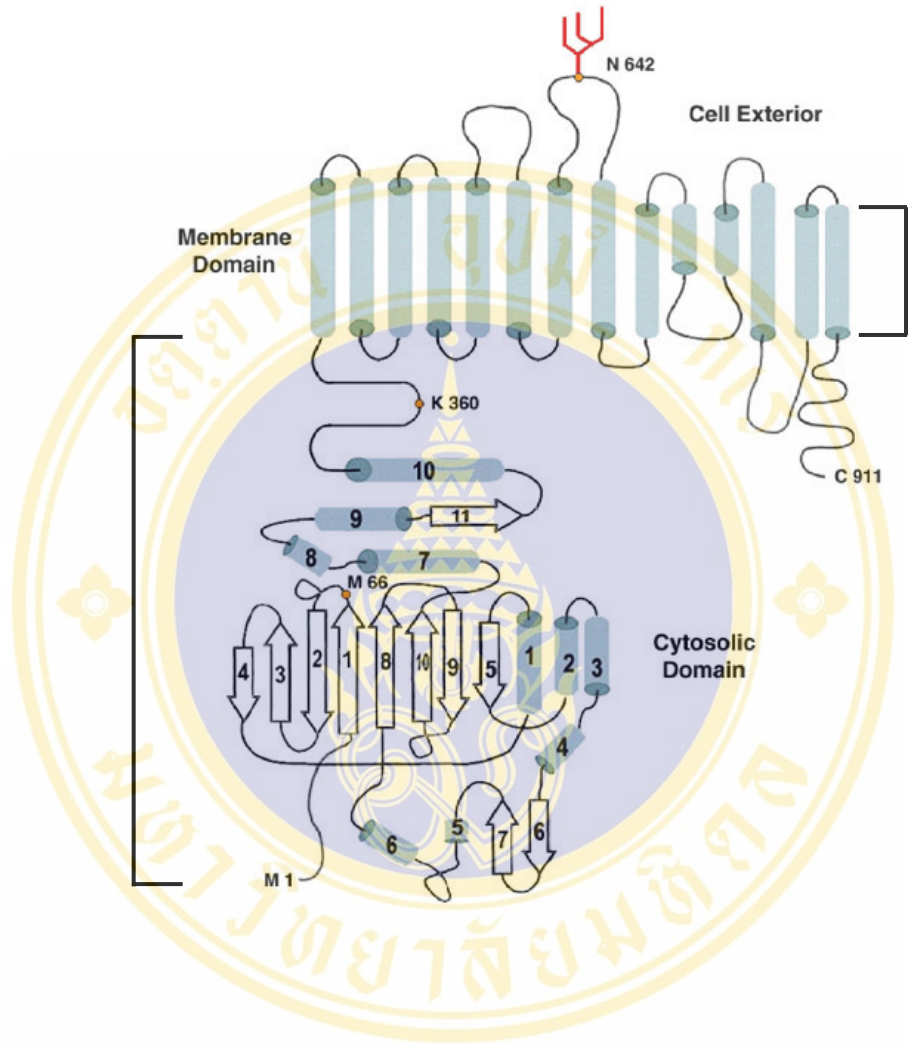


Figure 3.3 **Structural model of human AE1 (modified from Zhang *et al.* 2000)** (44).

The AE1 protein consists of two domains, amino-terminal cytoplasmic and carboxyl-terminal membrane domains. The amino-terminal cytoplasmic domain (M1-K360) comprises 11 beta-strands and 10 helical segments. The carboxyl-terminal membrane domain (G361-E882) spans the membrane 12 times. The fourth extracellular loop of membrane domain carries N-link glycosylation at the position N642. A short acidic carboxyl tail (L883-V911) is cytoplasmic.

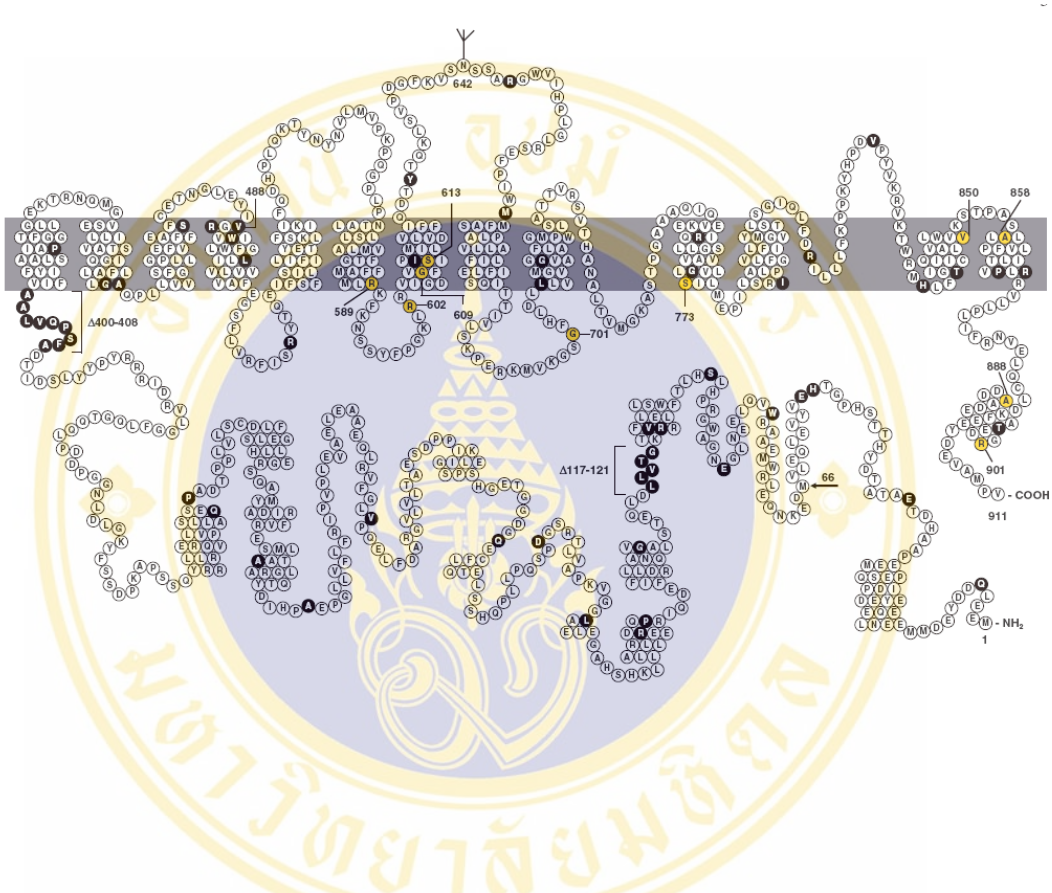


Figure 3.4 **The proposed topology model of the transmembrane domain of human kAE1 (modified from Alper 2002 and Zhu *et al.* 2003)** (51, 52)**.**

This AE1 topological model contains 13 transmembrane spans. The branched structure at N642 in the fourth extracellular loop represents N-linked glycosylation.

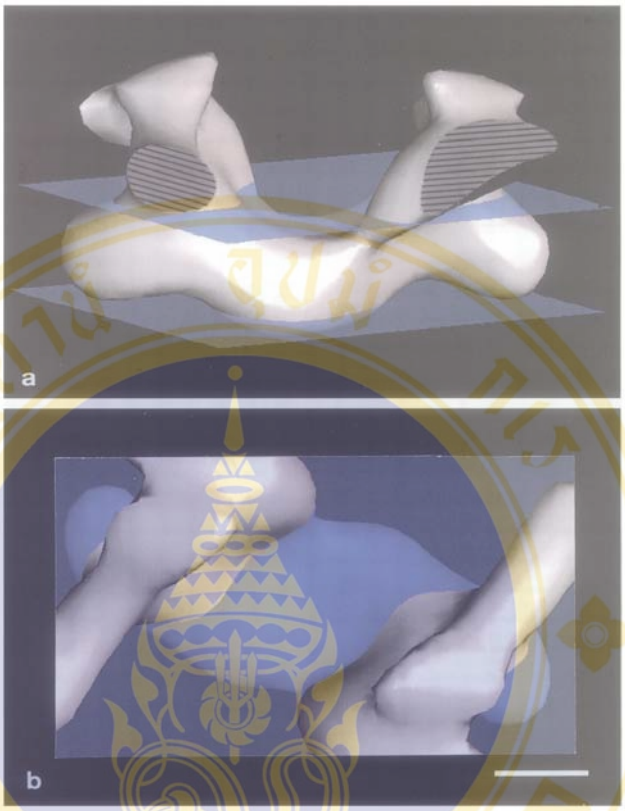


Figure 3.5 **Three dimension map of the dimeric transport domain of the Band 3 protein (taken from Wang *et al.* 1994)** (54)**.**

(a) Perspective view. The bulky basal domain is probably embedded in the lipid bilayer, indicated by the two blue planes which are separated by 35 Å. The longest dimension of the basal domain is 110 Å. The two domains on the cytosolic side form two sides of a canyon that leads to a depression which is probably the entrance to a pore. Striped areas represent cuts through the map above the putative membrane surface which form protein-protein contacts in the crystals.

(b) Top view, showing the open canyon on the cytoplasmic side. The two sides of the canyon narrow down on the surface of the basal domain. The lower part of the protein thought to be embedded in the membrane appears blue. The bar represents 20 Å.

3.2.4 Targeting of AE1

Trafficking of eAE1 from its biosynthesis site and intracellular compartments to the red cell membrane is facilitated by glycophorin A (GPA), a 36 kDa glycoprotein of the red cell membrane (56). The importance of the N-terminus of kAE1 in protein targeting has been described (50). Truncated kAE1 protein lacking amino residues 1-360 that was expressed in polarized MDCK cells was localized to the apical membrane. This indicates that the elements within the N-terminal sequence are required for the basolateral targeting of kAE1. A potential tyrosine-targeting motif in the N-terminus of kAE1 is $Y_{359}KGL_{362}$. A tyrosine-based signals, YXXØ motif (where Y is tyrosine, X is any amino acid, and Ø is hydrophobic amino acid) (57) is known to be involved in localization to coated pits and clathrin mediated endocytosis, and also in basolateral sorting (58-60).

Many membrane proteins are usually sorted to the basolateral surface through interactions of tyrosine-based signal motifs in their cytoplasmic domains with adaptor-protein complexes (61, 62). $Y_{904}DEV_{907}$ motif conforms to a subset of YXXØ motif presenting on the cytoplasmic C terminal tail of human AE1 and plays an important role in kAE1 distribution in polarized cells. Tyr904 in the YXXØ motif of kAE1 has been examined for the basolateral signal in MDCK cells and rat inner medullary collecting duct (IMCD) cells (20, 50). kAE1 with substitution of Tyr904 by alanine (Y904A) or with an 11 amino acid deletion (R901X) is mis-targeted to the apical membrane of MDCK cells (20, 50). The YXXØ motif is recognized by the μ subunit of the adaptor protein (AP) complexes of AP-1, AP-2, AP-3, and AP-4, (61, 63, 64), especially AP-1B which is specific to polarized epithelial cells (62, 65). However, in study using renal epithelial cell line from porcine kidneys (LLC-PK1) lacking the μ1B subunit showed that AP-1B may not be involved in kAE1 targeting (20, 50). The acidic patch (DE) at positions 905-906 followingTyr904 and the last 4 amino acids ($A_{908}MPV_{911}$) at the C-terminus may also be required for trafficking and localization of kAE1. The $A_{908}MPV_{911}$ motif has been proposed as a potential PDZ protein binding domain (XØXØ; where Ø represents a hydrophobic amino acid). Replacement of the acidic patch (DE) with two alanines (AA), or deletion of the last 4 amino acids at the C-terminus (AE1 R908-911) showed non-polarized distribution to

apical membrane and some intracellular localization in transfected polarized MDCK and IMCD cells (66). In addition, in transiently transfected HEK 293 cells and non-polarized-LLC-PK1 cells expressing a deletion of 5 amino acids at the C-terminus of AE1 (AE1 R907-911) also showed the similar result (67). The amino acid sequences of C-terminus of AE1 and the motifs are shown in Figure 3.6

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. Amino acid residues in the boxes represent known or putative interacting motifs for carbonic anhydrase II (CA-II), AP and PDZ.

3.3 *AE1* mutations associated with human diseases

AE1 mutations are found to be associated with two human diseases, red cell abnormalities and dRTA.

3.3.1 *AE1* mutations associated with red cell abnormalities

Hereditary spherocytosis (HS) is the most common congenital hemolytic anemia, characterized by spherocytic red cells with increased osmotic and mechanical fragility and splenomegaly (68). HS can be found to be associated with abnormalities in any of the red cell skeletal components, which are involved with stabilizing the lipid bilayer, including spectrin, ankyrin, band 3, and protein 4.2 (69, 70). About 20% of the cases of HS result from AE1 defects (69-72). The reported *AE1* mutations associated with HS are listed in Table 3.1. These mutations are located in both cytoplasmic and membrane domain occurring form missense, nonsense and frameshift mutations (73). The spectrum of AE1 deficiency in HS ranges from 20–40% decrease to complete absence of red cell AE1 content. Several *AE1* mutations that alter amino acids in the cytoplasmic domain of AE1 may change binding sites for ankyrin, band 4.2, α- and β-spectrins (74, 75). *AE1* E40K mutation affecting the cytosolic domain of AE1 is associated with a decreased band 4.2 without loss of AE1 content (76, 77). A mouse model of AE1 deficiency showed that AE1 is essential for stability of membrane lipid bilayer but not for assembly of the membrane skeleton (78). This would cause the reduction of surface area to volume ratio and the spheroidal shape of red cells. The seven *AE1* HS-missense mutations (L707P, R760Q, R760W, R808C, H834P, T837M, and R870W) locating in transmembrane domains were examined for their effect on the biosynthesis and functional expression in HEK 293 cells (79). All seven HS mutations caused the AE1 protein to misfold and be retained intracellularly. However, there was no change in the oligomeric state or the half-life of the mutant AE1. The intracellular retention of AE1 HS mutant might result in destruction of the protein during erythroid development.

Table 3.1 ***AE1*** **mutations associated red cell abnormalities (modified from the Human Gene Mutation Database Website - http://www.hgmd.org).**

No.	Mutation	Codon number	Mutation type	References
1	cGAG>AAG Glu>Lys	40	missense	Rykicki (1993) Blood 81, 2155
2	ACCCG^GGTACcCACAAG_E4I4_GTGA	53	small deletion	Miraglia del Giudice (1997) Br J Haematol 96, 70
3	GGTACC^CACAaG_E4I4_GTGAGGACC	55	small deletion	Kanzaki (1997) Blood 90S1 6b
4	gCTG>ATG Leu>Met	73	missense	Miraglia del Giudice (1997) Br J Haematol 96, 70
5	GGCGGCG^CGCtGGGTGCAACT	80	small deletion	Dhermy (1997) Br J Haematol 98, 32
6	TGGg>TGA Trp>Term	81	nonsense	Jarolim (1996) Blood 88, 4366
7	gGAG>AAG Glu>Lys	90	missense	Bracher (2001) Br J Haematol 113, 689
8	CCGCAC^CTCTtCTCACCTCAC	99	small insertion	Miraglia del Giudice (1997) Br J Haematol 96, 70
9	CGTAGA^GTCTtCACCAAGG_E5I5_GT	113	small deletion	Dhermy (1997) Br J Haematol 98, 32
10	tGGA>AGA Gly>Arg	130	missense	Ideguchi (1994) Int J Hematol 59, 255A
11	gCCT-TCT Pro-Ser	147	missense	Alloisio (1997) Blood 90, 414
12	cCGA>TGA Arg>Term	150	nonsense	Eber (1996) Nat Genet 13, 214
13	AGGCC^CTGGGgGGGTGTGAAG	170	small insertion	Jarolim (1996) Blood 88, 4366
14	GCTCT^GGGGAaTCCTTCACAG	182	small insertion	Kanzaki (1997) Blood 90S1 6b
15	CCTGAG^GCCCcCCCACATCGA	273	small insertion	Jarolim (1996) Blood 88, 4366
16	GCT>GAT Ala>Asp	285	missense	Jarolim (1996) Blood 88, 4366
17	CCC-CGC Pro-Arg	327	missense	Jarolim (1992) Blood 80, 523
18	gCAG>TAG Gln>Term	330	nonsense	Jenkins (1996) J Clin Invest 97, 373
19	GCACTG^TCACcCGCCATCACC	418	small deletion	Eber (1996) Nat Genet 13, 214
20	GCCCTG^CTGGgGGCTCAGCCC	454	small deletion	Dhermy (1997) Br J Haematol 98, 32
21	GGG>GAG Gly>Glu	455	missense	Jarolim (1996) Blood 88, 4366
22	gGGG>AGG Gly>Arg	455	missense	Yawata (2000) Int J Hematol 71, 118
23	cGTG>ATG Val>Met	488	missense	Alloisio (1997) Blood 90, 414
24	cCGC>TGC Arg>Cys	490	missense	Dhermy (1997) Br J Haematol 98, 32
25	CGC>CAC Arg>His	490	missense	Lima (1999) Eur J Haematol 63, 360
26	GATCGGC^TTCtGGCTCATCCT	495	small deletion	Dhermy (1997) Br J Haematol 98, 32
27	cCGC>TGC Arg>Cys	518	missense	Eber (1996) Nat Genet 13, 214
28	CCATC^CTGATcATGGTCCTGG	615	small deletion	Jarolim (1996) Blood 88, 4366
29	TACa>TAA Tyr>Term	628	nonsense	Jarolim (1996) Blood 88, 4366
30	TCCTCA^GCCCgGGGCTGGGTC	645	small deletion	Jarolim (1996) Blood 88, 4366
31	cGAG>AAG Glu>Lys	658	missense	Bruce (1995) Blood 85, 541
32	TCCCATC^TGGatgATGTTTGCCT	662	small deletion	Eber (1996) Nat Genet 13, 214
33	CTG>CCG Leu>Pro	707	missense	Jarolim (1996) Blood 88, 4366
34	tGGG>AGG Gly>Arg	714	missense	Kanzaki (1997) Br J Haematol 99, 522
35	CGG>CAG Arg>Gln	760	missense	Jarolim (1995) Blood 85, 634
36	gCGG>TGG Arg>Trp	760	missense	Jarolim (1995) Blood 85, 634
37	GGC>GAC Gly>Asp	771	missense	Maillet (1995) Br J Haematol 91, 804
38	ATC>AAC Ile>Asn	783	missense	Miraglia del Giudice (1997) Br J Haematol 96, 70
39	cCGC>TGC Arg>Cys	808	missense	Jarolim (1995) Blood 85, 634
40	CGC>CAC Arg>His	808	missense	Kanzaki (1997) Blood 90S1 6b
41	CACCCA^GATGcacccagatgTGCCCTACGT	821	small insertion	Jarolim (1994) J Clin Invest 93, 121
42	CAC>CCC His>Pro	834	missense	Jarolim (1996) Blood 88, 4366
43	ACG>AGG Thr>Arg	837	missense	Yawata (2000) Int J Hematol 71, 118
44	ACG>ATG Thr>Met	837	missense	Jarolim (1996) Blood 88, 4366
45	cACG>GCG Thr>Ala	837	missense	Iwase (1998) Acta Haematol 100, 200
46	CCG>CTG Pro-Leu	868	missense	Bruce (1993) Biochem J 293, 317
47	gCGG>TGG Arg>Trp	870	missense	Jarolim (1995) Blood 85, 634
48	CCAAG^GCAACcTTTGATGAGG	893	small deletion	Perrotta (1997) Blood 90S1 270
49	AGGGACCCTGAGGCTCGTGAGCAGGGAC CC(G>A)CGGTGCGGGTTATGCTGGGGGCT CAGATCA >62 relative to initiation codon		regulatory	Alloisio (1996) Blood 88, 1062
50	Duplication of 69 bp IVS 12 as >2 to nt. 1499 (described at genomic DNA level)		gross insertion	Bianchi (1997) Br J Haematol 98, 283
51	IVS5 >3 C>A		splicing	Jarolim (1996) Blood 88, 4366
52	IVS 8 +1 G>T		splicing	Lima (1997) Blood 90, 2810
53	IVS 12 +1 G>A		splicing	Jarolim (1996) Blood 88, 4366

Southeast Asian ovalocytosis (SAO) is a common *AE1* mutation which is widely distributed in Southeast Asia regions including the South of Thailand, Malaysia, Indonesia, Papua New Guinea, and Philippines (80). SAO is found in areas where malaria has been endemic since it is believed to afford protection against cerebral malaria in children (72). The molecular defect of SAO has been shown to be AE1 containing deletion of nine amino acid residues at the positions 400-408 due to deletion of 27 bp in exon 11 of the *AE1* gene. This mutation is linked to band 3 Memphis I polymorphism (81). The deleted nine amino acids are at the junction between amino terminal domain and the first transmembrane span of the AE1 protein. The condition is dominant; all individuals with SAO are heterozygous for the mutant allele. Apart from ovalocytic red cells which are more rigid than normal (82), individuals with SAO are generally asymptomatic, without anemia or dRTA (27). However, the homozygous state of SAO may be embryonic lethal (83). Several studies have been carried out in *Xenopus* oocytes and transfected mammalian cell lines to understand the biosynthesis of AE1 SAO. In oocytes, AE1 SAO was found to be able to traffic to the surface in the absence of normal AE1 or glycophorin A (GPA), but it is inactive in anion transport. Co-expressing AE1 SAO and GPA or wild-type AE1 did not effect on its trafficking to the oocytes surface (30, 84). In human erythroleukemia K562 cells that expresses GPA (85), AE1 SAO has been found to be stably expressed and interacted with GPA (86). There was no difference in the cell surface expression between AE1 and AE1 SAO in transfected K562 cells. In transfected HEK cells, AE1 SAO was less stable and retained intracellularly (31). In polarized MDCK cells, wild-type kAE1 localized at the basolateral membrane while kAE1 SAO retained intracellularly. When kAE1 SAO was co-expressed with kAE1 in MDCK cells, kAE1 SAO largely retained intracellularly; however, it also co-localized with kAE1 at the cell surface. Thus, in the heterozygous state of SAO patients, dimers of wild-type kAE1 and heterodimers of kAE1 SAO and wild-type kAE1 are sufficient to maintain adequate bicarbonate reabsorption and proton secretion without dRTA (31).

3.3.2 *AE1* mutations associated with dRTA

AE1 mutations can result in either autosomal dominant (AD) dRTA or autosomal recessive (AR) dRTA. The *AE1* mutations associated with dRTA are listed in Tables 3.2 and 3.3.

(1) Autosomal dominant dRTA (AD dRTA)

Several papers reported the association between *AE1* mutations and AD dRTA (15, 16, 87-90). Missense mutations at Arg589, a conserved residue located in the cytoplasmic loop between transmembrane (TM) segments 6 and 7 of AE1 protein, are common cause of AD dRTA (15, 16, 87, 88). Arg589 is predominantly substituted by histidine (R589H) or cysteine (R589C) and rarely by serine (R589S) (Table 3.2). Other mutations associated with AD dRTA are S613F (15), R901X (87), A888L+889X (91), and G609R (90).

