Identification of the Cellular and Molecular Mechanisms Governing the Post-translational Regulation of the Neuron-specific Potassium/Chloride Cotransporter KCC2

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TABLE OF CONTENTS

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ABSTRACT

The neuron-specific K^+/Cl^- cotransporter 2 (KCC2) is one of the major cation Cl^- cotransporter (CCC) proteins in the central nervous system that controls Cl^- homeostasis via mediating Cl^- extrusion. KCC2 has been established as an essential protein in promoting the maturation of synaptic inhibition during development and controlling neuronal excitibility in the adult central nervous system (CNS). Although various mechanisms are known to regulate KCC2 gene expression, accumulating evidence has shown KCC2 activity can be altered on much shorter time scales in a manner of 10-20 minutes. This suggested post-translational mechanisms, which take place much more rapidly than gene expression, may also be an important means by which KCC2 function can be regulated. However, what these post-translational mechanisms are and how they contribute to the control of KCC2 function remain elusive.

In this thesis, I set out to characterize the molecular mechanisms governing the post-translational regulation of KCC2, including its endocytosis, quaternary assembly, and cell surface expression. Specifically, my results suggest: 1) Endogenous KCC2 interacts with the clathrin-mediated endocytosis (CME) machinery; 2) KCC2 is constitutively endocytosed via a CME-dependent mechanism; 3) A di-leucine motif, $_{657}LL_{658}$, is essential for both KCC2 constitutive endocytosis and the binding of KCC2 to the CME adaptor protein AP-2 complex; 4) Two regions within KCC2 proximal and central carboxyl terminus respectively mediate KCC2 dimerization, and in particular mutating the $_{657}LL_{658}$ residues to alanines completely abolishes KCC2 dimerization; 5) The $_{657}LL_{658}$ sequence is highly conserved amongst closely related K^+/Cl^- cotransporter proteins, but absent from the more distant Na^+/Cl^- cotransporters controlling Cl^- uptake, suggesting an evolutionarily conserved mechanism may regulate the constitutive endocytosis and dimerization of functionally homologous KCC members; and 6) KCC2 expression at the neuronal surface can be dynamically regulated by cellular tyrosine phosphatase activity and brain-derived neurotrophic factor (BDNF) and epidermal growth factor (EGF) dependent signalling pathways. In conclusion, my findings provide insight into the molecular mechanisms regulating KCC2 constitutive endocytosis and

dimerization. My studies have also shed a light on how KCC2-related CCC member proteins may be regulated at the post-translational level.

RÉSUMÉ

Le co-transporteur d'ions K^+ et Cl^- (KCC2), spécifique aux neurones, est l'un des plus importants co-transporteurs de cations chlorure (CCC) du système nerveux central. Il contrôle l'homéostasie de la cellule en régulant l'extrusion d'ions chlorure. KCC2 est reconnu comme étant une protéine essentielle à la maturation de l'inhibition synaptique au cours du développement et au contrôle de l'excitabilité des neurones dans le système nerveux central de l'adulte. Bien que les mécanismes régulant l'expression de KCC2 soient connus, de nouvelles données suggèrent que l'activité de KCC2 peut aussi être modifiée sur de plus courtes périodes, de l'ordre de 10 à 20 minutes. Ceci suggère qu'un contrôle post-traductionnel, agissant beaucoup plus rapidement qu'un contrôle transcriptionnel, pourrait aussi être important dans la régulation de l'activité de KCC2. Toutefois, la nature de ces mécanismes post-traductionnels et la façon par laquelle ils contribuent à la fonction de KCC2 demeurent inconnus.