Bruce *et al.* (15) found that all affected members of 4 families with AD dRTA were heterozygous for R589H, R589C, or S613F mutation, which had normal red cell morphology and eAE1 content. The patients with R589 had slightly reduced red cell sulfate transport (approximately 80% of normal) whereas the patients with S613F mutation had markedly increased sulfate transport (262% of normal) but almost normal red cell iodide transport. The mutant eAE1 and kAE1 proteins were expressed in *Xenopus* oocytes. eAE1 R589H showed decreased (40% of normal) chloride influx activity while kAE1 R589H showed normal activity. The kAE1 R589C and S613F mutants did not show a significantly different transport function compared with wild-type kAE1. Co-expression of wild-type and mutant AE1 in oocytes did not show any effect on the chloride uptake function. It was concluded that dominantly inherited dRTA was associated with mutations in AE1 but both the disease and its autosomal dominant inheritance were not related simply to the anion transport activity of the mutant proteins. Arg589 is conserved in all known vertebrate sequences of AE1, AE2, and AE3, suggesting that it is functionally important. Arg589 is located in a cluster of basic residues which may form part of the cytoplasmic anion binding site of AE1. The S613F change may distort the conformation of the cytoplasmic loop between TM6 and TM7 which contains the putative anion binding site. The other group later reported three unrelated families with *AE1* R589H mutation associated with AD

Table 3.2 *AE1* mutations associated with AD dRTA (modified from the Human Gene Mutation Database Website - http://www.hgmd.org).

No.	Mutation	Codon number	Mutation type	References
1	IVS12+1G>T	477	splicing	Rysava (1997) Nephrol Dial Transp 12, 1869
2	CGC-CAC→Arg-His	589	missense	Karet (1998) Proc Natl Acad Sci U S A 95, 6337 Bruce (1997) J Clin Invest 100, 1693 Jalorim (1998) J Biol Chem 273, 6380
3	gCGC-AGC→Arg-Ser	589	missense	Karet (1998) Proc Natl Acad Sci U S A 95, 6337
4	gCGC-TGC→Arg-Cys	589	missense	Bruce (1997) J Clin Invest 100, 1693 Yenchitsomanus (2003) Southeast Asian J Trop Med Public Health 34, 651
5	GGG-AGG→ Gly-Arg	609	missense	Rungroj (2004) J Biol Chem 279, 13833
6	TCC-TTC→Ser-Phe	613	missense	Bruce (1997) J Clin Invest 100, 1693
7	GCC-GAC→Ala-Asp[1]	858	missense	Bruce (2000) Biochem J 350, 41
8	20 bp deletion within exon 20	888→889X	gross deletion	Cheidde (2003) Pediatrics 112, 1361
9	AGGAG^GAAGGatgagg aggaaggTCGGGATGAA	899→901X	small insertion	Karet (1998) Proc Natl Acad Sci U S A 95, 6337

[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* mutations associated with AR dRTA (modified from the Human Gene Mutation Database Website - http://www.hgmd.org).

No.	Mutation	Codon number	Mutation type	Reference
1	27 bp deletion within exon 11[1]	400-408	gross deletion	Liu (1990) New Eng J Med 323, 1530
2	GTG-ATG→Val-Met	488	missense	Riberiro (200) Blood 96,1602
3	CGT-CAT→Arg-His	602	missense	Sritippayawan (2004) 44, 64
4	GGC-GAC→Gly-Asp	701	missense	Tanphaichitr (1998) J Clin Invest 102, 2173 Bruce (2000) Biochem J 350, 41 Vasuvattakul (1999) Kidney Int 56, 1674 Yenchitsomanus (2003)J Hum Genet.48, 451
5	gTCC-CCC→Ser-Pro	773	missense	Sritippayawan (2004) 44, 64 Yenchitsomanus (2003) Southeast Asian J Trop Med Public Health 34, 651
6	GCTGTGG^GTGgtgAAGTCC ACGC	849→del 850	small deletion	Bruce (2000) Biochem J 350, 41

[1]SAO, Southeast Asian ovalocytosis - an in-frame nine-amino acid deletion (ΔAla400-Ala408), is regarded as a mutation causing AR dRTA because its compound heterozygous conditions with G701D or other recessive mutations results in dRTA with ovalocytic red cells. Homozygous SAO has not been reported, and is believed to be lethal *in utero*.

dRTA (16). The red cells of the affected individuals showed slightly decrease (~20%) in sulfate influx activity. In oocytes, kAE1 R589H showed 20-50% reduction in Cl^-/Cl^- and Cl^-/HCO_3^- exchange but were not different when co-expressed with wild-type eAE1. It was suggested that no dominant negative behavior on the anion transport activity. Furthermore, the presence of mutations at the same position in several populations with allelic heterogeneity and at least two *de novo* mutations (87, 89) makes it unlikely that this frequently observed R589H mutation will result from founder effect but is more likely to occur from recurrent mutations. This led to the suggestion that codon 589 (CGC) is a 'mutational hotspot' of *AE1* (89), and the mechanism of recurrent mutations probably involves methylation and deamination that alter cytosine (C) to thymine (T) in the CpG dinucleotides (89, 92).

A nonsense *AE1* R901X mutation (band 3 Walton) is caused by a intragenic 13-bp duplication which created a premature stop codon at the position 901 resulting in deletion of least 11 amino acids of AE1 (87). Transport function of both eAE1 and kAE1 R901X were examined. eAE1 R901X had normal sulfate transport and kAE1 R901X had normal chloride transport function, either in the absence or presence of GPA. *AE1* A888L+A889X, a truncate AE1 protein by 23 amino acids, due to 20 bp deletion in exon 20 of *AE1* gene leading to mutation in codon 888 followed by a premature stop codon at the position 889 (91). Two affected brothers with this mutation had dRTA, nephrocalcinosis, and failure to thrive, but their father who carried the same mutation had incomplete dRTA (91). Recently, a novel missense *AE1* G609R mutation causing AD dRTA was reported in a Caucasian family who all exhibited dRTA with prominent nephrocalcinosis and progressive renal impairment (90). The kAE1 G609R had a normal transport function and were expressed at the surface of oocytes.

The *AE1* mutations associated with AD dRTA did not result in significantly functional change as examined in red cells and *Xenopus* oocytes. This indicated that the defects are not simply a loss of anion transport activity but probably caused by other mechanisms. Additionally, these two cell types might not serve as good systems for studying the defects because the mutant AE1 proteins could move to the surface of the red cells and oocytes, mediating normal transport activities. It was

found in the subsequent studies using transfected cell systems that trafficking defects of mutant kAE1 proteins are the major pathogenic mechanisms.

(2) Autosomal recessive dRTA (AR dRTA)

AR dRTA in Southeast Asian and Melanesian populations is frequently associated with *AE1* mutations. The homozygous *AE1* G701D mutation was first described in a Thai family (22) and has been shown to be a frequent cause of AR dRTA in Thai children (23). The patients in the first reported family had dRTA with hemolytic anemia and abnormal red cells with xerocyte-like morphology but transport function of eAE1 in red cells was normal (22). When expressed in *Xenopus* oocytes, both eAE1 G701D and kAE1 G701D were functionally inactive owing to intracellular protein retention. Co-expression of either eAE1 G701D or kAE1 G701D with GPA in oocytes showed normal transport activity and surface expression, suggesting that GPA completely rescued surface expression and anion transport activity of the mutant AE1. The absence of the erythroid-specific GPA in the kidney strongly suggests that AE1 G701D polypeptide is likely to also retain intracellularly in the α-intercalated cells, resulting in impaired urinary acidification in these patients (22).

The presence of dRTA and SAO was observed in the conditions of compound heterozygosities for *AE1* SAO and the recessive mutations including G701D, R602H, ΔV850 and A858D (24, 27, 28). Two cases of dRTA and SAO resulted from compound heterozygous *AE1* SAO/G701D mutations were originally reported in two Thai families from southern Thailand (27). These patients had SAO in addition to a little RBC morphological change but were not anemic. Moreover, they had a decrease of red cell sulfate flux of about 40%. The presence of Thai families with compound heterozygous *AE1* SAO/G701D mutations most likely occur from a mixture between the northeastern Thai population that has a high frequency of the G701D mutation, and the southern Thai population that has a high frequency of *AE1* SAO (13). The southward migration of the *AE1* G701D allele from the Northeast of Thailand seems to reach as far as Malaysia.

The patients with AR dRTA with compound heterozygous *AE1* SAO/G701D SAO/ΔV850, ΔV850/ΔV850, and ΔV850/A858D were observed in patients from Malaysian and Papua New Guinean families (28). These patients had hemolytic

anemia and abnormal red cell properties. AE1 ΔV850/A858D, SAO/V850 and SAO/A858D mutant proteins showed greatly decreased anion transport in *Xenopus* oocytes. The heterozygous A858D mutation was found to be associated with incomplete dRTA but complete dRTA was observed in compound heterozygous (ΔV850/A858D or SAO/A858D) genotypes. The mutant AE1 A858D protein seems to possess a greater structural or functional abnormality than other mutant AE1 proteins resulting in AR dRTA. However, *AE1* A858D has been assigned as a mutation causing AD dRTA by the first group who reported this mutation (28).

Homozygous V488M (band 3 Coimbra) mutation causing severe HS and dRTA was reported in a young Portuguese child (93), supporting the presence of AR form of dRTA associated with *AE1* mutations. Two additional *AE1* mutations, R602H and S773P with AR dRTA were reported from 2 unrelated Thai families (24). In the first family, the patient carried *AE1* G701D/S773P mutations had dRTA, rickets, failure to thrive and nephrocalcinosis. In the second family, the patient and his sister who had dRTA and SAO with different clinical severities carried *AE1* SAO/R602H mutations. The patient had rickets, nephrocalcinosis, hypokalemia, metabolic acidosis, and inappropriately high urine pH while his sister was asymptomatic and not acidotic but her urine pH level could not be lowered to below 5.5 after a short acid load i.e. incomplete dRTA. The cause of these different clinical severities is unknown. It might be an example of the variable expressivity of dRTA phenotype attributable to a modifier gene.

3.4 Molecular defects of AE1 associated with dRTA

3.4.1 Molecular defects of AE1 associated with AD dRTA

The molecular defects of AD dRTA associated with *AE1* R589H and R901X mutations have been examined in HEK 293 cells (17, 18). In the transfected HEK 293 cells, eAE1, eAE1 R589H, and kAE1 were expressed at the cell surface while kAE1 R589H was retained intracellularly (17). Co-expression of kAE1 R589H reduced the cell surface expression of wild-type kAE1 and eAE1 resulting from heterodimer formation and a dominant negative effect. eAE1 and kAE1 R589H were not grossly misfolded as they could bind to an inhibitor affinity resin. Similarly, other substitutions at Arg589 of *AE1*, R589C and R589S, were not reached to the plasma

membrane, indicating that the normal Arg589 is important for proper trafficking. kAE1 R589H and kAE1 S613F were retained in ER of the non-polarized cells MDCK cells and were distributed throughout the cells with a failure to reach the cell surface in the polarized cells MDCK cells (26, 50). Co-expression of kAE1 R589H retains the wild-type kAE1 in the cytoplasm of MDCK cells, probably through heterodimer formation (26).

The properties of kAE1 R901X was examined in *Xenopus* oocytes, non-polarized MDCK, and HEK 293 cells (18, 19). kAE1 R901X had a normal chloride transport activity when expressed in *Xenopus* oocytes. In cultured kidney cell lines, kAE1 R901X was retained intracellularly indicating that the C-terminal tail of AE1, which is truncated in kAE1 R901X, is required for its movement to the cell surface in kidney cells. It was proposed that kAE1 R901X gives rise to dominant dRTA by inhibiting the movement of normal kAE1 to the cell surface, resulting from the association of the normal and mutant proteins in kAE1 hetero-oligomers (19). It was later shown that kAE1 R901X had a dominant negative effect due to hetero-oligomers formation of the co-expressed mutant and wild-type proteins (18). eAE1 R901X also showed this effect because it detained the wild-type protein in a medial Golgi compartment of HEK 293 cells. A series of truncations at the C-terminus of AE1 also resulted in impaired ER exit, depending on the extent of the truncation (67). Subsequently, AE1 R901X was examined in polarized MDCK cells and rat IMCD cells (which express AE1) (20). In both cell types, the wild-type AE1 localized to the basolateral plasma membrane domain whereas AE1 R901X was found at both the basolateral and apical cell surfaces as well as intracellularly. This suggests that AD dRTA is caused not only by a decrease of AE1 protein at the basolateral surface but also by its inappropriate presence at the apical surface. In stably transfected polarized MDCKI cells, the mis-targeting to the apical membrane of kAE1 R901X was also confirmed (50). The apical chloride and bicarbonate flux would alter electrochemical balance across the cell, impairing both unidirectional basolateral bicarbonate extrusion and apical proton secretion. The non-polarized distribution of mutant AE1 could be explained by the loss of a basolateral targeting signal present in the deleted portion of its C terminus. The $Y_{904}DEV_{907}$ motif present in the acidic tail of AE1 conforms to a subset of tyrosine based targeting motifs, YXXØ. When Tyr904 was

mutated to alanine (Y904A), the distribution of protein was similar to that of AE1 R901X. In addition, in the absence of the N-terminus of kAE1, the C-terminus was not sufficient to localize kAE1 to the basolateral membrane, suggesting that a determinant within the kAE1 N-terminus cooperates with the C-terminus for kAE1 basolateral localization (21, 50).

A G609R missense mutation caused AD dRTA found in a Caucasian extended family (90). In *Xenopus* oocytes, G609R had normal transport function and expressed on cell membrane. G609R was partially located at the apical and sub-apical membrane, and also at the basolateral membrane in polarized MDCK cells, similar to the kAE1 R901X. This suggests that Gly609 may be involved in the protein trafficking or sorting process (90).

3.4.2 Molecular defects of AE1 associated with AR dRTA

The foundation of kAE1 G701D study in *Xenopus* oocytes (22, 28) demonstrated a loss of function and a failure to cell surface expression. The molecular mechanism of AR dRTA caused by *AE1* mutations was proposed in two possible models (14). In first model, kAE1 mutant in heterozygous state is unable to form homodimers and heterodimers and would result in impaired trafficking to cell surface. Thus, only the mutant kAE1 is defective while the wild-type kAE1 is still intact. The normal kAE1 would be able to dimerize and traffic to the cell surface which is sufficient to maintain the normal transport function. The other possibility, kAE1 mutant in heterozygous state might be able to form homodimers and heterodimers but only homodimer of kAE1 mutant would be impaired trafficking. In this second model, the heterodimer of the mutant and wild-type kAE1 proteins would be able to traffic to the cell surface, not exhibit the dominant-negative effect.

A compound heterozygous *AE1* G701D/S773P mutation was found in Thai patient with AR dRTA (24) and was further study to elucidate the molecular mechanism of AR dRTA in HEK 293 cells and LLCPK1 cells (25). The biosynthesis kAE1 S773P showed protein reduction, misfolding, short half-life and protein degradation by proteasome. In the protein trafficking study, kAE1 S773P could not be detected at the cell surface and showed prominently in ER of both HEK 293 and LLCPK1 cells. kAE1 G701D also exhibited defective trafficking to the plasma

membrane. The kAE1 S773P was able to form homodimers and heterodimers with wild-type kAE1 or with kAE1 G701D. The heterodimers of wild-type kAE1 with kAE1 S773P or G701D was able to express plasma membrane in contrast to kAE1 R589H. Thus, the second proposed model is likely to be the true one, and the wild-type kAE1 seems to show a 'dominant-positive effect' in rescuing the recessive mutant kAE1 trafficking to the plasma membrane, in contrast with the dominant mutant kAE1 resulting in a 'dominant-negative effect' when heterodimerized with the wild-type kAE1.

kAE1 S773P and G701D were further examined in MDCK cells (26) showing distinct trafficking defects. kAE1 S773P was retained in the ER in non-polarized MDCK cells but also partially targeted to the basolateral membrane in polarized cells, while kAE1 G701D was retained in the ER and Golgi in both non-polarized and polarized cells. kAE1 mutants with the substitutions of G701 to E and R, but not to A or L, were also localized at the Golgi in both non-polarized and polarized cells (26). This co-expression study in MDCK cells confirmed the previous study in HEK 293 cells and provided more details. Additionally, while the dominant mutant kAE1 R589H retained wild-type kAE1 protein intracellularly, the recessive kAE1 mutants did not. 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. Also, no kAE1 G701D was detected at the cell surface, suggesting that kAE1 S773P did not assist the intracellularly retained 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 AE1 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.

3.5 The outline of experimental study in this thesis

To elucidate the molecular defects of compound heterozygous *AE1* SAO/G701D mutations causing AR dRTA, the expression and co-expression of wild-type kAE1, kAE1 SAO, and kAE1 G701D would be examined. The expression vectors containing wild-type kAE1 cDNA tagged with HA, 6xHis and Myc would be

constructed and kAE1 SAO and kAE1 G701D would be generated by site-directed mutagenesis. The plasmids containing wild-type or mutant kAE1 cDNA tagged with HA or 6xHis or Myc would be transfected into HEK 293 cells and examined for the expression of wild-type and mutant kAE1 proteins by Western-blot analysis. To study the effect of the mutant protein on the wild-type protein and *vice versa*, the wild-type *kAE1* would be co-transfected with either kAE1 SAO or kAE1 G701D. To mimic the condition of compound heterozygous *AE1* SAO/G701D mutations that was found in dRTA patients, the kAE1 SAO and kAE1 G701D would be co-expressed in HEK 293 cells. Protein-protein interaction would be determined by co-immunoprecipitation and co-purification procedures, and protein trafficking and subcellular localization would be investigated by confocal microscopy and FACS analysis.

CHAPTER IV

MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals and reagents

The chemicals and reagents used in the experimental studies 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.

4.1.3 Enzymes

(1) Restriction endonucleases

The restriction endonucleases including, *Not*I (10 U/µl), *Xho*I (20 U/µl), *Dpn*I (20 U/µl) and *Hpa*II (10 U/µl) were purchased from Promega, Madison, WI, USA.

(2) Polymerase and other modifying enzymes

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

(3) Ribonuclease

RNaseA was purchased from USB, USA

4.1.4 DNA and protein markers

(1) DNA markers

ϕx174-*Xho*I digested and λ-*Hind*III digested DNA-markers were purchased from New England Biolabs, Beverly, MA, USA.

Table 4.1 Oligonucleotide primers

Primer name	Nucleotide sequences	Annealing Tm (°C)
kAE1 *Not*I Fp[a]	5'-TCGGC'GGCCGCATGGACGAAAAGAACCAGGA-3'	55
kAE1 *Xho*I Rp[a]	5'-TCGCTCGAGTCACACAGGCATGGCCACTTC-3'	55
SAO Fp[b]	5'-CCTGAGTGACATCACAGATGTCATCTTCATCTACTACTTTGC-3'	60
SAO Rp[b]	5'-GCAAAGTAGATGAAGATGACATCTGTGATGTCACTCAGG-3'	60
G701D Fp[b]	5'-GATGGTTCAAGGGCTCCGACTTCCACCTGGACCTGC-3'	60
G701D Rp[b]	5'-GCAGGTCCAGGTGGAAGTCGGAGCCCTTGAACCATC-3'	60
WTmyc Fp[b]	5'-CCACTACAGAAGACTTATAACTACAACGTGTACCCATACGATG TTCCAGATTACGCTTTGATGGTGCCCAAACCTCAGGGCCCCCTG-3'	55
WTmyc Rp[b]	5-'CAGGGGGCCCTGAGGTTTGGGCACCATCAAAGCGTAATCTGGA ACATCGTATGGGTACACGTTGTAGTTATAAGTCTTCTGTAGTGG-3'	55
R2[c]	5'-CAGCTTCACCCCCCCAGG-3'	55
NL[c]	5'-TCACTCGAGATGAGAGTCTTCACCAAGGGTACTG-3'	55
L4[c]	5'-CGGTGCTGGGCTTCGTGAGG-3'	55
L6[c]	5'-TGCATTCAGCCCCCAGGTCC-3'	55
L8[c]	5'-TGCCCAACACAGCCCTCCTC-3'	55
L10[c]	5'-GTAGTAGCATGGGTGGGGT-3'	55
L5[d]	5'-TGCTGCCTCCCACCGATGCC-3'	55
R5[d]	5'-CGAAGGTGATGGCGGGTGAC-3'	55
L9[d]	5'-GGTGTCCAACTCCTCAGCCC-3'	55
R9[d]	5'-GGCATGGGTGACGGAACGCA-3'	55

Notes:
- [a]Primers for amplifications of *kAE1* cDNA for cloning.
- [b]Primers for site-directed mutagenesis.
- [c]Primers for sequencing of *kAE1* gene**.**
- [d]Primers for colony screening by PCR or PCR/RFLP**.**
- Underlined sequences are recognition sites of restriction enzymes, indicated in the primer names.
- Underlined bases in the middle of primers for site-directed mutagenesis is the mutated bases to be generated.

(2) Protein marker

PageRuler™ prestained protein ladder was purchased from Fermentas, Hanover, MD, USA.

4.1.5 Eukaryotic expression vector and recombinant plasmids

(1) pcDNA3.1/HisB

This vector was purchased from Invitrogen, Carlsbad, CA, USA. pcDNA3.1/His B was used as a vector backbone for DNA cloning and expression in HEK293 cell. The structure of pcDNA3.1/His B is shown in Figure 4.1.

(2) pcDNA-*kAE1* WT

Wild-type kidney isoform of *AE1* (*kAE1* WT) construction was generated using pcDNA3 as a vector backbone, namely pcDNA3-*kAE1* WT, was a gift from Professor Reinhart A Reithemeier, Canadian Institutes of Health Research Group in Membrane Biology, Department of Biochemistry and Medicine, University of Toronto, Toronto, Ontario, Canada.

(3) pcDNA-*kAE1* WT-HA

pcDNA-*kAE1* WT-HA was constructed by introducing hemagglutinin (HA) epitope tag downstream of the last codon before the stop codon of the gene. This construct was generous gift from Miss Thitima Keskanokwong, the Institute of Molecular Biology and Genetics, Mahidol University.

(4) pcDNA-*kAE1* WT-Myc

kAE1 WT Myc containing a Myc epitope was constructed by site-directed mutagenesis to insert the following sequences: TAC CCA TAC GAT GTT CCA GAT TAC GCT between the positions 557 and 558 using WTMyc Fp/WTMyc Rp primer pair as shown in Table 4.1. This construct was a gift form Miss Wandee Udomchaiprasertkul, Division of Medical Molecular Biology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University.

A.

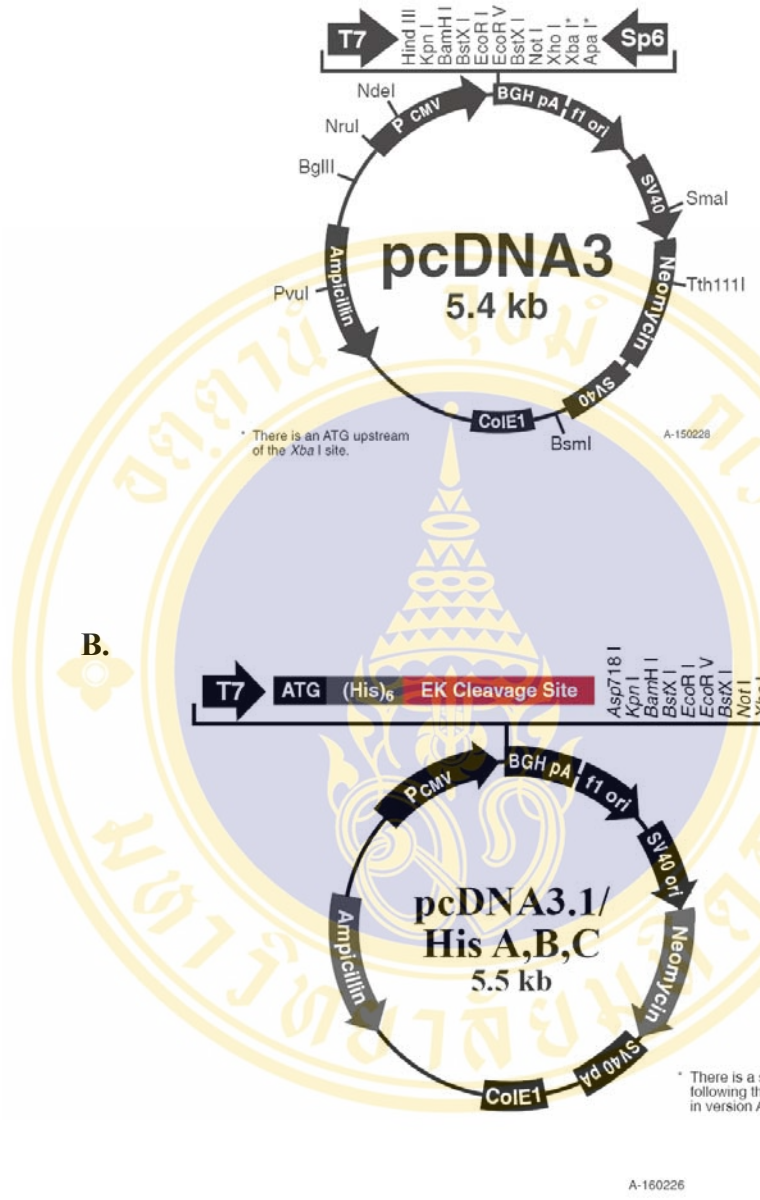


Figure 4.1 **Physical maps of mammalian expression vectors, pcDNA3 and pcDNA3.1/His A, B, C.**

A. pCDNA 3 is untagged vector containing T7 and SV40 promoters. kAE1 WT was constructed by inserting full-length kAE1 cDNA into this vector (Invitrogen).

B. pCDNA 3.1/His B contains both poly-histidine tagged and Xpress epitopes with T7 and SV40 promoters. The wild-type and mutant *kAE1* cDNA were constructed using this vector (Invitrogen).

4.1.6 *E. coli* DH5α competent cells

The genotype of this *E. coli* strain is supE44 [Delta] lacU169 ([Phi] 80 lacZ [Delta] M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1.

4.1.7 Human embryonic kidney 293 (HEK 293) cell line

Human embryonic kidney 293 cells (HEK 293) were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Life Technologies, Gaithersburg, MD, USA) supplemented with 10% bovine calf serum (PERBIO) and 1.2% penicillin and streptomycin in 5% CO^2 at 37 °C. These cells were maintained in the laboratory of the Division of Medical Molecular Biology, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University.

4.1.8 Antibodies

(1) Murine monoclonal anti-6xHistidine antibody was obtained from Amersham Biosciences, Sunnyvale, CA, USA.

(2) Rabbit polyclonal anti-HA antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

(3) Monoclonal anti-Myc IgG1 antibody from hybridoma 9E10 was a gift from Dr. Juthathip Mongkolsapaya, Division of Medical Molecular Biology, Department of Research and Development, which was provided through Miss Prapapun Ong-ajchaowlerd.

(4) Rabbit anti-mouse immunoglobulins (Igs) conjugated with horseradish peroxidase (HRP) was obtained from, Dako, Glostrup, Denmark.

(5) Swine anti-rabbit immunoglobulins (Igs) conjugated with horseradish peroxidase (HRP) was obtained from Dako, Glostrup, Denmark.

(6) Donkey anti-rabbit IgG (H+L) conjugated with Cy3 was purchased from Jackson Immunoresearch Laboratories, West Grove, PA., USA.

(7) Goat anti-mouse IgG (H+L) conjugated with Alexa Fluor® 488 *highly cross-adsorbed* was obtained from Molecular Probes, Eugene, OR, USA.

4.1.9 Miscellaneous materials

(1) Lipofectamine ™ 2000 was purchased from Invitrogen, Carlsbad, CA, USA.

(2) QIAquick Gel Extraction kit, QIAquick PCR purification kit, and QIAprep spin MiniPrep kit were purchased from QIAGEN, Hilden, Germany.

(3) Protran nitrocellulose transfer membrane was purchased from, Schleicher & Schuell, Keene, NH, USA.

(4) SuperSignal West Pico Chemiluminescent Substrate was purchased from PIERCE, Pierce, Rockford, IL, USA.

4.2 Methods

4.2.1 Experimental design

To examine expression, trafficking, and subcellular localization of kAE1 WT, kAE1 SAO, and kAE1 G701D individually expressed or co-expressed in HEK 293 cells, the recombinant plasmid containing either *kAE1* WT, *kAE1* SAO or *kAE1* G701D cDNA, tagged with the sequence of either His, HA or Myc would be required. To construct pcDNA-*kAE1* WT-His, the *kAE1* cDNA would be amplified by PCR form pcDNA3-*kAE1* WT and cloned into pcDNA3.1/HisB. Then, pcDNA-*kAE1* SAO-His and pcDNA-*kAE1* G701D-His would be generated by site-directed mutagenesis using pcDNA-*kAE1* WT-His as a template. pcDNA-*kAE1* WT-HA and pcDNA-*kAE1* WT-Myc, which were obtained from other investigators, would be used as template to, respectively, generate pcDNA-*kAE1* SAO-HA and pcDNA-*kAE1* G701D-HA, and pcDNA-*kAE1* SAO-Myc and pcDNA-*kAE1* G701D-Myc by site-directed mutagenesis. These recombinant plasmids would then be transfected into HEK 293 cells and examined for the protein expression by Western blotting method. To study the expression and subcellular localization of wild-type, mutant SAO, and mutant G701D kAE1 proteins, the pcDNA-*kAE1* WT-Myc, pcDNA-*kAE1* SAO-Myc, and pcDNA-*kAE1* G701D-Myc would be separately transfected into HEK 293 cells. The protein expression and subcellular localization would be examined by FACS analysis.

To examine the heterodimerization between wild-type kAE1 and kAE1 SAO or kAE1 G701D, and between kAE1 SAO and kAE1 G701D, pcDNA-*kAE1* WT-His would be co-transfected into HEK 293 cells with pcDNA-*kAE1* SAO-HA or pcDNA-*kAE1* G701D-HA, and pcDNA-*kAE1* SAO-His co-transfected into the cells with pcDNA-*kAE1* G701D-HA, and in addition pcDNA-*kAE1* SAO-HA co-transfected with pcDNA-*kAE1* G701D-His. The protein dimerization would be examined by co-purification and co-immunoprecipitation.

To study the effect of mutant kAE1 on wild-type kAE1 and *vice versa* in trafficking and subcellular localization in the cells, the HEK 293 cells would be co-transfected with pcDNA-*kAE1* WT-Myc and pcDNA-*kAE1* SAO-HA or pcDNA-*kAE1* G701D-HA. The protein subcellular localization and trafficking would be

examined by immunofluorescence and FACS analyses. To similarly study and mimic compound heterozygous *AE1* SAO/G701D condition observed in the patients with dRTA, the HEK 293 cells would be co-transfected with pcDNA-*kAE1* SAO-Myc and pcDNA-*kAE1* G701D-HA, or with pcDNA-*kAE1* SAO-HA and pcDNA-*kAE1* G701D-Myc. The protein subcellular localization and trafficking would be investigated by immunofluorescence and FACS analysis. The flowchart of experimental design is shown in Figures 4.2 and 4.3.

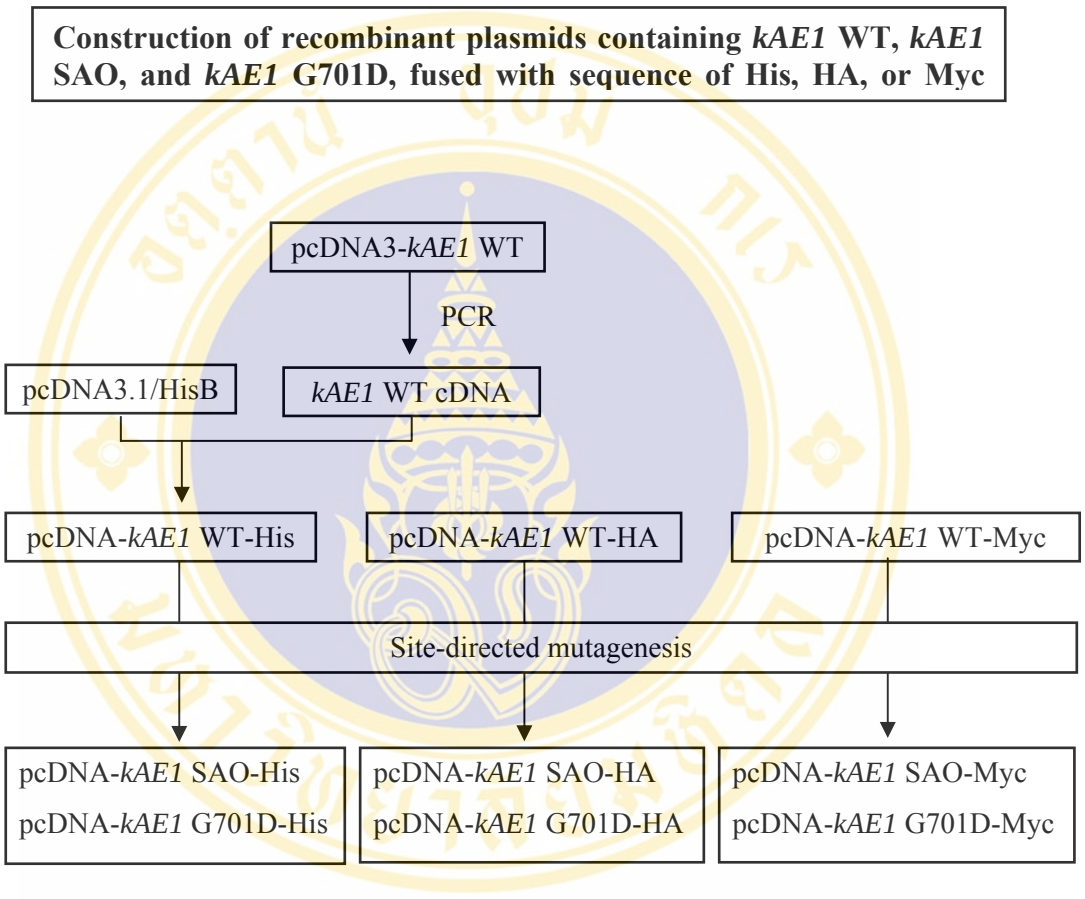


Figure 4.2 Experimental design: Part I - construction of recombinant plasmids.

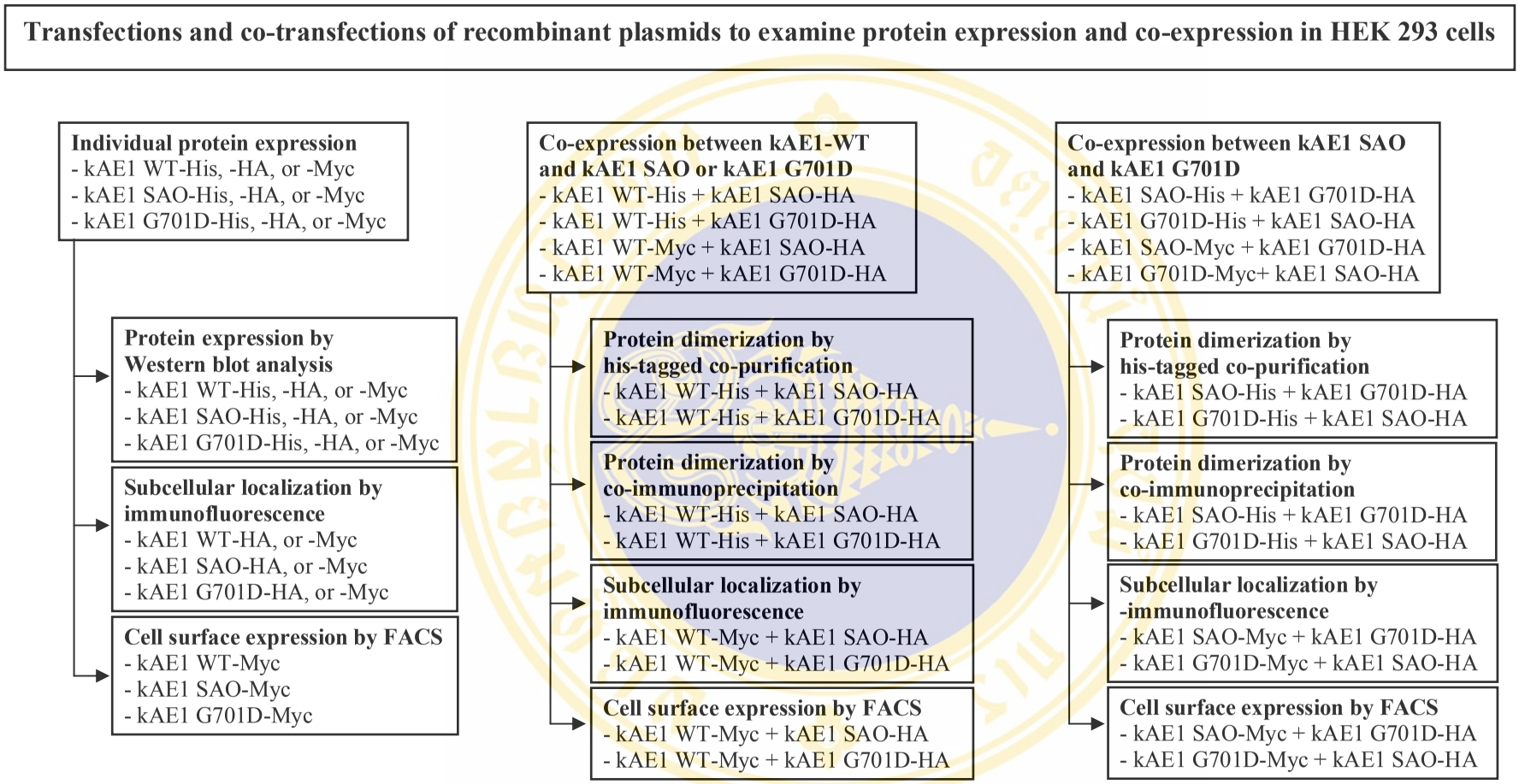


Figure 4.3 Experimental design: Part II – protein expression and co-expression in HEK 293 cells.

4.2.2 Construction of pcDNA-*kAE1* WT-His

To construct pcDNA-*kAE1* WT-His, a full-length *kAE1* cDNA isolated from pcDNA3-*kAE1* WT by PCR was cloned into pcDNA3.1/HisB vector. After cloning, nucleotide sequence was examined by sequencing. The details of cloning procedure are described below.

(1) Amplification of full-length *kAE1* WT cDNA by PCR

The recombinant plasmid containing human full-length *kAE1*, pcDNA3-*kAE1* WT, which was a gift from Professor Reithmeier's laboratory, University of Toronto, Canada, was used as DNA template for PCR amplification using *kAE1* specific primers containing two different restriction enzyme sites, kAE1*Not*I and kAE1*Xho*I, as shown in Table 4.1. PCR product with the size of 2.5 kb was generated from the amplification with these primers. The PCR reaction was performed in a total volume of 50 µl consisting of 100 ng of plasmid DNA, 12.5 pmole of each primer, 10 mM dNTPs, 1x *Pfx* buffer, 50 mM $MgSO_4$, and 1.25 unit of Platinum® *Pfx* DNA polymerase. After gently mixing and a brief centrifugation, the reaction mixture was placed in a thermal cycler GeneAmp®PCR system 9700 (Perkin Elmer Cetus, USA). PCR cycles were set as follows: *(i)* denaturation at 94 °C for 15 sec, *(ii)* annealing at 55 °C for 30 sec, *(iii)* extension at 68 °C for 2 min 30 sec, followed by another 7 min of extension at 68°C in the final step. An aliquot of *kAE1* PCR product was analyzed on 1% agarose gel electrophoresis. After staining with ethidium bromide solution, the PCR product was visualized on a UV transilluminator (Vilber Lourmat ETX-20-M, Vilber Lourmat, Marine la Vallee CDX 1, France). The *kAE1* PCR product was purified by using QIAquick Gel Extraction Kit (QIAGEN) following manufacturer's protocol.

(2) Isolation of pcDNA3.1/HisB plasmid

pcDNA3.1/HisB was isolated from *E. coli* harboring this plasmid by using QIAGEN plasmid mini kit (QIAGEN, Germany) according to the manufacturer's instruction. Briefly, *E. coli* containing pcDNA3.1/HisB was cultured in 5 ml LB broth containing 100 µg/ml of ampicillin for 12-16 h. Bacterial cells were harvested by centrifugation at 3,000 xg 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. 350 µl of chilled buffer P3 were added and mixed immediately by inverting 4-6 times and centrifuged at 13,000 xg for 10 min at 4 °C to clarify the cell lysis solution. The clear solution was loaded onto QIAGEN mini column and briefly centrifuged at 13,000 xg for 30-60 sec. The flow through was discarded and 750 µl of buffer PE was added into the column and let stand for 1 min. After centrifugation at 13,000 xg for 30-60 sec, flow-through was discarded and centrifuge for an additional 1 min to remove residual buffer PE. The column was placed on a clean 1.5 ml. microcentrifuge tube. The DNA was eluted by adding 30-40 µl of buffer EB (10 mM Tris-HCl, pH 8.5), letting stand for 1 min, and centrifugation 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 transilluminator comparing with the known amount of standard molecular weight DNA markers. Another way to estimate the DNA concentration, DNA was diluted to 50-200 times and measured at OD 260 and 280 nm by a spectrophotometer (Shimadzu UV-160A, Shimadzu, Tokyo, Japan). The DNA concentration was calculated by the formula: OD260 x 50 = DNA concentration in µg/ml. The ratio of OD260/OD280 was calculated to observe DNA purity (the ratio of pure DNA is 1.8).

(3) Restriction endonuclease digestion of *kAE1* PCR product and pcDNA3.1/HisB vector

kAE1 PCR product and pcDNA3.1/HisB vector, prepared as described respectively in sections (1) and (2), were separately digested with *Not*I and *Xho*I restriction enzymes. The digestion reaction (20 µl) contained either 200 ng of PCR product or 2 µg of vector, 1x reaction buffer D, and 5 units each of *Not*I and *Xho*I, incubated at 37 °C for 12-16 h. The digested *kAE1* PCR product and pcDNA3.1/His B were each purified with QIAquick Gel Extraction kit (QIAGEN, Germany). The concentrations of digested PCR product and plasmid vector were determined by comparing with known concentration of standard DNA markers after running on agarose gel electrophoresis and staining with ethidium bromide solution.

(4) Cloning of *kAE1* cDNA into pcDNA3.1/HisB expression vector

To clone *kAE1* cDNA into pcDNA3.1/HisB vector, the digested *kAE1* cDNA [from section (3)] was mixed with the cleaved pcDNA3.1/His B vector [also from section (3)] to generate pcDNA-*kAE1* WT-His at the molar ratio of 2:1. The ligation reaction was performed in a volume of 20 µl. This reaction consisted of 100 ng of *kAE1* WT cDNA, 100 ng of pcDNA3.1/HisB, 2 units of T4 DNA ligase and 1x ligation buffer. The ligation mixture was incubated in a waterbath at 16 °C for 16 h. Then, 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. The 900 µl of LB broth medium were added and incubated at 37 °C in a shaking incubator for 1 h. The mixture was centrifuged at 3,000 xg 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 spread on LB plate containing 100 µg/ml ampicillin and incubated at 37 °C for 12-16 h.

(5) Screening of colonies harboring pcDNA-*kAE1* WT-His recombinant plasmid

The positive clones of *E. coli* colonies harboring *kAE1* WT-His recombinant plasmid were screened by PCR method using kAE1*Not*I and kAE1*Xho*I primers. The clones with positive PCR results were selected for plasmid preparation by using QIAprep Spin Miniprep Kit (Qiagen, Germany). The presences of *kAE1* WT-His insert in the recombinant plasmids were confirmed by *Not*I and *Xho*I digestions. The digestion reaction was carried out in 12 µl of volume containing 300-500 ng of pcDNA-*kAE1* WT-His, 5 units of *Not*I and *Xho*I in an appropriate buffer. The reaction mixture was incubated in a waterbath at 37 °C for 3 h. The digested *kAE1* WT-His fragment was detected by agarose gel electrophoresis.

(6) Automated DNA sequencing of pcDNA-*kAE1* WT-His

The recombinant plasmid, pcDNA-*kAE1* WT-His was verified by DNA sequencing. The sequencing reaction was performed by using ABI PRISM^{TM} Big

Dye™ Terminator Cycle Sequencing Kit and analyzed by ABI PRISM™ 310 DNA sequencer. The reaction mixture was made up in the volume of 20 µl containing 5 µl of Big Dye premix (terminator ready mix), 0.32 pmole of a sequencing primer and 500 ng of the recombinant 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. In each washing, the pellet was collected by centrifugation and ethanol was discarded. The pellet was then briefly dried at room temperature. After that, the pellet was resuspended in 25 µl of template suppression reagent (TSR), heated at 95 °C for 2 min to denature the sequencing product, followed by chilling on ice and analyzed by automated DNA sequencer ABI PRISM™ 310.

4.2.3 Generations of recombinant plasmids containing *kAE1* mutants by site-directed mutagenesis

pcDNA-*kAE1* WT-His, pcDNA-*kAE1* WT-HA, and pcDNA-*kAE1*-Myc The recombinant plasmids, pcDNA-*kAE1* SAO-His, pcDNA-*kAE1* G701D-His, pcDNA-*kAE1* SAO-HA and pcDNA-*kAE1* G701D-HA, were constructed from by site-directed mutagenesis. The recombinant plasmids containing *kAE1* SAO and *kAE1* G701D were screened by PCR and PCR/RFLP, respectively. They were all confirmed by DNA sequencing.