Dans cette thèse, je vise à caractériser les mécanismes moléculaires qui gouvernent la régulation post-traductionnelle de KCC2, incluant son expression à la surface cellulaire, son endocytose et son assemblage quaternaire. Plus spécifiquement, mes résultats suggèrent que: 1) Le KCC2 endogène interagit avec la machinerie d'endocytose par clathrines (CME) ; 2) Le KCC2 est endocyté constitutivement par un mécanisme dépendant des clathrines; 3) Le motif di-leucine $_{657}LL_{658}$ est essentiel non seulement à l'endocytose constitutive de KCC2, mais aussi à l'interaction entre KCC2 et le complexe protéique adaptateur AP-2; 4) Deux régions à l'intérieur de KCC2, l'une proximale et l'autre centrale à l'extrémité C-terminale, sont responsables de la dimérisation de KCC2 qui peut être abrogée par la substitution des leucine 657 et 658 par des alanines; 5) La séquence $_{657}LL_{658}$ est très conservée parmi les co-transporteurs K^+/Cl^- proches de KCC2, mais absente de ceux plus éloignés, ce qui suggère qu'un mécanisme évolutif conservé pourrait réguler l'endocytose constitutive et la dimérisation de protéines homologues à KCC; et 6) L'expression de KCC2 à la surface neuronale peut être régulée dynamiquement par une activité tyrosine phosphatase et par les voies de signalisation activées par le facteur neurotrophique dérivé du cerveau (BDNF) et le facteur de croissance de l'épiderme (EGF). En conclusion, mes découvertes ouvrent une voie sur les

mécanismes moléculaires qui régulent l'endocytose constitutive et la dimérisation de KCC2. Mes études ont aussi mis en lumière la façon par laquelle les membres de la famille de KCC2 pourraient être régulés au niveau post-traductionnel.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	alanine
ABCA1	ATP binding cassette transporter A1
AP-2	adaptor protein-2
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ACSF	artificial cerebrospinal fluid
AQP2	Aquaporin-2
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
C	carboxyl
CD	C-terminal deletion
CIP	cotransporter interacting protein
CCC	cation chloride cotransporter
Ca	calcium
$CaCl_2$	calcium chloride
CaMK	Ca^{2+}/calmodulin kinase
CME	clathrin-mediated endocytosis
Cl	chloride
$[Cl^-]_i$	intracellular chloride concentration
CNS	central nervous system
CKB	brain-type creatine kinase
CREB	cAMP response element-binding
CNS	central nervous system
DIOA	dihydroindenyloxy-alkanoic acid
DIV	day *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
DRG	dorsal root ganglion
DN	dominant-negative
E#	embryonic day #
E	glutamic acid
Egr4	early growth response 4
EDTA	ethylenediaminetetraacetic acid
E_{gly}	reversal potential for glycine
E_{GABA}	reversal potential for GABA
EGF	epidermal growth factor
Eps15	epidermal growth factor receptor pathway substrate 15
EH	Eps15-Homology
ER	endoplasmic reticulum
FBS	fetal bovine serum
FRS-2	fibroblast growth factor receptor substrate 2
GABA	γ-amino-butyric acid
$GABA_AR$	γ-amino-butyric acid type-A receptor
GlyR	glycine receptor
GFP	green fluorescent protein

GST	glutathione-S-transferase
GPCR	G-protein coupled receptors
H	hydrogen
HA	haemagglutin
HEK293	human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	hydrogen peroxide
IgG	immunoglobulin G
IP	immunoprecipitation
IB	immunoblot
IGF-1	insulin-like growth factor 1
JNK	Jun N-terminal kinase
K	potassium
KCl	potassium chloride
$[K^+]_o$	extracellular potassium concentration
KCC	potassium chloride cotransporter
L	leucine
LTP	long-term potentiation
LTD	long-term depression
LSO	lateral superior olive
Mg	magnesium
$MgCl_2$	magnesium chloride
mRNA	messenger ribonucleic acid
MLCK	myosin light-chain kinase
N	amino
Na	sodium
NH_4	ammonium
NaCl	sodium chloride
NaOH	sodium hydroxide
NaF	sodium floride
NCC	sodium chloride cotransporter
NKCC	sodium potassium chloride cotransporter
NRSE	neuron-restrictive silencer element
Na_3VO_4	sodium orthovanadate
NDI	nephrogenic diabetes insipidus
NEM	*N*-ethylmaleimide
NMDA	N-methyl D-aspartate
NMDAR	N-methyl D-aspartate receptor
NMR	nuclear magnetic resonance
P#	postnatal day #
PD	pull-down
PBS	phosphate buffer saline
PMSF	phenylmethylsulfonyl fluoride
PP1	protein phosphatase 1
PP2A	protein phosphatase 2 type A
PNS	peripheral nervous system
PTZ	pentylenetetrazole