(1) Site-directed mutagenesis to generate plasmids containing *kAE1* SAO or *kAE1* G701D mutants tagged with sequence of either His, HA, or Myc

a. Generations of pcDNA-*kAE1* SAO-His, pcDNA-*kAE1* G701D-His, pcDNA-*kAE1* SAO-HA, and pcDNA-*kAE1* G701D-HA

pcDNA-*kAE1* SAO-His and pcDNA-*kAE1* G701D-His were generated by using pcDNA-*kAE1* WT-His as a template while pcDNA-*kAE1* SAO-HA and pcDNA-*kAE1* G701D-HA were generated by using pcDNA-*kAE1* WT-HA as a template. The protocol for site-directed mutagenesis as described by the

QuickChange TM site-directed mutagenesis kit of Stratagene was followed. The plasmids containing *kAE1* SAO or *kAE1* G701D tagged with either HA or His were generated by using SAO Fp/SAO Rp and G701D Fp/G701D Rp primers (Table 4.1), respectively. PCR was performed in the reaction volume of 50 µl 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 eliminate methylated parental DNA templates. Fifteen µl of digested PCR reaction were transformed into 200 µl of competent DH5α cell. Eight hundred µl of LB broth medium were added and incubated at 37 °C in a shaking incubator for 1 h. The mixture was centrifuged at 3,000 xg 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 spread on LB plate containing 100 µg/ml ampicillin and incubated at 37 °C for 12-16 h.

b. Generations of pcDNA-*kAE1* SAO-Myc and pcDNA-*kAE1* G701D-Myc

pcDNA-*kAE1* WT-Myc containing a sequence of Myc epitope inserted at the position 557 of kAE1 which locates at the third extracellular loop was used as a template to generate pcDNA-*kAE1* SAO-Myc and pcDNA-*kAE1* G701D-Myc, according to the protocol of the QuickChange TM site-directed mutagenesis kit from Stratagene, using SAO Fp/SAO Rp and G701D Fp/G701D Rp primers (Table 4.1). The method of site-directed mutagenesis was as same as that described in the last section. These recombinant plasmids were constructed and sequenced by the assistance from Miss Wandee Udomchaiprasertkul, Division of Medical Molecular Biology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University.

(2) Screening of colonies harboring pcDNA-*kAE1* SAO-His, pcDNA-*kAE1* G701D-His, pcDNA-*kAE1* SAO-HA, and pcDNA-*kAE1* G701D-HA

The *E. coli* colonies possibly harboring the recombinant plasmid containing either *kAE1*-SAO or *kAE1*-G701D were screened by PCR using L5/R5 primers for *kAE1*-SAO and by PCR using L9/R9 primers followed by *Hpa*II digestion for *kAE1*-G701D. The *kAE1* PCR products from pcDNA-*kAE1* SAO-HA and pcDNA-*kAE1* SAO-His were determined by 2% agarose gel electrophoresis whereas *kAE1* PCR products from pcDNA-*kAE1* G701D-HA and pcDNA-*kAE1* G701D-His were determined by digestion with *Hpa*II. The PCR product of *kAE1* WT could be digested with *Hpa*II while the PCR product of *kAE1* G701D could not. The digested product was detected on 2% agarose gel electrophoresis. The positive colonies were grown and plasmids were purified by QIAprep Spin Miniprep Kit (Qiagen, Germany).

(3) Sequencing analyses of recombinant plasmids

The recombinant plasmids, pcDNA-*kAE1* SAO-His, pcDNA-*kAE1* G701D-His, pcDNA-*kAE1* SAO-HA, and pcDNA-*kAE1* G701D-HA, were verified by DNA sequencing. The DNA sequencing was performed by a commercial company, Macrogen (Korea) following the standard protocol of ABI Prism ® BigDye ™ Terminator Cycle Sequencing Ready Reaction Kits v3.1 and using an Automatic DNA sequencer ABI3730.

4.2.4 Transfection of constructed plasmids into HEK 293 cells

HEK 293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (PERBIO), 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium). The cell culture was maintained in 25-cm^2 flask at 37 ºC with 5% CO_2 and 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 1 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 containing the cells was

incubated normally at 37 ºC with 5% CO_2.

To maintain long-term stocks, the cells were kept frozen in liquid nitrogen. The cells were subcultured as previously described 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 a 37 ºC waterbath. 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 was carried out again.

The day before transfection, the HEK 293 cells were collected by trypsinization and seeded in 6-well plates. DEAE-Dextran or lipofection transfection method was used to transiently transfect the cells with the recombinant plasmids.

(1) Transfection by using DEAE-Dextran method

The DEAE-Dextran transfection method was used for protein expression and heterodimerization studies. This method is simple, reproducible, and inexpensive. However, it is toxic to cells, especially when DMSO is used as a supplementary chemical shock to increase gene transfer efficiency, leading to abnormal cell shape and cell death. As an efficient method, it is appropriate for the transfection study that cell morphology is not considered. DEAE-Dextran-diethylaminoethyl-dextran (DEAE-Dextran) is a polycatonic derivative of the carbohydrate polymer, dextran. Because of its positive charge, DEAE-Dextran is able to bind to the anionic phosphodiester backbone of DNA. The resultant complex maintains an overall cationic charge and is able to bind to negatively charged cell membrane surfaces. Subsequently, the complex is internalized, presumably by endocytosis.

HEK 293 cells were grown in 6-well plate for a day. The cells with 50% confluence were transfected with 1 μg of a recombinant plasmid. The transfection

mixture consisted of 1 μg of recombinant plasmid, 10 μl of 10 mM chloroquine, 50 μl of 10 mg/ml DEAE-Dextran, and serum-free DMEM in a total volume of 1 ml. The mixture was slightly overlaid onto cells and the cells were incubated for 3 h. After incubation, cells were shocked with 1 ml of 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) Transfection by using lipofection method

The lipofection is probably the most commonly used transfection method for protein localization study because it has a low toxicity to cells; the physical cell shape is not changed or destroyed. Cationic transfection lipids are typically composed of a positively charged head group, such as an amine, a flexible linker group such as an ester or ether, and two or more hydrophobic tail groups. When combined with DNA, cationic lipids spontaneously act to form structures known as lipoplexes, which are much more complex than simple liposomes. When prepared under appropriate conditions, lipoplexes maintain an overall positive charge, enabling them to efficiently bind to negatively charged cell surfaces. Subsequently, the lipoplexes enter cells via the endocytotic pathway. This pathway would normally result in fusion with lysosomes and degradation of the DNA. However, neutral “helper” lipids, such as dioleoylethanolamine (DOPE), are typically included with the cationic lipid, allowing entrapped DNA to escape the endosomes. From there, the DNA can make its way to the nucleus and gain access to the transcriptional machinery of the cell.

HEK 293 cells were grown in 6-well plate for a day. The cells with 50% confluence were transfected with transfection mixture containing 1 μg of a recombinant plasmid in the transfection mixture composed of solution A and solution B. The recombinant DNA was initially diluted in 50 μl of serum-free DMEM as a solution A. The solution B was a 4 μl of lipofectamine™ 2000 diluted in serum-free DMEM in a total volume of 50 μl. The solutions A and B were incubated at room temperature for 5 min. After incubation, the solution A was combined with solution B, mixed gently and incubated for 20 min at room temperature. The serum-free DMEM was added to the combination mixture in a total volume of 1 ml and mixed

gently. The culture medium in 6-well plate containing HEK 293 cells was replaced with the transfection mixture and incubated at 37 ºC with 5% CO_2. After incubation for 4-6 h of incubation, 2x complete medium (20 % FBS, 200 U/ml penicillin and 200 µg/ml streptomycin) was added and the incubation was extended to 42-44 h.

4.2.5 Studies of wild-type and mutant kAE1 proteins expressed in HEK 293 cells

After transfection of the recombinant plasmids into HEK 293 cells, expression of wild-type and mutant kAE1 proteins was examined by Western-blot analysis. Co-transfections of the recombinant plasmids were performed to study heterodimerization of kAE1 proteins by co-immunoprecipitation and co-purification procedures and to examine kAE1 protein subcellular localization and trafficking by confocal microscopy and by FACS analysis, respectively.

(1) Protein expression study by Western blot analysis

To study kAE1 protein expression, HEK 293 cells were individually transfected with the plasmid construct containing *kAE1* fused with the sequence of an epitope tag. Two days after transfection, the transiently transfected HEK 293 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 lysis buffer [1 mM EDTA, 0.5% (v/v), Igepal (Nonidet P-40 detergent), 150 mM NaCl, 10 mM Tris-HCl pH 7.5] 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 the proteins were transferred to a sheet of nitrocellulose membrane. The membrane was blocked for 1 h in 5% skim milk in TBST (TBS with 0.1% Tween 20) and then incubated with a specific antibody reacting to an epitope tag that was fused to kAE1 at appropriate dilution in TBST containing 5% skim milk (anti-HA; 1:5000, anti-HIS; 1:5000, anti-Myc cultured supernatant; undiluted) for 2 h. After washing 3 times for 5 min each in TBST, the membrane was incubated with 1: 1,000 dilution of secondary antibody conjugated to horseradish peroxidase for 1 h. After washing 3 times in TBST, the band of specific protein was detected by SuperSignal West Pico Chemiluminescent Substrate

(PIERCE) plus Western Blotting Detection System for 5 min according to manufacturer's instruction. To detect a chemiluminescence signal, the membrane was exposed with an X-ray film in a cassette for various time points and the exposed X-film was developed by a film-developing machine.

(2) Study of heterodimerization of wild-type and mutant kAE1 proteins

To examine whether kAE1 WT could interact with kAE1 SAO or kAE1 G701D, or with each other, HEK 293 cells were co-transfected with the recombinant plasmids and heterodimerizations of the proteins were investigated by co-immunoprecipitation and his-tagged protein co-purification procedures.

a. His-tagged protein co-purification

Affinity (his-tagged protein) co-purification was performed by taking an advantage of 6xHis tag at the N-terminus of kAE1. HEK 293 cells were co-transfected with pcDNA-*kAE1* WT-His and pcDNA-*kAE1* SAO-HA or pcDNA-*kAE1* G701D-HA (transfections with each of plasmid constructs were performed as controls). Two days after transfection, the transfected cells were detached with 1 ml of phosphate buffer saline (PBS) (150 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and collected by centrifugation at 3,000 xg for 5 min. Cells were lysed with 500 µl of IPB^+ buffer [1 mM EDTA, 0.5% (v/v), Igepal (Nonidet P-40 detergent), 150 mM NaCl, 2% (w/v) bovine serum albumin, 10 mM Tris-HCl pH 7.5 and protease inhibitors cocktail] on ice for 15 min. The insoluble fraction was removed by centrifugation at 12,000 xg for 10 min at 4°C. After removing the insoluble materials, an aliquot 400 µl of cell lysate was incubated with 40 µl of Co^{2+} chelate resins (BD Bioscience) at 4°C for 12-16 h with rotation. Before use, the resins were washed twice with sterile water to remove ethanol. After incubation, the resins were collected by centrifugation at 12000 xg for 5 min and washed thoroughly with washing buffer 1 (0.1 mM EDTA, 0.1% Igepal, 150m M NaCl, 10 mM Tris-HCl, pH 7.5) and washing buffer 2 (0.1 mM EDTA, 10 mM Tris-HCl, pH 7.5). Bound kAE1 proteins were eluted with 2x SDS-PAGE sample loading buffer containing 2% (v/v) 2-mercaptoethanol and heated at 65°C for 5 min. The samples were subjected to SDS-PAGE and immunoblotting method as same as that was described for the

Western blot analysis. The presence of kAE1 in the eluates were detected by anti-HA antibody.

b. Co-immunoprecipitation

In co-immunoprecipitation, the co-transfected HEK 293 cells (and the transfected cell controls) were processed as same as described above. After the cell lysate was centrifuged to remove insoluble materials, the supernatant was transferred to a 1.5 ml clean microcentrifuge tubes containing 50 µl of Protein G-Sepharose resin mixture to pre-clear the cell lysates. Tubes were incubated for 2 h at 4 ˚C, with constant rotation. The resins were then removed by centrifugation at 7,500 xg for 5 min. One hundred µl of the supernatant were taken to be a total fraction and 400 µl of supernatant was immunoprecipitated with 2 µl of rabbit anti-HIS antibody and 60 µl of protein G-Sepharose beads. The immunoprecipitated complexes were incubated on a shaker for 12-16 h at 4°C. After incubation, the resins was collected by centrifugation and washed thoroughly with washing buffers 1 and 2 as mentioned above. Proteins were eluted with 2x SDS-PAGE sample loading buffer containing 2% (v/v) 2-mercaptoethanol and heated at 65°C for 5 min. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting method as previously described.

(3) Subcellular localization of wild-type and mutant kAE1 proteins

Indirect immunofluorescence (IF) and fluorescence activated cell sorting (FACS) analyses were performed to examine subcellular localization and trafficking of wild-type and mutant kAE1 proteins expressed in HEK 293 cells. The indirect immunofluorescence-stained cells were visualized by a Zeiss LSM 510 META confocal microscope and the protein trafficking was analyzed by FACSort™ flow cytometry. The experiments were performed with the cells that were individually transfected and co-transfected with the recombinant plasmids.

a. Indirect immunofluorescence examination

Subcellular localization of the expressed kAE1 protein in HEK 293 cells was examined by using an antibody specific to epitope tag fused to kAE1 and a secondary antibody labeled with a fluorochrome, recognizing the primary antibody. The images were captured by confocal microscope. The HEK 293 cells were grown on glass

coverslips in a 6-well plate. The cells were individually transfected with pcDNA-*kAE1* WT-HA, pcDNA-*kAE1* WT-Myc, pcDNA-*kAE1* SAO-HA, pcDNA-*kAE1* SAO-Myc, pcDNA-*kAE1* G701D-HA or pcDNA-*kAE1* G701D-Myc. In co-transfection experiments, the cells were co-transfected with pcDNA-*kAE1* WT-Myc plus *kAE1* SAO-HA or pcDNA-*kAE1* WT-Myc plus *kAE1* G701D-HA. They were also co-transfected with pcDNA-*kAE1* SAO-Myc plus pcDNA-*kAE1* G701D-HA, or with pcDNA-*kAE1* G701D-Myc plus pcDNA-*kAE1* SAO-HA. Two days post-transfection, the cells on the coverslips were washed once with 1x PBS and fixed with 4% paraformaldehyde in 1x PBS at room temperature for 20 min. After washing twice with 1x PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (1x PBS^{++}), coverslips were incubated in quenching buffer (100 mM glycine in 1x PBS) for 5 min. Then, cells were permeabilized in 0.2% Triton X-100 at room temperature for 15 min followed by rinsing twice with 1x PBS^{++}. The coverslips were blocked with 1% BSA in 1x PBS^{++} for 30 min and then incubated with primary antibodies (1:1000 dilution of rabbit anti-HA polyclonal antibody and undiluted cultured supernatant of mouse anti-Myc monoclonal antibody to detect kAE1-HA and kAE1-Myc, respectively) for 60 min at room temperature. The coverslips were washed thrice with 1x PBS^{++} for 5 min each following the incubation in a mixtures of 1:8000 dilution and 1:1000 of Cy3-conjugated donkey anti-rabbit IgG and Alexa Fluor® 488-conjugated goat anti-mouse IgG, respectively (Molecular Probes, Inc. Canada) to visualize kAE1-HA and kAE1-Myc, respectively. The coverslips were washed twice with 1x PBS^{++} and then mounted with 50% glycerol in 1x PBS. Fluorescence images were captured by Ziess LSM 510 META confocal microscopy. The pixels of cell-surface and total kAE1 protein expression were estimated by LSM 510 META software; then, the percentage of relative cell surface kAE1 protein expression was calculated as follows: relative cell surface expression = pixel of total expression / pixel of surface expression. Statistical analysis was performed by an unpaired Student's *t test* using Intercooled Stata 9.0 for Windows (StataCorp LP, College Station, TX, USA). The value of $p < 0.05$ was considered to be statistically significant.

b. Fluorescence activated cell sorting (FACS) analysis

FACS is a mean to analyze physical characteristics of single cells or particles. The properties analyzed include a relative size, relative granularity or internal complexity, and relative fluorescence intensity. Thus, amount of protein expression can be measured by this method after fluorescence staining. kAE1-Myc was used for this analysis because the Myc epitope was inserted at the third extracellular loop of kAE1 so that it would expose to an extracellular area when it was expressed. Thus, expression and trafficking of kAE1-Myc to the cell surface could be determined. Transfected HEK 293 cells expressing kAE1 WT-Myc, kAE1 SAO-Myc or kAE1 G701D-Myc, or co-expressing kAE1 WT-Myc and either kAE1 SAO-HA or kAE1 G701D-HA, or co-expressing kAE1 SAO-Myc and kAE1 G701D-HA, or co-expressing kAE1 G701D-Myc and kAE1 SAO-HA were investigated.

The transfected cells were collected by centrifugation at 2000 xg, 4 ˚C for 5 min. They were resuspended in chilled DMEM containing with 2% fetal bovine serum, 1% BSA and 10 mM NaN_3 and permeabilized (for total protein) or non-permeabilized (for surface protein) with 0.2% Triton X-100/PBS before incubated with undiluted cultured supernatant of mouse anti-Myc antibody for 1 h. After incubation, the cells were washed twice with chilled DMEM containing with 2% fetal bovine serum, 1% BSA and 10 mM NaN_3. Then, 1:1000 goat anti-mouse Alexa 488 (Molecular Probes, Eugene, OR, USA) was used to probe mouse anti-Myc antibody for 30 min, on ice. All samples were washed and analyzed by using FACSort™ flow cytometer (Becton Dickinson, Mountain View, CA, USA.). The intensity of the fluorescent signal was determined to quantify the level of the cell surface expression or total expression of kAE1; then, the percentage of relative cell-surface expression of kAE1 was calculated from percentage of positive cells as follows: relative cell-surface expression = percentage of positive cells with cell-surface expression of kAE1 / percentage of positive cells with total kAE1 expression. And, relative fluorescence intensity was also calculated from mean fluorescence intensity (MFI) as follows: relative fluorescence intensity = (MFI of surface expression - MFI of vector control) / MFI of vector control. Statistical analysis was performed by an unpaired Student's *t test*. The value of $p < 0.05$ was considered to be statistically significant.

CHAPTER V

RESULTS

5.1 Construction of pcDNA-*kAE1* WT-His

pcDNA-*kAE1* WT-His was constructed for studying protein heterodimerization by his-tagged protein co-purification and co-immunoprecipitation procedures. *kAE1* WT cDNA was amplified and inserted into pcDNA3.1/HisB. Transformant *E. coli* clones containing pcDNA-*kAE1* WT-His were screened by PCR. The pcDNA-*kAE1* WT-His isolated was verified by DNA sequencing.

5.1.1 Isolation of *kAE1* WT cDNA by PCR and cloning into pcDNA3.1/HisB

A full-length wild-type *kAE1* cDNA with the size of 2.5 kb was amplified from the pcDNA-*kAE1* WT (a generous gift from Professor Reinhart A, Department of Biochemistry and Medicine, University of Toronto, Canada) by using kAE1 *Not*I Fp and kAE1 *Xho*I Rp primers. The result is shown in Figure 5.1. The PCR products were digested with combination of *Not*I and *Xho*I and purified by QIAGEN PCR purification kit. The *kAE1* fragment was then ligated into pcDNA3.1/HisB vector digested with *Not*I/*Xho*I to generate pcDNA-kAE1 WT-His and transformed to *E. coli* DH5α competent cells.

5.1.2 Screening of *E. coli* containing pcDNA-*kAE1* WT-His

The transformant *E. coli* clones containing pcDNA-*kAE1* WT-His were screened by PCR method using kAE1 *Not*I Fp and kAE1 *Xho*I Rp primers. The transformants that gave positive results showing the PCR products with the size of 2.5 kb were selected (Figure 5.2). The plasmids were purified by QIAGEN MiniPrep kit and confirmed by digestion with either *Not*I alone or with combination of *Not*I and *Xho*I. The plasmid digested with *Not*I alone showed one fragment of a linear pcDNA-*kAE1* WT-His with the size of 7.9 kb while that digested with *Not*I and *Xho*I gave to two

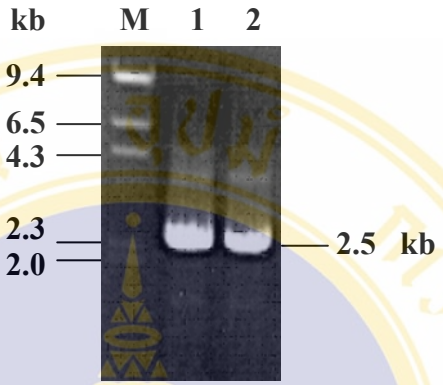


Figure 5.1 Gel electrophoresis of *kAE1* cDNA amplification.
The full-length wild-type *kAE1* cDNA with the size of 2.5 kb was amplified with kAE1 *Not*I Fp and kAE1 *Xho*I Rp primers from pcDNA-*kAE1* WT. Lane M is *Hind*III-digested λ DNA markers. The amplified *kAE1* cDNA is shown in lanes 1 and 2.

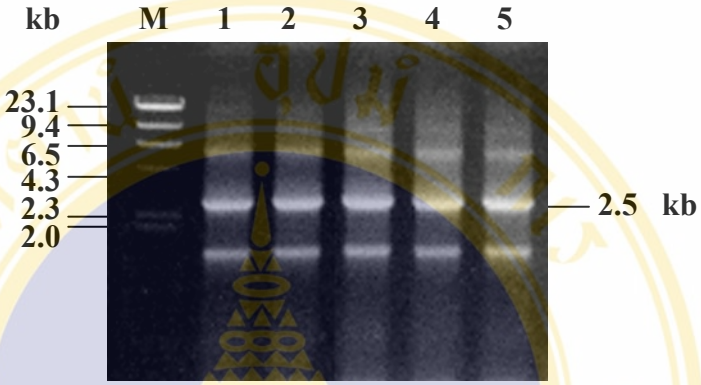


Figure 5.2 Screening of transformant *E. coli* clones containing pcDNA-*kAE1* WT-His by PCR.

The transformant clones containing pcDNA-*kAE1* WT-His were screed by PCR using kAE1 *Not*I Fp and kAE1 *Xho*I Rp primers to amplify *kAE1* cDNA fragment. Lane M is *Hind*III-digested λ DNA markers. Lanes 1-5 are amplifications of *kAE1* cDNA from clone numbers 1-5 showing the PCR product with the size of 2.5 kb.

Figure 5.3 **Screening of pcDNA-*kAE1* WT-His by restriction endonuclease digestion.**

The purified pcDNA-*kAE1* WT-His was digested with either *Not*I alone or with combination of *Not*I and *Xho*I. Lane M is *Hind*III-digested λ DNA markers. Lanes 1, 3, 5, 7, and 9 are pcDNA-*kAE1* WT-His digested with *Not*I gave only one fragment of linear pcDNA-*kAE1* WT-His with the size of 7.9 kb. Lanes 2, 4, 6, 8, and 10 are pcDNA-*kAE1* WT-His digested with *Not*I and *Xho*I gave to two fragments, pcDNA3.1/HisB vector and *kAE1* cDNA insert with sizes of 5.4 kb and 2.5 kb, respectively.

fragments, pcDNA3.1/HisB vector and *kAE1* cDNA insert with sizes of 5.4 kb and 2.5 kb, respectively (Figure 5.3).

The clone number 1 containing pcDNA-*kAE1* WT-His was selected for a medium scale of plasmid preparation and verified by DNA sequencing. This plasmid construct would be used for site-directed mutagenesis to generate pcDNA-*kAE1* SAO-His and pcDNA-*kAE1* G701D-His.

5.1.3 Automated DNA sequencing

The purified pcDNA-*kAE1* WT-His was analyzed by a custom-service direct DNA sequencing performed by Macrogen (Korea). The entire region of *kAE1* cDNA was sequenced by using primers as showed in Table 4.1. The result showed that the regions of *kAE1* cDNA, its junction region, and the sequence of His tag were all correct (data not shown).

5.2 Creations of pcDNA-*kAE1* SAO-His, pcDNA-*kAE1* G701D-His, pcDNA-*kAE1* SAO-HA, and pcDNA-*kAE1* G701D-HA

The plasmids containing mutant *kAE1* tagged with the sequences of His or HA, pcDNA-*kAE1* SAO-His, pcDNA-*kAE1* G701D-His, pcDNA-*kAE1* SAO-HA, and pcDNA-*kAE1* G701D-HA, were constructed by site-directed mutagenesis and transformed into *E. coli*. The transformant colonies containing either pcDNA-*kAE1* SAO-His or pcDNA-*kAE1* SAO-HA were screened by PCR and gel-electrophoresis whereas the transformant colonies containing either pcDNA-*kAE1* G701D-His or pcDNA-*kAE1* G701D-HA were screened by PCR followed by restriction enzyme – *Hpa*II digestion and electrophoresis. The presences of SAO and G701D mutations in *kAE1* cDNA and the tagged sequences were examined by direct DNA sequencing.

5.2.1 Site-directed mutagenesis

pcDNA-*kAE1* WT-His and pcDNA-*kAE1* WT-HA (the latter was a gift from Miss Thitima Keskanokwong, Institute of Molecular Biology and Genetics, Mahidol University) were used as a template for site-directed mutagenesis to generate pcDNA-*kAE1* SAO-His, pcDNA-*kAE1* G701D-His, and pcDNA-*kAE1* SAO-HA and pcDNA-*kAE1* G701D-HA, respectively. The plasmids containing *kAE1* SAO and *kAE1* G701D were generated by using SAO Fp and SAO Rp primers (Figure 5.4, lanes 1-3),

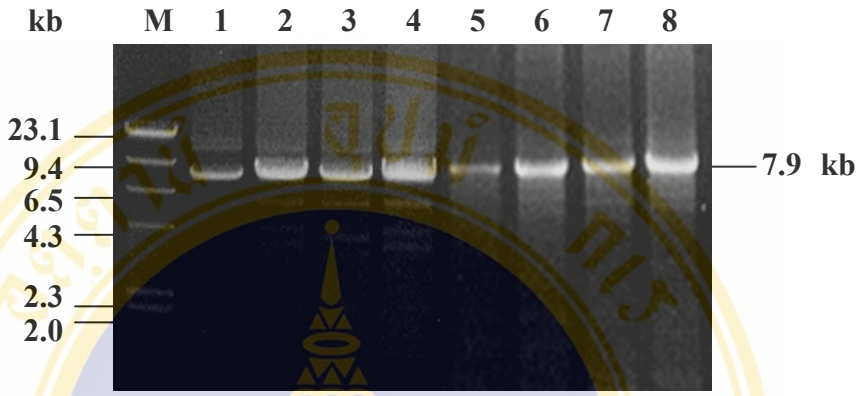


Figure 5.4 **Agarose gel electrophoresis of PCR products of pcDNA-*kAE1* SAO-His (or –HA) and pcDNA-*kAE1* G701D-His (or HA) created from pcDNA-*kAE1* WT-His (or –HA) by site-directed mutagenesis using PCR.**

Lane M is *Hind*III-digested λ DNA markers. Lanes 1 and 2 are PCR products of pcDNA-*kAE1* SAO-His and pcDNA-*kAE1* G701D-His, respectively. Lanes 3 and 4 are PCR products of pcDNA-*kAE1* SAO-HA and pcDNA-*kAE1* G701D-HA, respectively. Lanes 5-8 are PCR products of pcDNA-k*AE1* SAO-His, pcDNA-*kAE1* G701D-His, pcDNA-k*AE1* SAO-HA, and pcDNA-*kAE1* G701D-HA after digestion with *Dpn*I. The sizes of all plasmids were about 7.9 kb.

and by using G701D Fp and G701D Rp (Figure 5.4, lanes 2-4), respectively. To eliminate original template before transformation into *E. coli*, the amplified plasmids were digested with *Dpn*I (Figure 5.4, lanes 5-8).

5.2.2 Screening of *E. coli* clones containing recombinant mutant plasmids

The *E. coli* clones containing pcDNA-*kAE1* SAO-His and pcDNA-*kAE1* SAO-HA were screened by PCR using L5/R5 primers followed by electrophoresis, and those containing pcDNA-*kAE1* G701D-His and pcDNA-*kAE1* G701D-HA were screened by PCR using L9/R9 primers followed by *Hpa*II digestion and electrophoresis. Figure 5.5 shows the PCR products amplified from pcDNA-*kAE1* WT-His, pcDNA-*kAE1* SAO-His, and pcDNA-*kAE1* SAO-HA. The PCR product from the wild-type *kAE1* (318 bp) and *kAE1* SAO with a 27 bp-deletion (291 bp) could be differentiated by 2% agarose gel electrophoresis. The PCR product of *kAE1* SAO with a shorter fragment moved faster than that of wild-type on the electrophoresis.

The PCR products amplified from pcDNA-*kAE1* WT-His and pcDNA-*kAE1* G701D-His (or –HA) by L9/R9 primers were digested with *Hpa*II to differentiate from each other. The size of PCR products after the amplification was 290 bp. The PCR product amplified from pcDNA-*kAE1* WT-His could be digested but that of pcDNA-*kAE1* G701D-His (or –HA) could not be digested with *Hpa*II at the mutated site. The PCR product amplified from pcDNA-*kAE1* WT-His was digested with *Hpa*II at two positions giving three fragments with the sizes of 164, 106, and 20 bp (the latter could not be observed on the gel). The PCR product amplified from pcDNA-*kAE1* G701D could not be digested with *Hpa*II at the mutated size but could be cut at one site outside, generating fragments with the sizes of 270 and 20 bp. Figure 5.6 shows examples of *Hpa*II digestion of the PCR product amplified from pcDNA-*kAE1* G701D-His and pcDNA-*kAE1* G701D-HA. From these screenings, two each of positive clones containing pcDNA-*kAE1* SAO-His, pcDNA-*kAE1* SAO-HA, pcDNA-*kAE1* G701D-His, and pcDNA-*kAE1* G701D-HA were obtained.

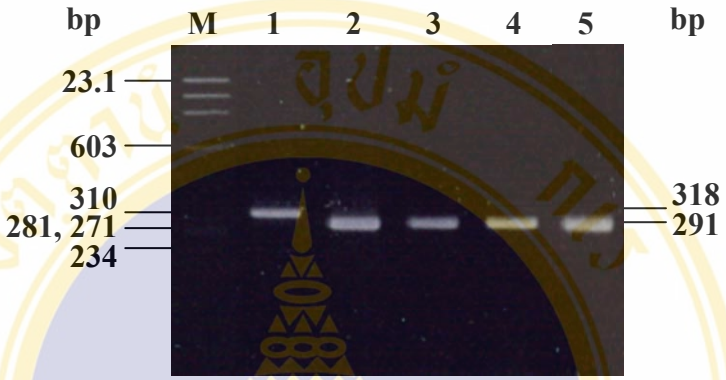


Figure 5.5 **Agarose gel electrophoresis of *kAE1* amplified by PCR from pcDNA-*kAE1* SAO-His and pcDNA-*kAE1* SAO-HA generated by site-directed mutagenesis.**

kAE1 SAO partial fragment was amplified by PCR using L5/R5 primers and the PCR products were examined on 2% agarose gel electrophoresis. Lane M is *Hae*III-digested ϕX174 DNA markers. Lane 1 is the PCR product of *kAE1* WT amplified from pcDNA-*kAE1* WT, served as positive control. Lanes 2-3 are PCR products of *kAE1* SAO amplified from pcDNA-*kAE1* SAO-His obtained from clone numbers 1-2, generated by site-directed mutagenesis. Lanes 4-5 are PCR products of *kAE1* SAO amplified from pcDNA-*kAE1* SAO-HA from clone numbers 1 and 2.

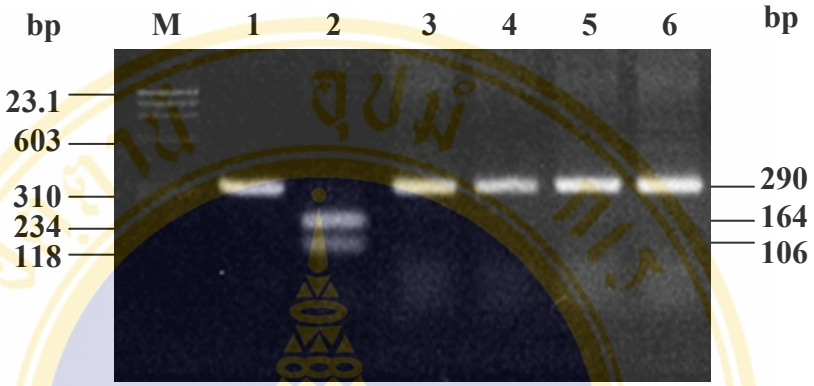


Figure 5.6 **Agarose gel electrophoresis of *Hpa*II digested *kAE1* G701D amplified by PCR from pcDNA-*kAE1* G701D-His and pcDNA-*kAE1* G701D-HA generated by site-directed mutagenesis.**

kAE1 G701D partial fragment was amplified by PCR using L9/R9 primers and digested with *Hpa*II. Lane M is *Hae*III-digested ϕX174 DNA markers. Lane 1 is undigested PCR product of *kAE1* WT with the size of 290 bp. Lane 2 is PCR product of *kAE1* WT that could be digested with *Hpa*II at two positions giving product sizes of 164, 106 and 20 bp (the latter is undetectable). Lanes 3-4 and lanes 5-6 are *Hpa*II digested PCR products from *kAE1* G701D amplified using pcDNA-*kAE1* G701D-His (clone numbers 1-2) and pcDNA-*kAE1* G701D-HA (clone numbers 1-2), respectively, as DNA templates. The PCR product of *kAE1* G701D could not be digested with *Hpa*II at the mutated site, but be digested at the other site; generating the fragments with sizes of 270 and 20 bp (the latter is undetectable).

5.2.3 DNA sequencing of *kAE1* SAO and *kAE1* G701D mutant plasmids

The entire *kAE1* region of *kAE1* SAO-His (from clone number 1), *kAE1* SAO-HA (from clone number 1), *kAE1* G701D-His-(from clone number 1) and *kAE1* G701D-HA (from clone number 1) were analyzed by DNA sequencing. All the recombinant plasmids harbored the *kAE1* SAO or *kAE1* G701D mutation as expected. Figure 5.7 shows chromatograms of DNA sequences of *kAE1* WT and *kAE1* SAO in the region encoding amino acid residues 400-408. The chromatograms of *kAE1* WT from pcDNA3-*kAE1* WT-His (Figure 5.7A) maintains DNA sequence in this region. pcDNA-*kAE1* SAO-His (Figure 5.7B) and pcDNA-*kAE1* SAO-HA (Figure 5.7C) harbors a deletion of 27 nucleotides encoding for nine amino acids (residues 400-408) as designed.

The sequencing analysis shows that pcDNA-*kAE1* WT-His contains a GGC codon for glycine (Figure 5.8A) and pcDNA-*kAE1* G701D-His and pcDNA-*kAE1* G701D-HA comprise a CAG codon for aspartic acid as designed (Figure 5.8B and 5.8C).

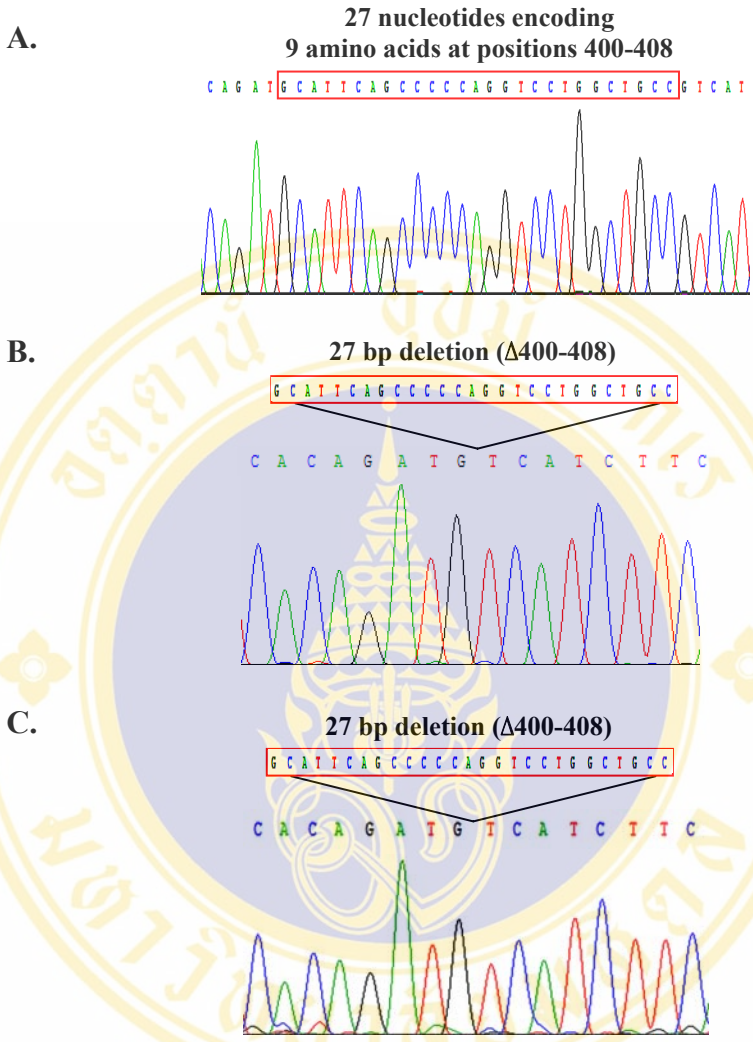


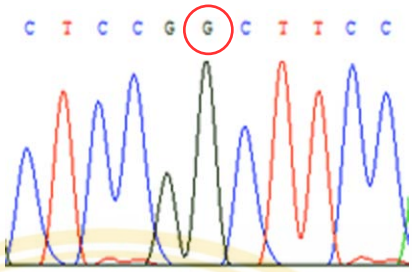
Figure 5.7 **Partial sequencing profiles in the mutated regions of pcDNA-*kAE1* SAO-His (or –HA).**

A. The nucleotide sequence of pcDNA-*kAE1* WT-His in the *kAE1* region of codons 400-408 corresponding to the mutated region that is shown in B.

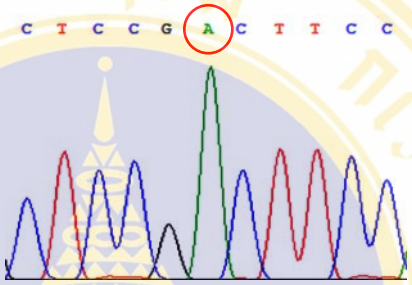
B. The nucleotide sequence of pcDNA-*kAE1* SAO-His in the mutated region of *kAE1*, codons 440-408. The sequence in the mutated region harbors a deletion of 27 nucleotides encoding for 9 amino acids.

C. The nucleotide sequence of pcDNA-*kAE1* SAO-HA in the mutated region of *kAE1*, codons 440-408. The sequence in the mutated region harbors a deletion of 27 nucleotides encoding for 9 amino acids.

A.



B.



C.

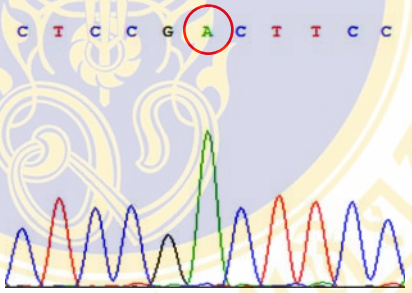


Figure 5.8 **Partial sequencing profiles in the mutated regions of pcDNA-*kAE1* G701D-His (or –HA).**

A. The nucleotide sequence of pcDNA-*kAE1* WT-His in the *kAE1* region corresponding to the mutated region in B. The codon 701 is GGC encoding for glycine.

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

C. The nucleotide sequence of pcDNA-*kAE1* G701D-HA in the mutated region of *kAE1*. The codon 701 is changed to GAC encoding for aspartic acid.

5.3 Studies of wild-type and mutant kAE1 proteins expressed in HEK 293 cells

The recombinant plasmids containing *kAE1* WT, *kAE1* SAO, or *kAE1* G701D were individually transfected or co-transfected into HEK 293 cells to examine protein expression, heterodimerization and subcellular localization.

5.3.1 Expression of wild-type and mutant kAE1 proteins

To study expression of wild-type and mutant kAE1 proteins in HEK293 cells, 1 μg each of pcDNA-*kAE1* WT-His, pcDNA-*kAE1* WT-HA, pcDNA-*kAE1* WT-Myc and all of *kAE1* SAO and *kAE1* G701D plasmid constructs were individually transfected in HEK 293 cells by using DEAE-Dextran transfection protocol. The protein expression was examined by Western blot analysis using antibody to 6xHis for kAE1-His, antibody to HA for kAE1-HA and antibody to Myc for kAE1-Myc proteins. The HEK 293 with pcDNA3.1/HisB vector transfection was used as control and it was found that there was no endogenous kAE1 protein expression in HEK 293 cells that could be detected by the antibodies used (data not shown). The cells transfected with the recombinant plasmid containing either *kAE1* WT, *kAE1* SAO, or *kAE1* G701D cDNA fused with the epitope tag (His, HA, or Myc) expressed the kAE1 fusion proteins with molecular weight (MW) of ~96 kDa, close to that was calculated (Figure 5.9).

5.3.2 Study of heterodimerization of wild-type and mutant kAE1 proteins

(1) His-tagged protein co-purification

To examine whether kAE1 SAO and kAE1 G701D could form a heterodimer with kAE1 WT, and whether kAE1 SAO could form a heterodimer to kAE1 G701D. The pcDNA-*kAE1* WT-His and pcDNA-*kAE1* WT-HA plasmids expressing kAE1-His and kAE1-HA, respectively, were used in this study. The co-expressions were performed in HEK 293 cells in the following combinations: kAE1 WT-His co-expressed with either kAE1 SAO-HA or kAE1 G701D-HA, kAE1 SAO-His co-expressed with kAE1 G701D-HA, and kAE1 SAO-HA co-expressed with kAE1 G701D-His kAE1. Additionally, the expression of only kAE1 WT-HA, kAE1 SAO-HA or kAE1 G701D-HA in the presence of pcDNA3.1/HisB was also performed as controls. The complexes of kAE1 WT-His and any associated proteins, i.e. kAE1

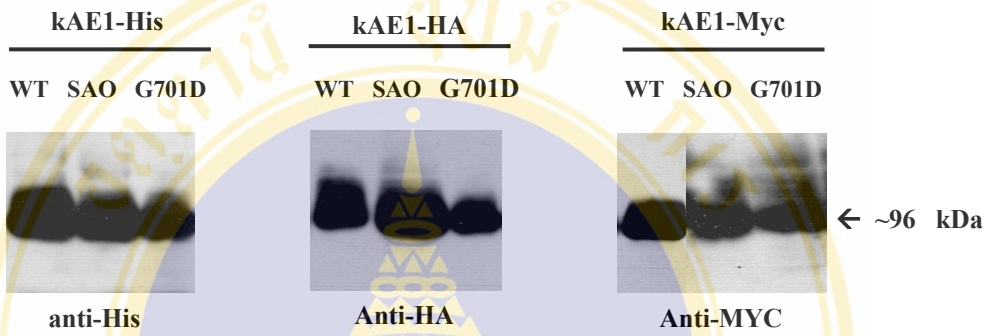


Figure 5.9 Western blot analysis of wild-type and mutant kAE1 proteins expressed in transfected HEK 293 cells.

The kAE1 WT, kAE1 SAO, and kAE1 G701D proteins expressed in transfected HEK 293 cells were analyzed by Western blot method using antibodies to epitope tags (anti-HA, -His ,and -Myc). The left panel shows the results of transfected cells expressing kAE1 WT-His, kAE1 SAO-His, and kAE1 G701D-His probed by anti-His antibody. The middle panel shows the results of transfected cells expressing the wild-type and mutant kAE1-HA proteins detected by anti-HA antibody. And, the right panel shows the results of transfected cells expressing the wild-type and mutant kAE1-Myc proteins detected by anti-Myc antibody.

WT-HA or kAE1 SAO-HA or kAE1 G701D-HA that bind to Co^{2+} resin using polyhistidine residues were eluted with 4x sample loading buffer. The eluted kAE1 WT-His and kAE1 WT-HA or kAE1 SAO-HA or kAE1 G701D-HA proteins were detected by immunoblot using anti-His and anti-HA antibody, respectively (Figure 5.10).

kAE1 WT-HA, kAE1 SAO-HA, and kAE1 G701D-HA could be expressed in the presence of pcDNA3.1/HisB vector and were detected by anti-HA antibody (Figure 5.10, lanes 1-3). However, as kAE1-His was not co-expressed, these kAE1-HA proteins could not be purified by the Co^{2+}-affinity resin (Figure 5.10, lanes 1-3). On the contrary, kAE1 WT-His could be expressed in the presence of pcDNA3.1/HisB vector and be purified by the Co^{2+}-affinity resin (Figure 5.10, lane 4). However, as kAE1-HA was not co-expressed, kAE1 WT-His could not be observed by staining with anti-HA antibody but it could be detected by anti-His antibody, confirming the effective method (Figure 5.10, lane 4). kAE1 WT-HA was expressed and could be detected using anti-HA antibody when co-expressed with kAE1 WT-His (Figure 5.10). kAE1 WT-His either was expressed in combination with kAE1 WT-HA, or other mutant HA-tagged proteins (kAE1 SAO and kAE1 G701D). The result was shown in Figure 5.10. Co-expression of kAE1 WT-His with kAE1 WT-HA revealed the dimerization after co-purification (Lane 5); kAE1-HA was visualized by anti-HA antibody. Likewise, kAE1 SAO-HA and kAE1 G701D-HA could be co-purified with kAE1 WT-His (Lanes 6-7, respectively). Thus, heterodimerization between kAE1 WT and SAO or G701D was demonstrated. To mimic compound heterozygote SAO/G701D caused dRTA, kAE1 SAO and G701D with different tag were co-expressed in HEK 293 cells to understand mechanism of dRTA occurred from this mutations. The interaction between kAE1 SAO and kAE1 G701D was studied by using histidine tagged co-purification (Figure 5.10, lanes 8-9, respectively). The results demonstrated that kAE1 SAO or kAE1 G701D interacted with kAE1 WT, and kAE1 SAO interacted with kAE1 G701D. Thus, the mutant proteins were not only capable of interacting with the wild-type protein but also with each other.