PLCγ	phospholipase C γ
PTK	protein tyrosine kinase
PKA	protein kinase A
PKC	protein kinase C
RVI	regulatory volume increase
RVD	regulatory volume decrease
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SLC	solute carrier
Shc	src homology 2 domain containing transforming protein
siRNA	small interference RNA
SNR	substantia nigra pars reticulata
SPAK	Ste20-related proline-alanine-rich kinase
Tac	interleukin 2 receptor α-chain
TfR	transferrin receptor
TAL	thick ascending limb
TG	trigeminal ganglion
TRK	tyrosine receptor kinase
TrkB	tyrosine receptor kinase B
TL	temporal lobe
TLE	temporal lobe epilepsy
TM	transmembrane
TBS	theta-burst stimulation
VDCC	voltage-dependent calcium channel
WB	western blot
WNK	with no lysine

CONTRIBUTIONS OF AUTHORS

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Contribution to figures:

B. Zhao	Figures 2.1A and B, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8
A. Wong	Figure 2.1C and D

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B. Zhao	Figures 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8

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LITERATURE REVIEW

Maintenance of cellular homeostasis, such as intracellular ion concentration, cell volume, and pH, are fundamental for normal intracellular events to occur. This cellular homeostasis is maintained by a large number of membrane proteins capable of conducting ions and water, such as ion transporters and cotransporters, ion channels, and aquaporins. The proper function of individual proteins as well as the coordination of their function ensures cells respond to their microenvironment precisely.

A cotransporter is a transmembrane protein involved in the transport of two or more different molecules or ions across the membrane. It acts as a secondary active transporter as its transport activity does not require the hydrolysis of adenosine-5'-triphosphate (ATP) as does a primary active transporter. Rather, the driving force for the transport activity of a co-transporter is derived from the electrochemical gradient of one solute (maintained by the primary active transporters), which couples the movement of the other molecule(s) or ion(s) against its electrochemical gradient across the membrane. The cotransporter family includes the cation chloride cotransporters, the sodium/phosphate cotransporter and the sodium-glucose cotransporters, etc.

The cation chloride cotransporter (CCC) family, solute carrier 12 (*SLC12*), consists of genes encoding nine electroneutral cation chloride (Cl^-) cotransporter proteins that cotransport Na^+ or K^+ and Cl^- across the plasma membrane in a 1:1 stoichiometry. These proteins are expressed in a wide range of tissues and play important physiological roles in many intracellular events, such as the maintenance of Cl^- homeostasis, cell volume regulation, trans-epithelial ion absorption and secretion, and the control of inhibitory neurotransmission polarity. Consistent with these "housekeeping" roles of CCCs, loss-of-function mutations in the human genes of some family members are associated with specific inherited diseases, such as Gitelman's syndrome (Karolyi et al., 1998), Bartter's syndrome (Simon et al., 1996), and Anderman's syndrome (Howard et al., 2002). One of this family member proteins, the neuron-specific K^+/Cl^- cotransporter, KCC2, has been identified as an essential protein for controlling neuronal Cl^- homeostasis. It therefore plays a crucial role in controlling the strength of synaptic inhibition mediated by γ-amino-butyric acid type-A ($GABA_A$) and glycine receptors,

which are both Cl^- permeant ion channels. Disruption of KCC2 function has been linked to the neuropathology of epileptic seizures, neuronal trauma, and chronic pain. The first CCC member identified in 1993 was the Na^+/Cl^- cotransporter (NCC) (Gamba et al., 1993). It was isolated from the urinary bladder of the winter flounder *Pseudopleuronectes americanus* (Gamba et al., 1993). Since then, CCCs have been studied in various cell types and a variety of organisms, ranging from *C. elegans*, *D. melanogaster,* sheep, dog, rabbit, Guinea pig, rodent, to human. A large amount of information has been obtained regarding the factors that promote or inhibit the cotransport activity of CCCs, such as changes in the concentrations of sodium, potassium, chloride, or magnesium ions, osmolarity, tonicity, cell volume, pH, oxidants, thiol-alkylating agent, loop diuretics, kinase/phosphatase activities, urea, oxygenation, neurotrophic factors and neuronal activity (Gamba, 2005).