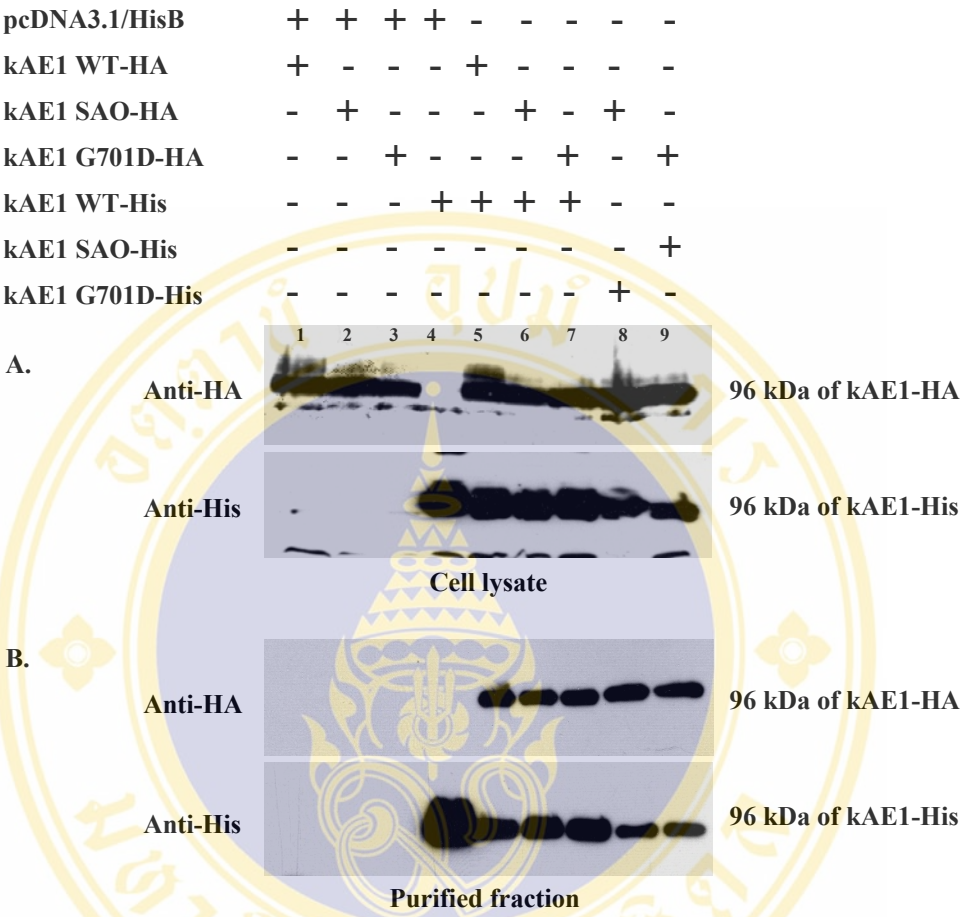


Figure 5.10 Co-purification between wild-type and mutant kAE1 proteins in HEK 293 cells.

HEK 293 cells were co-transfected with the plasmid constructs expressing kAE1-His and kAE1-HA. The co-transfected cells were lysed and his-tagged oligomers were purified by Co^{2+}-affinity resin. The purified proteins were eluted and analyzed by SDS-PAGE and immunoblotting.

A. The immunoblots of the cell-lysate probed with anti-HA (upper) and anti-His (lower), detecting kAE1-HA and kAE1-His expression.

B. The immunoblots of purified protein-complexes probed with anti-HA (upper) and anti-His (lower), detecting kAE1-HA co-purified with kAE1-His.

(2) Co-immunoprecipitation

To confirm kAE1 protein dimerization and heterodimerization by a different method, co-immunoprecipitation was also carried out by using the transfected and co-transfected HEK 293 cells that were used in the His-tagged protein co-purification described in the previous section. The transfected and co-transfected HEK 293 cells were lysed by a mild lysis solution (IPB^{++}) containing 0.5% NP-40 and 0.15 M NaCl to preserve protein-protein interaction. kAE1-His and associated proteins were immunoprecipitated by using anti-His antibody. The presences of kAE1-His and other associated proteins in the immunoprecipitates were determined by immunoblotting using anti-HA antibody for associated protein and anti-His antibody for precipitated kAE1-His protein (Figure 5.11). The results showed that kAE1 SAO-HA and kAE1 G701D-HA was immunoprecipitated with kAE1 WT-His. As similar with the results of the previous experiment, both kAE1 SAO-HA/kAE1 G701D-His and kAE1 SAO-His/kAE1 G701D-HA were co-immunoprecipitated, indicating the heterodimerization of kAE1 SAO and kAE1 G701D (Figure 5.11).

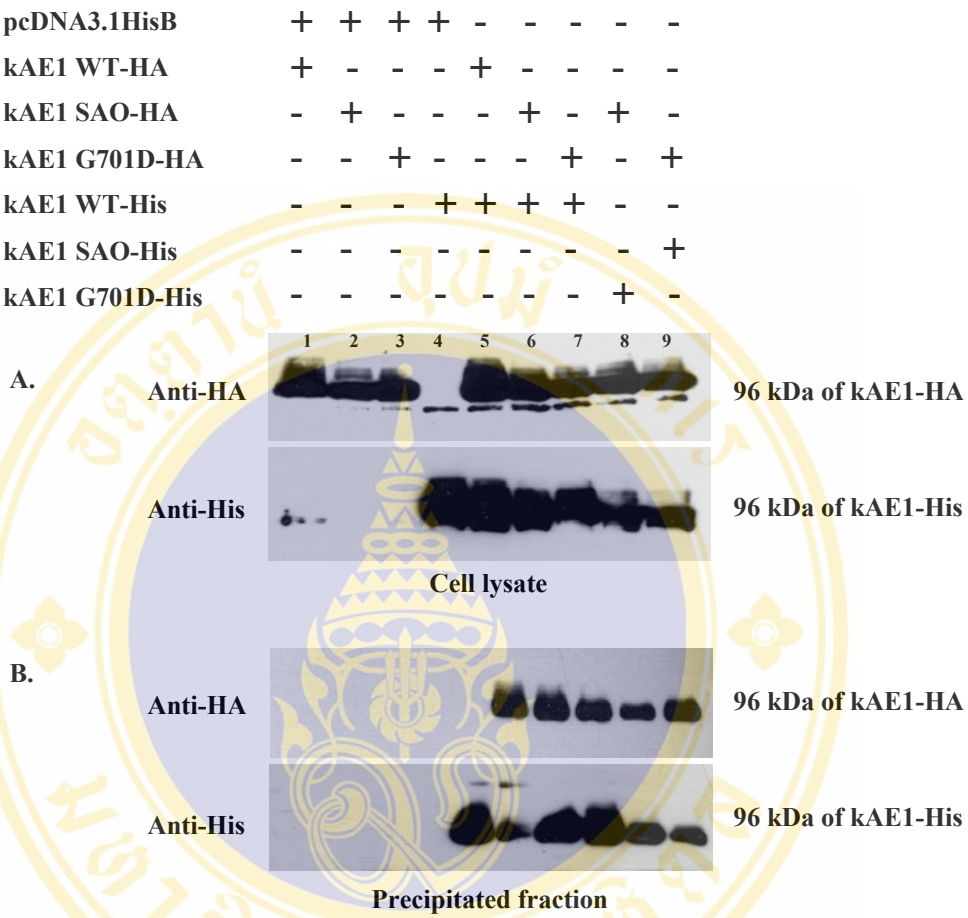


Figure 5.11 Co-immunoprecipitation of wild-type and mutant kAE1 proteins in HEK 293 cells.

HEK 293 cells transfected and co-transfected with the plasmid constructs expressing kAE1-His and kAE1-HA were lysed and immunoprecipitated with anti-His antibody. Immunoprecipitated proteins were eluted and analyzed by SDS-PAGE and immunoblotting.

A. The immunoblots of the cell-lysate probed with anti-HA (upper) and anti-His (lower), detecting kAE1-HA and kAE1-His expression.

B. The immunoblots of immunoprecipitated protein-complexes probed with anti-HA (upper) and anti-His (lower), detecting kAE1-HA co-immunoprecipitated with kAE1-His.

5.3.3 Subcellular localization of wild-type and mutant kAE1 proteins

(1) Subcellular localization study of wild-type and mutant kAE1 proteins by immunofluorescence and confocal microscopy

Subcellular localization of wild-type and mutant kAE1 proteins were examined by using indirect immunofluorescence and confocal microscopy. HEK 293 cells were either individually transfected with *kAE1*-Myc or co-transfected with pcDNA-*kAE1* WT-Myc/pcDNA-*kAE1* WT-HA, pcDNA-*kAE1* WT-Myc/pcDNA-*kAE1* SAO-HA, pcDNA-*kAE1* WT-Myc/pcDNA-*kAE1* G701D-HA, pcDNA-*kAE1* SAO-Myc/ pcDNA-*kAE1* G701D-HA, or pcDNA *kAE1* G701D-Myc/pcDNA-*kAE1* SAO-HA by using Lipofectamine 2000 transfection protocol. Two days after transfection, transfected HEK 293 cells were fixed and then stained with antibody against Myc or HA epitope tags, followed by Alexa 488 conjugated goat anti-mouse or Cy-3 conjugated donkey anti-rabbit antibody. The transfected HEK 293 cells were visualized under Zeiss LSM 510 META confocal microscope. Figure 5.12 (left and right panels) shows localizations of kAE1 WT-Myc, kAE1 SAO-Myc, and kAE1 G701D-Myc and localizations of kAE1 WT-HA, kAE1 SAO-HA, and kAE1 G701D-HA in HEK 293 cells, respectively. The representative transfected cells expressing kAE1 WT-Myc and kAE1 WT-HA shows their predominant expressions at the cell surface (Figure 5.12A-B and G-H). In contrast, the HEK 293 cells intracellularly expressed kAE1 SAO tagged with Myc or HA, or kAE1 G701D tagged with Myc or HA (Figure 5.12C-F and I-L). The numbers of total and cell-surface pixels of kAE1 expression were examined from representative cells by using LSM 510 META software and the percentages of relative cell-surface expression were calculated from the numbers of cell-surface pixels against total pixels. The percentages of relative cell-surface expressions of kAE1 WT-Myc was 74.9±9.6% (n=10), kAE1 SAO-Myc 0.9±0.4% (n=10, $p<0.0001$), and kAE1 G701D-Myc 1.2±0.6% (n=10, $p<0.0001$) (Table 5.1 and Figure 5.15). A similar results were obtained for cell-surface expressions of kAE1 WT-HA (78.3±5.6%, n=3), kAE1 SAO-HA (1.5±0.4%, n=3, $p<0.0001$), and kAE1 G701D-HA (1.4±0.2%, n=3, $p<0.0001$). Thus, cell-surface expressions of kAE1 SAO and kAE1 G701D were greatly decreased.

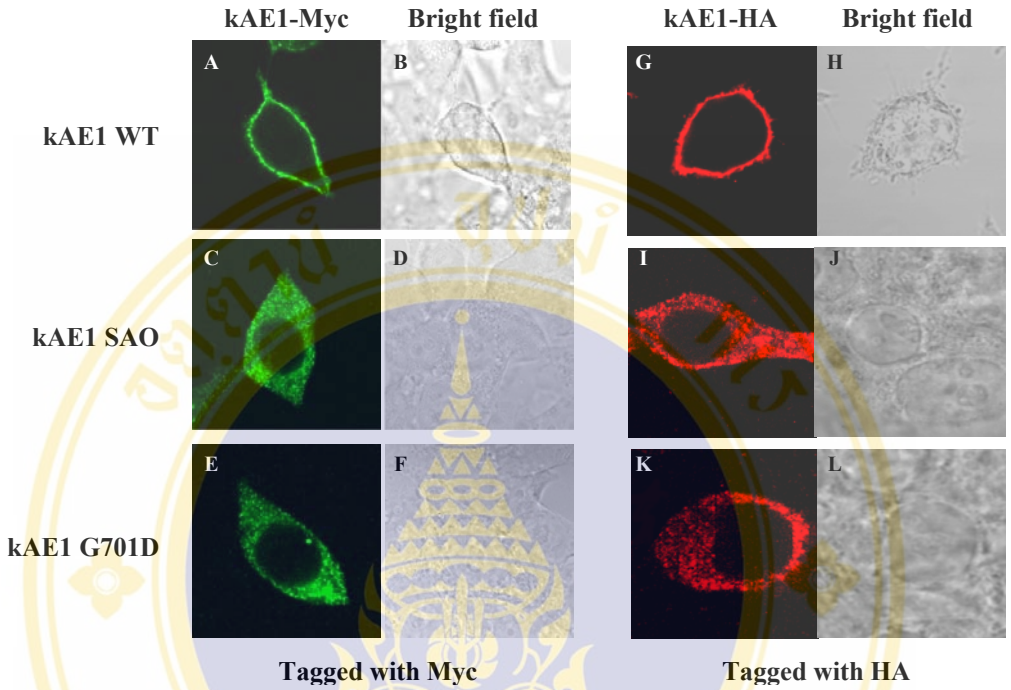


Figure 5.12 Subcellular localization of wild-type and mutant kAE1.

HEK 293 cells grown on coverslips were transfected with the recombinant plasmids expressing wild-type and mutant kAE1. Two days after the transfection, cells were fixed with 3.7% paraformaldehyde, permeabilized by incubation in 0.2% Triton X-100, and blocked with 1% BSA. kAE1 was stained with anti-Myc or anti-HA antibody. Alexa 488-conjugated mouse antibody or Cy3-conjugated antibody was used to stain kAE1 WT-Myc or kAE1 WT-HA. Immunofluorescence images were captured by using Zeiss LSM510 META confocal microscopy. A-B and G-H are kAE1 WT, C-D and I-J are kAE1 SAO, and E-F and K-L are kAE1 G701D. Left column is confocal images of kAE1-Myc and right column is confocal images of kAE1-HA.

Table 5.1 Percentages of cell-surface expression of kAE1 protein in transfected HEK 293 cells.

kAE1 fusion protein	Number of kAE1 pixel*		Percentage of cell-surface expression (%)	Average percentage of cell-surface expression of kAE1 (mean ± SD)
	Total	Surface		
kAE1 WT-Myc	48940	34061	69.60	74.9±9.6
	31298	28501	91.06	
	7909	6138	77.61	
	10178	8650	84.99	
	9547	6967	72.98	
	39417	30637	77.73	
	8480	6908	81.46	
	12485	8313	66.58	
	13920	8145	58.51	
	10937	7497	68.55	
kAE1 SAO-Myc	35460	185	0.52	0.9±0.4
	31738	126	0.40	
	17067	199	1.17	
	13739	83	0.60	
	13731	134	0.98	
	20482	374	1.83	
	17916	242	1.35	
	14513	167	1.15	
	56599	464	0.82	
	76612	401	0.52	
kAE1 G701D-Myc	17525	142	0.81	1.2±0.6
	18664	141	0.76	
	7934	55	0.69	
	12827	254	1.98	
	12459	215	1.73	
	9944	60	0.60	
	11066	261	2.36	
	29661	312	1.05	
	19306	185	0.96	
	7971	87	1.09	
kAE1 WT-HA	9780	7208	73.70	78.3±5.6
	15385	13013	84.58	
	12720	9745	76.61	
kAE1 SAO-HA	14431	150	1.04	1.5±0.4
	21482	390	1.82	
	17901	278	1.55	
kAE1 G701D-HA	11404	139	1.22	1.4±0.2
	9066	147	1.62	
	29582	397	1.34	

*The pixel numbers of kAE1-Myc were obtained from ten cells and kAE1-HA were obtained from three cells.

Sub-cellular localization of co-expressed kAE1 WT-Myc/kAE1 WT-HA, kAE1 WT-Myc/kAE1 SAO-HA, or kAE1 WT-Myc/kAE1 G701D-HA were examined to observe the effect of kAE1 WT on kAE1 SAO or kAE1 G701D, and *vice versa* in HEK 293 cells. Two days after co-transfections with recombinant plasmids, the transfected cells were fixed, stained with specific antibody and visualized by confocal microscope as previously described. Figure 5.13 shows confocal images of HEK 293 cells co-expressing of kAE1 WT-Myc with either kAE1 WT-HA or kAE1 SAO-HA or kAE1 G701D-HA. The co-expression of kAE1 WT-Myc with kAE1 WT-HA showed cell-surface expression of both kAE1 proteins (Figure 5.13A-C) with estimated percentages of 48.5±7% (Table 5.2). Co-expression of kAE1 WT-Myc with kAE1 SAO-HA or kAE1 WT-Myc with kAE1 G701D-HA, demonstrated obvious cell-surface expression of both wild-type and mutant kAE1 proteins (Figure 5.13E-G and I-K) with approximate percentages of 43.3±8% and 48.1±11%, respectively (Table 5.2).

To mimic kAE1 SAO/G701D compound heterozygous condition, kAE1 SAO-Myc/kAE1 G701D-HA, and kAE1 G701D-Myc/kAE1 SAO-HA were co-expressed in HEK 293 cells. The results showed a predominant retention of the stained kAE1 mutant proteins in cytoplasm (Figure 5.14, A-H) with very little cell-surface expression of 2.0±1% and 2.4±2%, respectively (Table 5.2). The percentages of kAE1 co-expression are showed in Figure 5.15. The co-expression kAE1 WT-Myc with either kAE1 WT-HA (n=10) or kAE1 SAO-HA (n=10, $p>0.05$) or kAE1 G701D-HA (n=10, $p>0.05$) showed vicinity levels. In contrast, co-expressions of kAE1 SAO-Myc/kAE1 G701D-HA, and kAE1 G701D-Myc/kAE1 SAO-HA showed almost no cell-surface expression with a high significance (n=10, $p<0.0001$), comparing with kAE1 WT-Myc/kAE1 WT-HA, kAE1 WT-Myc/kAE1 SAO-HA, and kAE1 WT-Myc/kAE1 G701D-HA co-expressions. The co-expression of kAE1 SAO-Myc/kAE1 G701D-HA showed similar result with that of kAE1 G701D-Myc/kAE1 SAO-HA, suggesting that Myc and HA epitope tags did not effect the protein expression.

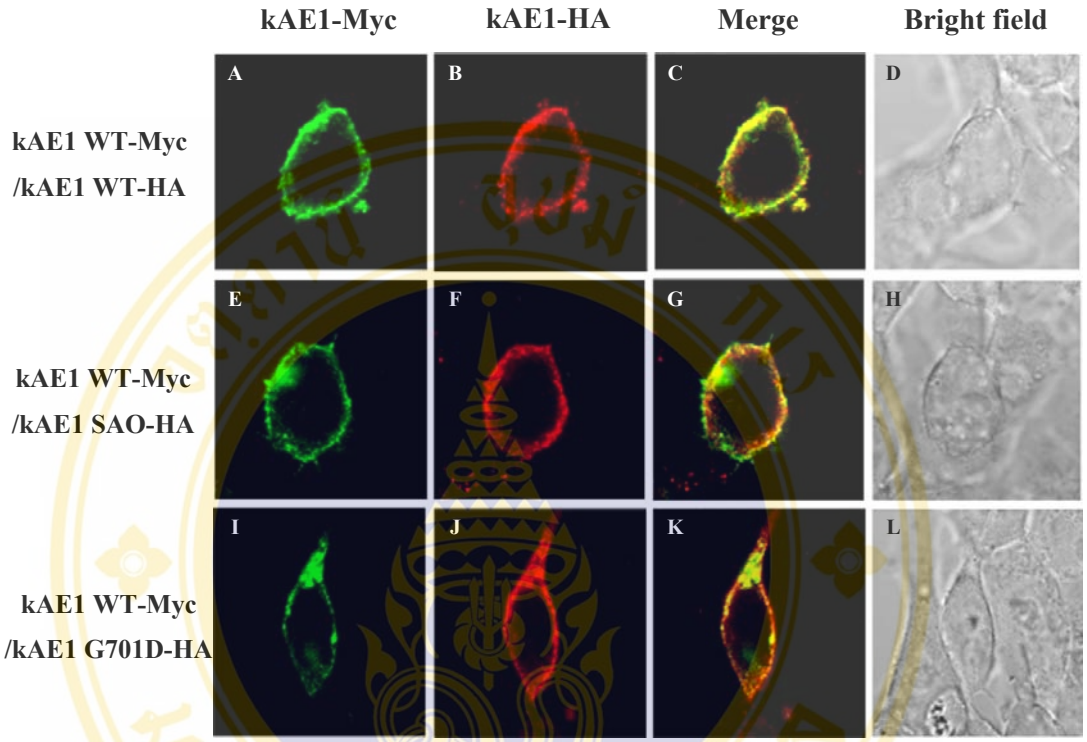


Figure 5.13 Co-localization of wild-type and mutant kAE1 proteins in HEK 293 cells.

HEK 293 cells co-expressing kAE1 WT-Myc with either kAE1 WT-HA **(A-D)**, or kAE1 SAO-HA **(E-H)** or kAE1 G701D-HA **(I-L)** were examined by indirect immunofluorescence and visualized by confocal microscopy. The first column stained with anti-Myc antibody and second column stained with anti-HA antibody. Merge and bright field images were shown in third and fourth column, respectively. The wild-type and mutant kAE1 proteins co-localized at the surface of HEK 293 cells.

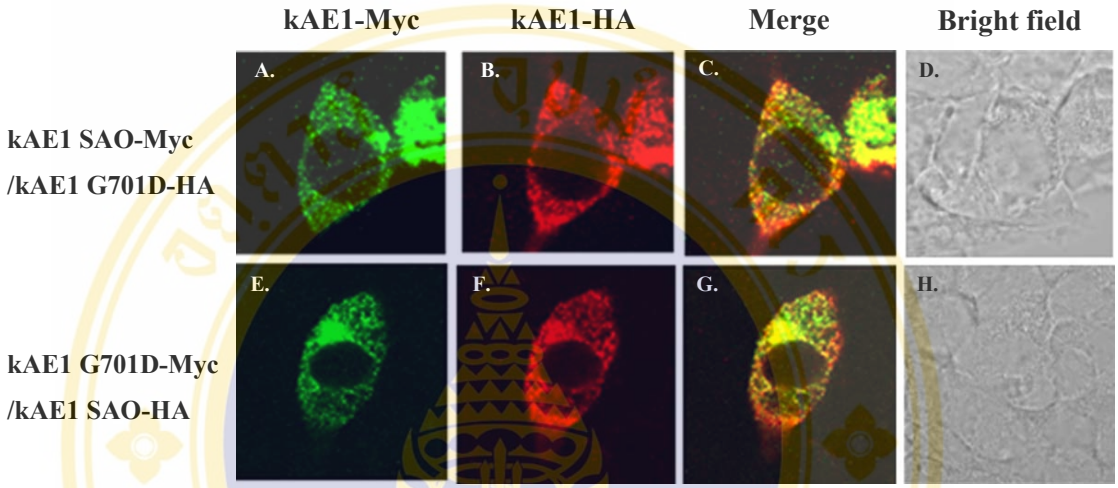


Figure 5.14 Co-localization of kAE1 SAO with G701D in HEK 293 cells.
HEK 293 cells were co-transfected with the recombinant plasmids. Two days after transfection, the cells were fixed in 3.7% paraformaldehyde and permeabilized by incubation in 0.2% Triton, blocked with 1% BSA and then stained with a mouse anti-Myc and rabbit anti-HA antibodies recognizing kAE1-Myc and kAE1-HA, respectively. Then, Alexa 488-conjugated goat anti-mouse and Cy3-conjugated donkey anti-rabbit antibodies were used to visualize kAE1-Myc and kAE1-HA, respectively, using a Zeiss LSM510 META confocal microscope. kAE1 SAO and kAE1 G701D were retained intracellularly.

Table 5.2 Percentages of cell-surface expression of co-expressing kAE1 in HEK 293 cells co-transfected with recombinant plasmids.

kAE1 fusion protein	Number of kAE1 pixel*		Percentage of cell-surface expression (%)	Average percentage of cell-surface expression of kAE1-Myc/kAE1-HA (mean ± SD)
	Total	Surface		
kAE1 WT-Myc/ kAE1 WT-HA	18842	9895	52.52	48.5±7
	11412	5149	45.12	
	22831	8909	39.02	
	11933	6452	54.07	
	18202	7311	40.17	
	13767	6285	45.65	
	17538	8017	45.71	
	10566	5695	53.90	
	10390	5089	48.98	
	10318	6174	59.84	
kAE1 WT-Myc/ kAE1 SAO-HA	8448	2918	34.54	43.3±8
	31543	9123	28.92	
	20555	11345	55.19	
	21642	10035	46.37	
	12197	5152	42.24	
	12818	5445	42.48	
	13052	5320	40.76	
	13065	6129	46.91	
	18468	9843	53.30	
	23581	9921	42.07	
kAE1 WT-Myc/ kAE1 G701D-HA	9339	6075	65.05	48.1±11
	10026	4989	49.76	
	24383	11061	45.36	
	38211	12144	31.78	
	18892	8553	45.27	
	9087	5056	55.64	
	5901	3481	58.99	
	28608	8488	29.67	
	23997	10888	45.37	
	68462	36806	53.76	
kAE1 SAO-Myc/ kAE1 G701D-HA	49671	494	0.99	2.0±1
	25367	345	1.36	
	93040	1095	1.18	
	70927	879	1.24	
	50563	808	1.60	
	41389	1103	2.66	
	110688	2289	2.07	
	90396	1868	2.07	
	20388	403	1.98	
	14569	643	4.41	
kAE1 G701D-Myc/ kAE1 SAO-HA	23784	942	3.96	2.4±2
	28795	400	1.39	
	11264	542	4.81	
	13965	617	4.42	
	74822	296	0.40	
	49747	277	0.56	
	80817	1236	1.53	
	26805	594	2.22	
	18196	557	3.06	
	21985	256	1.16	

*The pixel numbers were obtained from ten cells

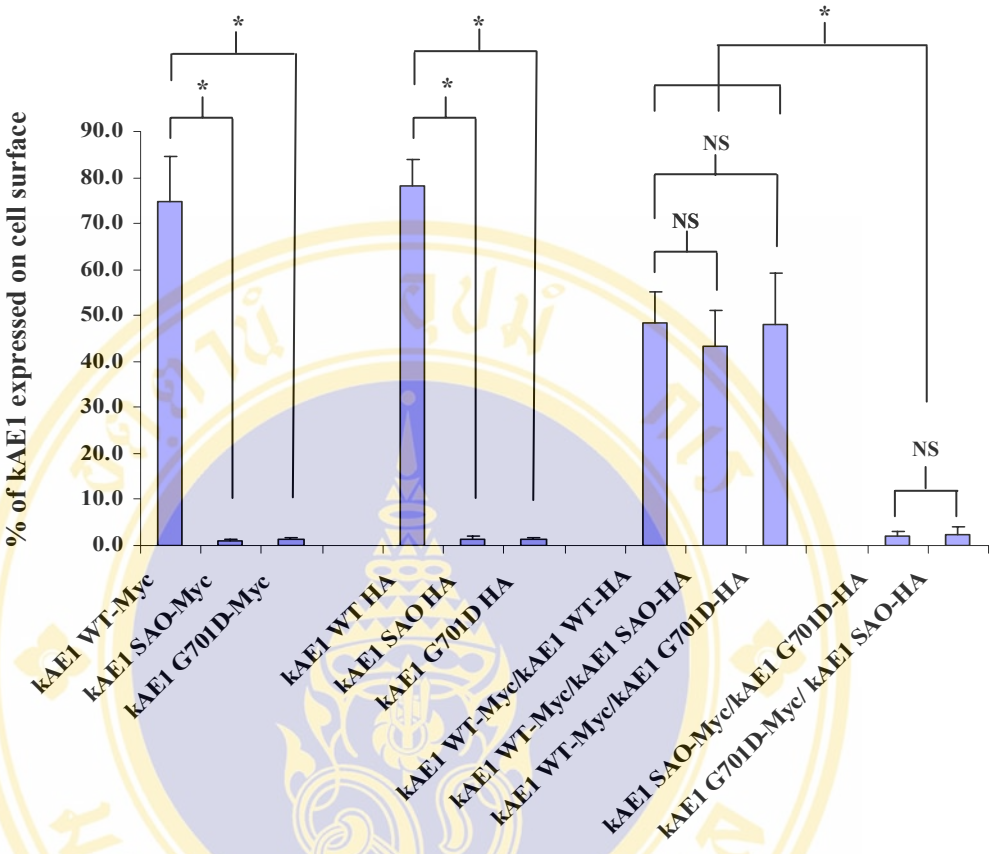


Figure 5.15 Percentages of kAE1 cell-surface expression in transfected HEK 293 cells.

The bar graph showed the percentages of individual kAE1 expression and co-expression on the surface of HEK 293 cells. The percentages were calculated from the numbers of kAE1 pixels located on the cell surface against total kAE1 pixels. Individual kAE1 WT showed cell surface expression whereas kAE1 SAO and kAE1 G701D were not expressed on plasma membrane. Both kAE1 WT and kAE1 SAO or kAE1 WT and kAE1 G701D could be expressed on cell surface. Co-expression of kAE1 SAO and kAE1 G701D had a low levels of cell-surface expression. The values are mean±SD from 10 cells. NS denotes 'not significant'. *Statistically significant difference with *p value* < 0.0001.

When the percentages of cell surface expression of kAE1 co-expression were calculated from only kAE1-HA pixels (Table 5.3 and Figure 5.16), co-expression of kAE1 WT-Myc/kAE1 WT-HA showed 60.7±7% of cell surface expression similar to that of co-expressions of kAE1 WT-Myc/kAE1 SAO-HA kAE1 and WT-Myc/kAE1 G701D-HA (54.2±11% and 58.9±14%, respectively). The co-expressions of kAE1 SAO-Myc/kAE1 G701D-HA and kAE1 SAO-HA/kAE1 G701D-Myc had almost no cell surface expression (1.90±1% and 1.83±1%, n=10, $p<0.0001$), which showed a highly statistical significance when they were compared with that of kAE1 WT-Myc/kAE1 WT-HA, kAE1 WT-Myc/kAE1 SAO-HA kAE1 or WT-Myc/kAE1 G701D-HA. This indicates that the co-expressed wild-type kAE1 could rescue both mutant kAE1 proteins from intracellular retention to the plasma membrane and co-expressed kAE1 SAO and kAE1 G701D fails to rescue each other to the cell surface. The subcellular localizations of individual expression and co-expression of kAE1 in HEK 293 cells were concluded in Table 5.4.

Table 5.3 Percentages of cell-surface expression of kAE1-HA in HEK 293 cells co-transfected with recombinant plasmids.

kAE1 fusion protein	Number of kAE1 pixel*		Percentage of cell-surface expression	Average percentage of cell-surface expression of kAE1-HA (mean ± SD)
	Total	Surface		
kAE1 WT-Myc/ kAE1 WT-HA	10367	6507	62.77	60.7±7
	7110	4549	63.98	
	12179	6054	49.71	
	6956	3976	57.16	
	11097	6380	57.49	
	10010	5471	54.66	
	10980	6361	57.93	
	4633	3169	68.40	
	6230	3875	62.20	
	8013	5835	72.82	
kAE1 WT-Myc/ kAE1 SAO-HA	23491	7830	33.33	54.2±11
	15187	8099	53.33	
	14568	9186	63.06	
	9218	5083	55.14	
	11688	6035	51.63	
	7524	4439	59.00	
	7749	4815	62.14	
	13464	8453	62.78	
	16080	10458	65.04	
	15164	5552	36.61	
kAE1 WT-Myc/ kAE1 G701D-HA	14593	8091	55.44	58.9±14
	5991	4667	77.90	
	5977	3449	57.70	
	18418	9478	51.46	
	51747	33999	65.70	
	21102	10702	50.72	
	14185	6504	45.85	
	6356	4440	69.86	
	3687	2903	78.74	
	30526	10895	35.69	
kAE1 SAO-Myc/ kAE1 G701D-HA	10534	395	3.75	1.90±1
	9931	318	3.20	
	52500	1264	2.41	
	69127	1578	2.28	
	42369	596	1.41	
	34035	818	2.40	
	54325	659	1.21	
	68997	517	0.75	
	21464	224	1.04	
	38718	191	0.49	
kAE1 G701D-Myc/ kAE1 SAO-HA	18479	171	0.93	1.83±1
	9867	309	3.13	
	19884	312	1.57	
	57754	364	0.63	
	42848	176	0.41	
	64222	121	0.19	
	12661	496	3.92	
	9458	339	3.58	
	25600	279	1.09	
	18664	537	2.88	

*The pixel numbers were obtained from ten cells

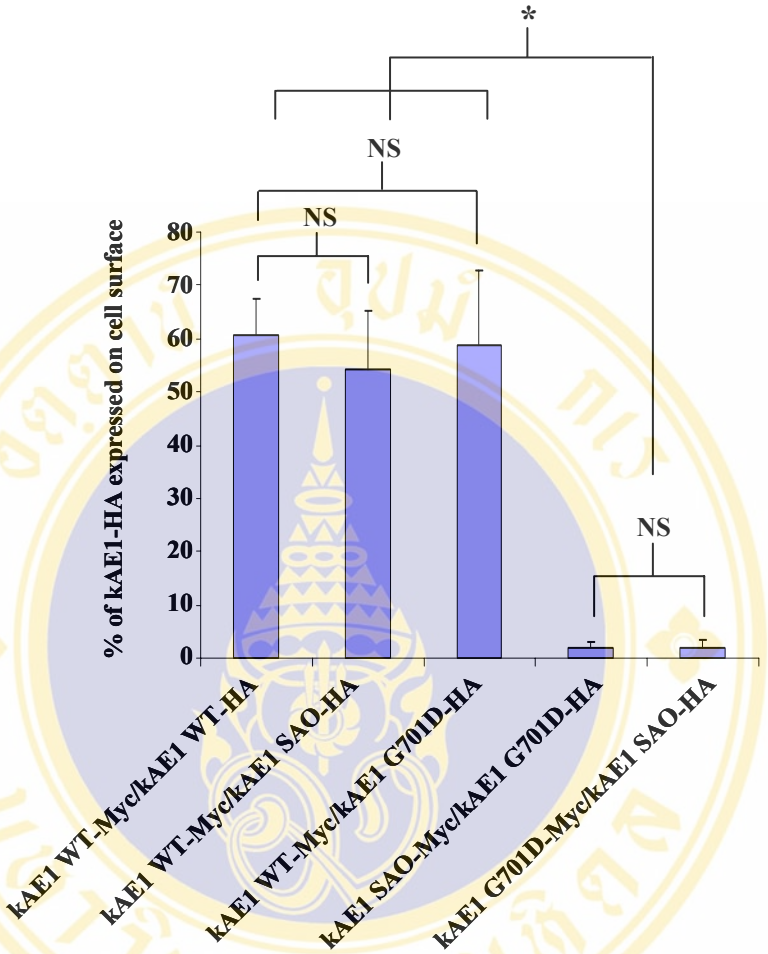


Figure 5.16 Percentages of kAE1-HA cell-surface expression in transfected HEK 293 cells.

The bar graph showed the percentages of kAE1-HA expressed on the surface of HEK 293 cells when co-expressed with kAE1-Myc. The percentages were calculated from the numbers of kAE1-HA pixels located on the cell surface against total kAE1-HA pixels. kAE1 SAO and kAE1 G701D could be rescued to express on cell surface when co-expressed with kAE1 WT. Co-expression of kAE1 SAO and kAE1 G701D failed to rescue each other to express on cell surface. The values are mean±SD from 10 cells. NS denotes 'not significant'. *Statistically significant difference with *p value* < 0.0001.

Table 5.4 Cellular localization of kAE1 in transfected HEK293 cells.

kAE1 fusion protein	Cellular localization
WT-Myc	cell surface
SAO-Myc	intracellular retention
G701D-Myc	intracellular retention
WT-My/WT-HA	cell surface
WT-Myc/SAO-HA	cell surface
WT-Myc/G701D-HA	cell surface
SAO-Myc/G701D-HA	intracellular retention
G701D-Myc/SAO-HA	intracellular retention

(2) Cell-surface expression study of wild-type and mutant kAE1 by FACS analysis

Cell surface expression of wild-type and mutant kAE1 proteins was also determined by using FACS analysis. The recombinant plasmids containing wild-type and mutant *kAE1*-Myc were used for this study. The sequence of Myc epitope was inserted in *kAE1* cDNA the third extracellular loop at the position 557 to allow the cell-surface expressed kAE1 protein to be detected by immunological staining method. HEK 293 cells were either transfected with pcDNA-*kAE1*-Myc alone or co-transfected with both pcDNA-*kAE1*-Myc and pcDNA-*kAE1*-HA. Two days after transfect or co-transfection, the cells were harvested, immunologically stained by using anti-Myc analyzed by flow cytometry as described in the Methods (4.2.5). The result showed that 58±12% and 23.6±2% of the numbers of HEK 293 cells expressed kAE1 WT-Myc in the total and cell-surface staining, respectively. While the number of HEK 293 cells that expressed kAE1 SAO-Myc and kAE1 G701D-Myc in the total staining were 28.89±2% and 36.73±11%, the cells with cell-surface expression were 0.86±1% and 0.75±1% of the total number of cells, respectively.

The relative expression of kAE1 (Table 5.5 and Figure 5.17) revealed that there were very low cell-surface expressions with a highly significant difference of kAE1 SAO-Myc (1.47±0%, n=3, $p<0.005$) or kAE1 G701D-Myc (0.92±0%, n=3, $p<0.005$) when compared with that of kAE1 WT-Myc (42.11±13%, n=3). The cell-surface expression of kAE1 WT-Myc co-expressed with either kAE1 SAO-Myc (5.45±2%, n=3, $p<0.005$) or kAE1 G701D-Myc (5.26±4%, n=3, $p<0.05$) showed a little increment but still low when compared with that of kAE1 WT-HA (22.36±6%, n=3). The co-expression of kAE1 SAO-Myc/kAE1 G701D-HA or kAE1 G701D-Myc/kAE1 SAO-HA did not show any cell-surface expression and had the signals at the background level (0.59±0% and 0.72±0%, n=3, $p<0.005$).

Table 5.5 Relative cell-surface expressions of wild-type and mutant kAE1 in transfected and co-transfected HEK 293 cells determined by FACS analysis.

kAE1 fusion protein	Expression*		Percentage of relative cell surface expression	Average percentage of relative cell surface expression (mean±SD)
	% of cell expressing kAE1	% of cell expressing kAE1 on cell surface		
WT-Myc	64.30	24.55	38.18	42.11±13
	66.58	21.29	31.98	
	44.48	24.99	56.18	
SAO-Myc	28.36	0.43	1.52	1.47±0
	30.98	0.47	1.52	
	27.32	0.38	2.49	
G701D-Myc	40.73	0.35	0.86	0.92±0
	45.07	0.32	0.71	
	24.40	0.29	2.42	
WT-Myc/WT-HA	60.43	12.74	21.08	22.36±6
	65.21	11.04	16.93	
	32.69	9.50	29.06	
WT-Myc/SAO-HA	58.48	2.97	5.08	5.45±2
	55.50	2.23	4.02	
	33.46	2.43	7.26	
WT-Myc/G701D-HA	65.33	3.54	5.42	5.26±4
	59.56	2.70	4.53	
	35.16	2.05	5.83	
SAO-Myc/G701D-HA	34.29	0.23	0.67	0.59±0
	42.87	0.14	0.33	
	20.49	0.16	0.78	
G701D-Myc/SAO-HA	49.84	0.21	0.42	0.72±0
	55.32	0.32	0.58	
	22.51	0.26	2.93	
pcDNA3.1 vector	0.89	0.00	0.00	0.95±1
	1.40	0.01	0.71	
	0.93	0.02	2.15	

*The values were obtained from 3 separate experiments.

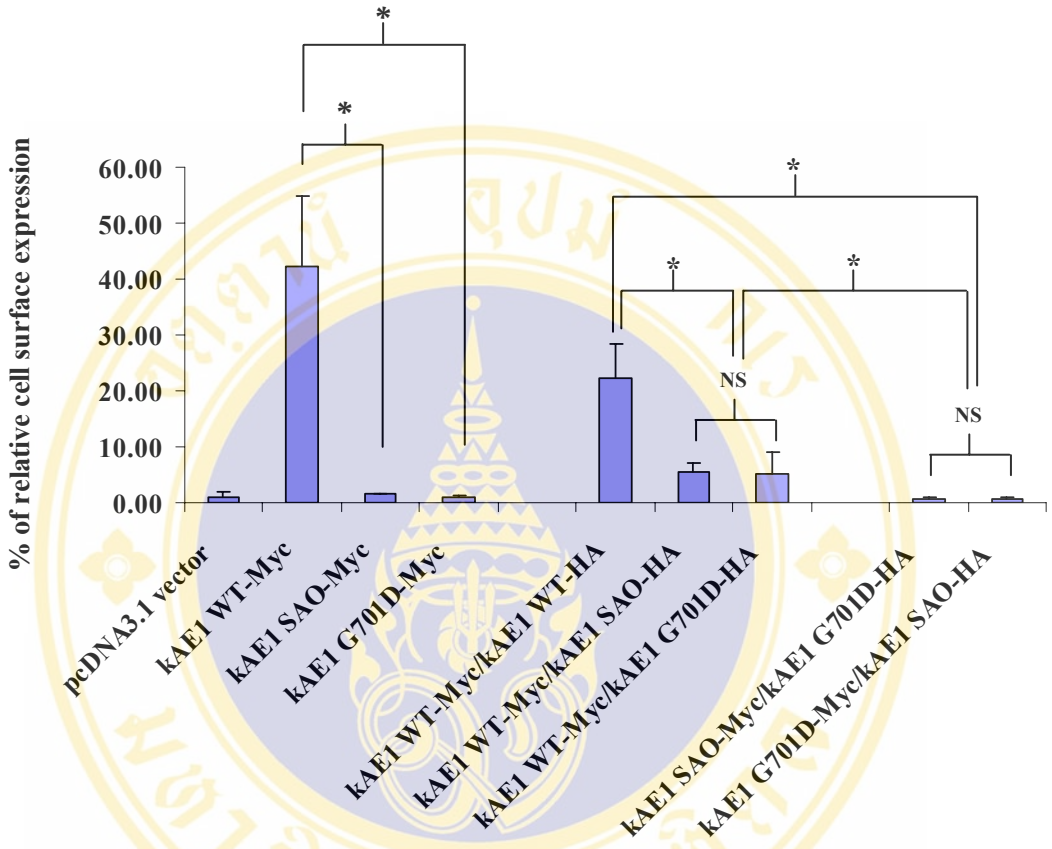


Figure 5.17 Relative cell-surface expressions of wild-type and mutant kAE1 in transfected and co-transfected HEK 293 cells determined by FACS analysis.

The bar graph showed the percentages of relative cell surface kAE1 expression which calculated form surface expression against total expression. Individual expression of kAE1 SAO or kAE1 G701D and their co-expression showed little cell-surface expression. The values are mean±SD from 3 separate experiments. NS denotes 'not significant'. *Statistically significant difference with *p value* < 0.005.

The results of relative fluorescence intensities were shown in Table 5.6 and Figure 5.18. The individual expression of kAE1 SAO and kAE1 G701D showed a lower value of relative fluorescence intensity than that of kAE1 WT. Additionally, co-expression of kAE1 SAO/kAE1 G701D had a less value of relative fluorescence intensity than those of kAE1 WT/kAE1 WT, kAE1 WT/kAE1 SAO, and kAE1 WT/kAE1 G701D. The co-expression of kAE1 SAO-Myc/kAE1 G701D-HA showed a low level of relative fluorescence intensity as same as that of kAE1 G701D-Myc/kAE1 SAO-HA. These results indicated again that kAE1 SAO and kAE1 G701D have trafficking impairment and failure to rescue each other to express at the cell surface. However, kAE1 WT could rescue kAE1 SAO and kAE1 G701D mutant proteins to present at the cell surface.

Table 5.6 Relative fluorescence intensity of wild-type and mutant kAE1 in transfected and co-transfected HEK 293 cells determined by FACS analysis.

kAE1 fusion protein	Mean fluorescence intensity	Relative fluorescence intensity (U)	Average relative fluorescence intensity (mean±SD)
WT-Myc	229.25	0.52	0.59±0.10
	249.54	0.55	
	264.27	0.71	
SAO-Myc	157.89	0.04	0.06±0.03
	169.25	0.05	
	169.24	0.10	
G701D-Myc	166.13	0.10	0.08±0.02
	170.86	0.06	
	166.12	0.08	
WT-Myc/WT-HA	176.54	0.17	0.29±0.13
	203.75	0.27	
	220.83	0.43	
WT-Myc/SAO-HA	162.41	0.07	0.12±0.05
	180.31	0.12	
	179.74	0.17	
WT-Myc/G701D-HA	163.37	0.08	0.12±0.05
	179.69	0.12	
	180.97	0.17	
SAO-Myc/G701D-HA	155.19	0.03	0.02±0.04
	158.52	-0.01	
	163.05	0.06	
G701D-Myc/SAO-HA	150.89	0.00	0.032±0.05
	159.65	-0.01	
	167.88	0.09	
pcDNA3.1 vector	151.30	0.00	0.00±0.00
	160.64	0.00	
	154.27	0.00	

*Relative fluorescence intensity = (MFI of surface expression - MFI of vector control) / MFI of vector control. The values were obtained from 3 separate experiments.

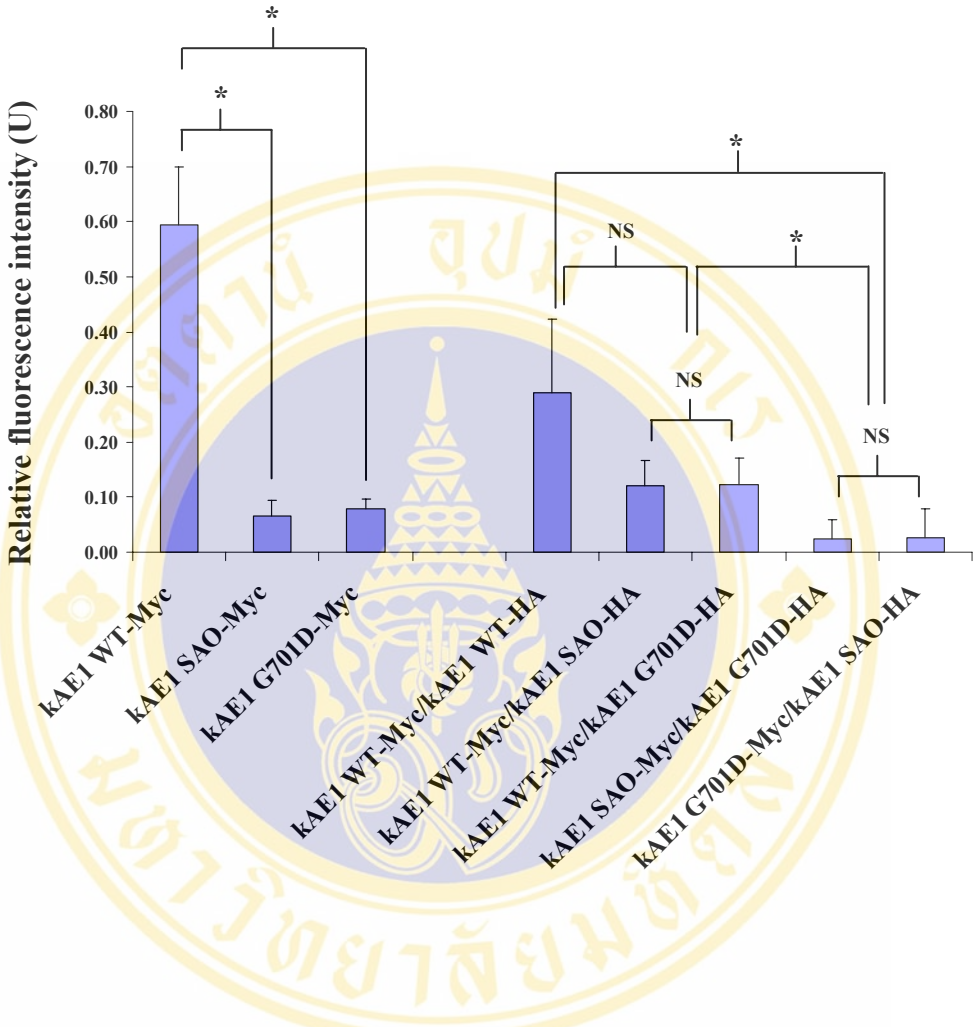


Figure 5.18 Relative fluorescence intensities of wild-type and mutant kAE1 in transfected and co-transfected HEK 293 cells determined by FACS analysis.

The bar graph showed the relative fluorescence intensity of kAE1 expression. Individual expression of kAE1 SAO or kAE1 G701D had a lower relative fluorescence intensity than that of kAE1 WT. Co-expression of kAE1 SAO/kAE1 G701D showed a less value than that of kAE1 WT/kAE1 WT, kAE1 WT/kAE1 SAO, and kAE1 WT/kAE1 G701D. The values are mean±SD from 3 separate experiments. NS denotes 'not significant'. *Statistically significant difference with *p value* < 0.05.

CHAPTER VI

DISCUSSION

Mutations in the *SLC4A1* or *AE1* gene may cause kAE1 defects leading to AR or AD dRTA (14). Normally, kAE1 protein located on basolateral membrane of the α-intercalated cells of kidney, functioning in Cl^-/HCO_3^- exchanger. A majority of AE1 defects result in impaired trafficking, leading to intracellular retention or mis-targeting of mutant protein to apical membrane, and a minority produces abnormal anion transport function (14, 21). *AE1* mutation associated with AR dRTA frequently found in Southeast Asia as homozygous and compound heterozygous conditions. The homozygous recessive *AE1* G701D mutation was first reported in two Thai sisters (22). Besides, *AE1* G701D mutation was also found in a compound heterozygous condition with *AE1* SAO mutation that firstly reported in Thai patients (14). The function and localization of eAE1 and kAE1 SAO and G701D have been studied in red blood cells, *Xenopus* oocytes, and cultured mammalian cell lines. eAE1 SAO on red blood cells of individuals with a heterozygous condition had a reduced anion transport activity (29) and presented at the cell surface but no anion transport function in *Xenopus* oocytes (30). In the cultured non-polarized and polarized MDCK cells, AE1 SAO was retained in the ER (31). AE1 G701D showed normal anion transport activity and cell surface expression in red cells from the patients with homozygous condition but reduced anion transport function and cell surface expression in *Xenopus* oocytes; however, these could be rescued by co-expression with GPA (22). The *AE1* G701D had no transport function in HEK 293 cells and had intracellular retention in HEK 293 and non-polarized LLC-PK1 cells (25). In non-polarized and polarized MDCK cells, *AE1* G701D was retained intracellularly and in Golgi apparatus (25). Moreover, red cells from patients with compound heterozygous *AE1* SAO/G701D also had a reduced anion transport function (27, 28) and a similar finding was also observed in the co-expression study in oocytes (28). Although there were some studies on expression and function of AE1 SAO and G701D on red cells and oocytes

in addition to individual expression study in cultured polarized and non-polarized mammalian cells, there was no data on co-expression of kAE1 SAO and G701D in the cultured mammalian cells that would mimic the compound heterozygous *AE1* SAO/G701D condition that has been observed in many patients with dRTA. The study in this thesis was thus an attempt to examine and elucidate the molecular defect of the compound heterozygous *AE1* SAO/G701D condition leading to dRTA in the patients. The kAE1 WT, kAE1 SAO and kAE1 G701D fused with either His, HA, or Myc epitope tag were studied in cultured HEK 293 cells for their expression, oligomerization, and subcellular localization, in both individual expression and co-expression situations. The protein expression and co-expression were examined by Western blot analysis, oligomerization and heterodimerization were studied by co-purification and co-immunoprecipitation, and subcellular localization and cell surface expression were investigated by IF and FACS, respectively.

The plasmid containing *kAE1* WT-His was constructed and used as a template to generate the plasmids containing *kAE1* SAO-His and *kAE1* G701D-His by site-directed mutagenesis. Similarly, the plasmid containing kAE1 WT-HA was used to create the plasmids containing *kAE1* SAO-HA or *kAE1* G701D-HA by site-directed genesis. In addition, the plasmid containing *kAE1* WT-Myc was generated by using a complementary pair of primers containing Myc sequence between amino acid positions 557 and 558, and *kAE1* WT-Myc was then used as a template to generate *kAE1* SAO-Myc and *kAE1* G701D-Myc by site-directed mutagenesis. All constructs were confirmed by DNA sequencing. They were individually transfected in HEK 293 cells and examined for the protein expression by Western blotting analyses using antibodies to the epitope tags. The result showed that the kAE1 WT, kAE1 SAO and kAE1 G701D fused with each of the epitope tags were expressed as the proteins with the size of about 96 kDa, as expected (Figure 5.9). This indicated that all recombinant plasmids could express kAE1 tagged with either His, HA or Myc epitope in the cultured HEK 293 to be further used for studies in the next experiments.

To determine whether kAE1 WT kAE1 could form heterodimer with kAE1 SAO or kAE1 G701D, and the two latter could form heterodimer with each other, the co-expression of kAE1 WT-His with either kAE1 SAO-HA or kAE1 G701D-HA, and the co-expression of kAE1 SAO-His with kAE1 G701D-HA, or kAE1 G701D-His

and kAE1 SAO-HA were carried out and examined by co-purification and co-immunoprecipitation methods. Anti-His and anti-HA antibodies were used to detect kAE1 WT-His and kAE1 WT-HA proteins, respectively. The results of co-purification and co-immunoprecipitation were similar which confirms each other. The cell lysates from transfected cells were detected with specific antibodies to the epitope tags revealing that the kAE1 proteins could be expressed. kAE1 WT-His was expressed alone or co-expressed with either kAE1 WT-HA, kAE1 SAO-HA, or kAE1 G701D-HA. In addition, kAE1 WT-HA was also expressed alone to serve as a control. As expected, kAE1 WT-HA could be detected by the anti-HA antibody (Figures 5.10A and 5.11A, lanes 1-3, upper panel) but not by the anti-His antibody (Figures 5.10A and 5.11A, lanes 1-3, lower panel) and *vice versa* (Figures 5.10A and 5.11A, lane 4, upper and lower panels). After His-tagged protein co-purification and co-immunoprecipitation with anti-His antibody, the protein (kAE1-HA) in the eluted fraction detected by anti-HA would be the one that dimerized with kAE1-His protein. The singly expressed kAE1 WT-HA could not directly bind to Co^{2+}; thus, no band was detected on the immunoblot using anti-HA or anti-His antibody (Figures 5.10A and 5.11B, lanes 1-3, upper and lower panels). The co-expression of kAE1 WT-His with either kAE1 WT-HA, kAE1 SAO-HA or kAE1 G701D-HA showed that the wild-type protein could from hetero-oligomers with the wild-type and two mutant proteins (Figures 5.10A and 5.11A, lanes 5-7). The heterodimer of kAE1 WT with kAE1 SAO has also previously been reported (94). When the interaction between kAE1 SAO and kAE1 G701D was studied by co-purification and co-immunoprecipitation, the results showed that kAE1 G701D-His could form hetero-oligomer with kAE1 SAO-HA, and kAE1 SAO-His could form hetero-oligomer with kAE1 G701D-HA (Figures 5.10A and 5.11A, lanes 8-9). Thus, the kAE1 SAO or kAE1 G701D was not only capable of interacting with kAE1 WT but also interacting with each other, indicating that these two mutations do not affect their interaction and dimerization or oligomerization property.

The expression and subcellular localization wild-type and mutant kAE1 were next examined. As His-tag and anti-His antibody did not give good and satisfactory results with cellular staining in indirect immunofluorescence (IF) and FACS analyses, kAE1-HA and kAE1-Myc were instead used in these studies. For kAE1-Myc, the

Myc epitope was inserted in the third extracellular loop (position 557) so that it would extracellulary express and be detected by anti-Myc antibody and FACS analysis. The result of IF and confocal imaging analysis of the transfected HEK 293 cells showed that kAE1 WT-Myc and kAE1 WT-HA predominantly expressed at the cell surface (Figure 5.12 A-B and G-H, respectively), as same as that has previously been studied (17, 25). The average percentage of cell-surface expression (mean ± SD) analyzed from the image pixels for kAE1 WT-Myc was 74.9±9.6 and that for kAE1 WT-HA was 78.3±5.6 (Table 5.1). In contrast, kAE1 SAO-Myc, kAE1 SAO-HA, kAE1 G701D-Myc, and kAE1 G701D-HA expressed mainly in the cytoplasm with very little or almost non on the cell surface (Figure 5.12 C-D, I-J, E-F and K-L, respectively). These results confirm that has previously been reported (25, 31). The average percentages of cell-surface expression for kAE1 SAO-Myc, kAE1 SAO-HA, kAE1 G701D-Myc, and kAE1 G701D-HA were 0.9±0.4, 1.5±0.4, 1.2±0.6, and 1.4±0.2, respectively (Table 5.1).

In the analysis of cell surface expression by FACS, kAE1 WT-Myc had a relative cell surface expression of 42.11±13% (Table 5.5), similar to that was previously described for kAE1 WT-HA557 expression (25). The relative cell surface expressions of kAE1 SAO-Myc and kAE1 G701D-Myc detected by FACS were 1.47±0% and 0.92±0%, respectively (Table 5.5). The results indicated that there was the highly significant difference of cell surface expression between kAE1 WT and kAE1 SAO or kAE1 G701D ($p<0.0001$ for IF and $p<0.005$ for FACS), revealing that kAE1 SAO and kAE1 G701D greatly decreased or had no cell surface expression while kAE1 WT prominently located at the plasma membrane.

To study the effect of kAE1 WT to either kAE1 SAO or kAE1 G701D, and kAE1 SAO to kAE1 G701D (and *vice versa*) in the protein expression and subcellular localization study using IF and FACS in HEK 293 cells, co-expression studies of kAE1 WT-Myc with either kAE1 SAO-HA or kAE1 G701D-HA, and kAE1 SAO-Myc with kAE1 G701D-HA or kAE1 G701D-Myc with kAE1 SAO-HA were examined. The representative transfected cells co-expressing kAE1 WT-Myc and kAE1 WT-HA shows predominantly expression at the cell surface (Figure 5.13 A-D), compared to the co-expression of kAE1 WT-Myc with either kAE1 SAO-HA or

kAE1 G701D-HA that also located at the plasma membrane (Figure 5.13.E-H and I-L, respectively). The cell surface expression estimated from image pixels showed no significant difference among the three co-expressions. The percentage of relative cell surface expression by FACS of the co-expression between kAE1 WT-Myc and kAE1 WT-HA was 22.36±6%, higher than that of the co-expression of kAE1 WT-Myc with either kAE1 SAO (5.45±2%) or kAE1 G701D (5.26±4%) (Table 5.5). The difference of percentage in the co-expression using FACS from IF may be explained by that FACS measured number of cells with surface expression against the total number of cells while in IF the percentage of cell surface expression compared with the total protein expression within the individual representative cells was calculated. When co-expressed with kAE1 WT-Myc, kAE1 SAO-HA or kAE1 G701D-HA could be rescued to express at cell surface in a limited number of cells as detected by FACS. There was about 5-folds reduction of the percentage of cell surface expression of kAE1 WT-Myc when co-expressed with either kAE1 SAO-HA or kAE1 G701D-HA, compared with co-expression of kAE1 WT-Myc with kAE1 WT-HA (Table 5.5 and Figure 5.17). Moreover, co-expression of kAE1 SAO-Myc with kAE1 G701D-HA or kAE1 G701D-Myc with kAE1 SAO-HA to mimic compound heterozygous *kAE1* SAO/G701D condition causing dRTA showed predominantly retention in cytoplasm of HEK 293 cells as examined by IF (Figure 5.14, A-H , Figure 5.15, and Table 5.2) and the results of FACS analysis were similar (Table 5.5 and Figure 5.17). The percentages of cell surface expression of co-expression kAE1 SAO-Myc with kAE1 G701D-HA, or kAE1 G701D-Myc with kAE1 SAO-HA were very low and significantly different ($p<0.005$) from that of co-expression between kAE1 WT-Myc and kAE1 WT-HA. These results indicate that kAE1 SAO and kAE1 G701D have trafficking impairment and failure to rescue each other to express at the plasma membrane. However, kAE1 WT could rescue kAE1 SAO and kAE1 G701D mutant proteins to present at the cell surface.

The dominant kAE1 R589H and kAE1 R901X mutation shows a dominant effect because of hetero-oligomerization with the normal protein, resulting in intracellular retention of the normal protein (17, 18). In contrast, from the results of this work and other studies (25, 26), wild-type kAE1 protein could rescue the trafficking of kAE1 SAO and kAE1 G701D mutants, allowing the movement of the mutant protein from

the cytoplasm to the plasma membrane, because of hetero-oligomerization, the so-called 'dominant positive effect' of the wild-type kAE1 on the mutant kAE1 proteins (14). The heterodimers between normal AE1 and AE1 SAO that are present at the red cell surface have been observed (94). The normal AE1 protein retains transport function, although it is associated with an inactive SAO subunit (29). Interestingly, although kAE1 SAO which is inactive in anion transport function is able to express at the surface of red cells and oocytes, it is unable to express at the cell surface of HEK 293 and MDCK cells (31).

kAE1 G701D is unable to express at plasma membrane of oocytes and mammalian cells (HEK 293, LLC-PK1 and MDCK) but able to express at the surface of red cells (26). Substitutions of G701 to E or R, but not to A or L, were localized to the Golgi in both non-polarized and polarized MDCK cells (26). E is a negative charge amino acid and R is a positive charge amino acid that showed retention of kAE1 protein in cytoplasm as similar to D substitution of G701. While A and L are neutral non-polar amino acid showed normal trafficking as wild-type. This suggests that the presence of a positively and negatively charged residue but not loss of glycine at this position induced the intracellular retention of kAE1 and blocked its trafficking to plasma membrane. The G701D, E and R substitutions were hypothesized that they may effectively shorten the hydrophobic span of the putative 9th transmembrane domain (26). The introduction of a charged residue may also disrupt its interaction with other transmembrane segments in the protein allowing the segment to become exposed and act as a Golgi retention signal (95). Another mechanism was proposed for Golgi retention of G701D is the formation of oligomers. It may be possible that the G701D mutation causes kAE1 to form large aggregates in the Golgi resulting in the retention of the protein. The G701D mutant cannot move from the Golgi to the plasma membrane and may recycle back to the ER and become trapped in the ER–Golgi compartments (26).

The rescuing findings in this work also confirmed the results that have recently been reported (25, 26, 31). The trafficking defect of kAE1 SAO and kAE1 G701D in the mammalian cells can be rescued by the co-expression of kAE1 WT, explaining the absence of dRTA phenotype in individuals with heterozygous *AE1* G701D or SAO mutation.

The co-expression kAE1 SAO with kAE1 G701D in HEK 293 cells that mimics the compound heterozygous *AE1* SAO/G701D condition detected in many patients with dRTA and SAO showed that the two proteins were intracellularly retained. This result was demonstrated by both IF and FACS methods. The hetero-dimerization of kAE1 SAO and kAE1 G701D failed to rescue each other to express at the cell surface. This result can explain the molecular pathology of the compound heterozygous *AE1* SAO/G701D condition as it will cause loss of transport function of kAE1 at the basolateral membrane of kidney cells resulting in inadequate anion exchange activity in the patients with dRTA.

CHAPTER VII

CONCLUSION

To elucidate molecular defect of dRTA caused by compound heterozygous SAO/G701D mutation, kAE1 SAO and G701D proteins were expressed and co-expressed in HEK 293 cells. Protein interaction was determined by co-immunoprecipitation and co-purification procedures, and protein trafficking and subcellular localization were investigated by immunoflourescence staining followed by confocal microscopy and FACS analysis. All nine fusion proteins including kAE1 WT-His, kAE1 SAO-His, kAE1 G71D-His, kAE1 WT-HA, kAE1 SAO-HA, kAE1 G701D-HA, kAE1 WT-Myc, kAE1 SAO-Myc and kAE1 G701D-Myc could be expressed and detected by Western blot analysis. The individual expression of kAE1 WT-Myc was presented at the cell surface while kAE1 SAO-Myc and kAE1 G701D-Myc were retained intracellularly in the transfected HEK 293 cells. Co-expression of kAE1 WT with either kAE1 SAO or kAE1 G701D demonstrated that they could interact to form heterodimer as examined by the co-precipitation and co-purification methods. Additionally, both wild-type and mutant kAE1 proteins were observed on the cell surface, indicating that the co-expressed wild-type kAE1 could rescue both mutant kAE1 proteins from intracellular retention to the plasma membrane. The co-expression of kAE1 SAO and kAE1 G701D showed that they could interact but were intracellularly retained, without or with little cell surface expression, suggesting their trafficking defect and failure to rescue each other to the plasma membrane. The trafficking impairment and intracellular retention of the co-expressed kAE1 SAO and kAE1 G701D mutant proteins may result in the absence of functional kAE1 at the basolateral membrane of kidney α-intercalated cells. It is most likely that the molecular mechanism of dRTA associated with compound heterozygous AE1 SAO/G701D mutation in the patients.

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APPENDIX

APPENDIX

Chemicals, solutions and buffers

1. Chemicals

Chemicals	Company
Absolute ethanol (C_2H_5OH)	BDH
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
Chloroquin ($C_{18}H_{26}ClN_3.2H_3PO_4$)	Sigma
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
Diethylaminoethyl-Dextran (DEAE)	Sigma
Dimethyl Sulfoxide (DMSO)	Sigma
Ethidium bromide	Bio-Rad
Ethylenediamine tetraacetic acid or EDTA ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	USB
Fetal Bovine Serum (FBS)	PERBIO
Glycerol ($C_3H_8O_3$)	BDH
Hydrochloric acid (HCl)	Merck
Isopropanol ($CH_3CHOHCH_3$)	BDH

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 or TEMED ($C_{14}H_{11}NO_3$)	Bio-Rad
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
Polyoxyethylenesorbitan monolaurate tween (Tween-20)	Sigma
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. Reagents for DNA extraction from *E. coli*

3.1 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.

3.2 Lysis buffer (P2 buffer)

Per 100 ml:

1 N NaOH	20	ml
20% SDS	5	ml

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

3.3 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.

4. Growth media

4.1 Bacterial media

(1) Low salt Luria-Bertani plate (LB plate)

Per liter:

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.

(2) Low salt Luria-Bertani broth (LB broth)

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

(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.

4.2 Mammalian cell line media

(1) Plain DMEM

Per liter:

Dulbecco's Modified Eagle Medium	13.4	g
NaHCO3	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

(2) Complete DMEM

Per liter:

Stock DMEM	888	ml
FBS	100	ml
60.79 U/ml of penicillin G/ 60 µg/ml of streptomycin	12	ml

5. Reagents for mammalian cell line transfection

5.1 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)

5.2 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).

5.3 Cell dissociation solution

(1) 2.5 mM EDTA in PBS

Per 500 ml:

$EDTA.Na_2.2H_2O$	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.

(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.

6. Buffer for protein sample preparation

10 mM EDTA	1	ml
5 % (v/v) Igepal (Nonidet P-40 detergent)	1	ml
1.5 M NaCl	1	ml
BSA	0.2	g
100 mM Tris-HCl pH 7.5	1	ml

The mixture was mixed and adjusted at a final volume to 10 ml

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

7.1 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)

7.2 3 M Tris-HCl: Resolving gel buffer pH 8.8

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.

7.3 10% (w/v) Sodium dodecyl sulphate

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

7.4 10% (w/v) Ammonium persulfate

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

7.5 0.5 M Tris-HCl: Stacking gel buffer pH 6.8

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.

7.6 10% SDS-polyacrylamide gel

Reagents	10% Separating gel/5 ml	4% Stacking gel/1.5 ml
30.8% (w/v) Acrylamide-bisacrylamide (ml)	1.67	0.25
3.0 M Tris-HCl pH 8.8 (ml)	0.63	-
0.5 M Tric-HCl pH 6.8 (ml)	-	0.50
Distilled water(ml)	2.63	1.22
10% ammonium persulfate (µl)	38.0	15.0
TEMED (µl)	2.50	1.50

The separating gel 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. Then, the stacking gel solutions was mixed and loaded into a protein gel electrophoresis set. Gel was completely set for 30-45 minutes.

7.7 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.

7.8 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.

7.9 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.

7.10 Blocking buffer: 5 % skimmed milk

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

7.11 TBST (20 mM Tris, , 137 mM NaCl, 0.1% Tween-20, pH 7.5)

Per liter:

Tris base	2.42	g
NaCl	8	g
Tween-20	1	ml

The mixture was dissolved and adjusted the final volume to 1 liter with distilled water.

BIOGRAPHY

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POSTER PRESENTATION

A part of result from this project was presented as a poster entitled "Molecular Defect of Distal Renal Tubular Acidosis Caused by Compound Heterozygous Anion Exchanger 1 Mutations" by **Nunghathai Sawasdee**, **Wandee Udomchaiprasertkul**, Nanyawan Rungroj, Sansanee Noisakran, Varaporn Akkarapatumwong, Pa-thai Yenchitsomanus. The Siriraj-Rama Congress, April 18-21, 2006. Queen Sirikit National Convention Center, Bangkok, Thailand. (The first and second authors contributed equally).