Many studies have been carried out to elucidate the molecular and cellular mechanisms controlling CCC function. While gene transcription and protein translation are indispensable for the initiation and maintenance of CCC functional expression, research in recent years has just started to reveal post-translational mechanisms also play a crucial role in regulating CCC activity. These post-translational mechanisms include protein maturation, intracellular protein trafficking, phosphorylation/dephosphorylation modifications and protein quaternary assembly. They provide the cells with "tools" by which the activity of CCCs can be regulated in a short-term manner so that cells can respond to their environment efficiently. Together, the coordination of all these regulatory mechanisms ensures that cellular homeostasis is well maintained.

In this chapter, I will first review several aspects of the CCC superfamily member proteins, including their protein structure, tissue distribution, physiological function, as well as their association to human diseases. This will be followed by a specific discussion about the physiological roles of KCC2 and the mechanisms involved in the control of KCC2 function, including transcriptional/translational and post-translational regulations. Finally, I will recapitulate the key points of this chapter and introduce the rationale of my PhD studies.

I. Overview of the cation-chloride cotransporter (CCC) family

The electroneutral cation-chloride cotransporter (CCC) gene family, solute carrier 12 (*SLC12*), composes a family of solute carriers in which the movement of sodium (Na^+) or potassium (K^+) across the plasma membrane is accompanied by chloride (Cl^-) in a 1:1 stoichiometry, and therefore the transport of these ions by CCCs does not alter the membrane potential. This family of cotransporter proteins are considered as secondary transporters because adenosine triphosphate (ATP) hydrolysis is not required for the transport of ions. Rather, the driving force for the translocation of these ions is derived from the electrochemical gradient of Na^+ and K^+ across the cell membrane generated by the primary transporter, Na^+/K^+ ATPase, which actively pumps 2 K^+ in and 3 Na^+ out of the cell using the energy derived from ATP hydrolysis.

To date, nine member proteins of the CCC family have been identified at the molecular level, and they are functionally categorized as two major branches based on the cation(s) coupled with Cl^- and the stoichiometry of the cation(s) *versus* Cl^- (Hebert et al., 2004; Fig. 1.1). One branch includes the Na^+-coupled Cl^- cotransporters, one thiazide-sensitive Na^+/Cl^- cotransporter (NCC) encoded by the gene *SLCA3* and two bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporters (NKCC1/2) encoded by the gene *SLCA2* and *SLCA1*, respectively. The other main branch includes the four dihydroindenyloxy-alkanoic acid (DIOA)-sensitive K^+-coupled Cl^- cotransporters, KCC1-4, which are encoded by the gene *SLCA4-7*. The remaining two family member proteins, cation-chloride cotransporter interacting protein (CIP) and cation-chloride cotransporter 9 (CCC9), are far less well understood in regards to their transport function. CIP, which is encoded by the gene *SLCA9* and shares 25% of homology with KCCs and NKCCs (Gamba, 2005), was initially identified due to its ability to physically interact with the endogenous NKCC when expressed in the human embryonic kidney (HEK) 293 cells and to inhibit the transport activity of NKCC1 when co-expressed in *Xenopus laevis* oocytes (Caron et al., 2000). However, when expressed alone in heterologous HEK293 cells or *Xenopus laevis* oocytes, no transport activity of CIP was observed (Caron et al., 2000). Therefore, the transport substrate of CIP remains elusive. Finally, the most recently identified gene of this family, *SLC12A8*, was identified as a psoriasis-susceptibility candidate gene in human (Hewett et al., 2002). *SLC12A8* encodes the protein CCC9,

which shares 30% of homology with NKCC2 (Mount et al., 2002). Like CIP, the function of CCC9 is yet unclear in that neither transport activity nor interaction with the other CCC family member proteins was observed. The following sections will mainly review some of the most relevant findings about the better-understood CCC members, NCC, NKCCs and KCCs.

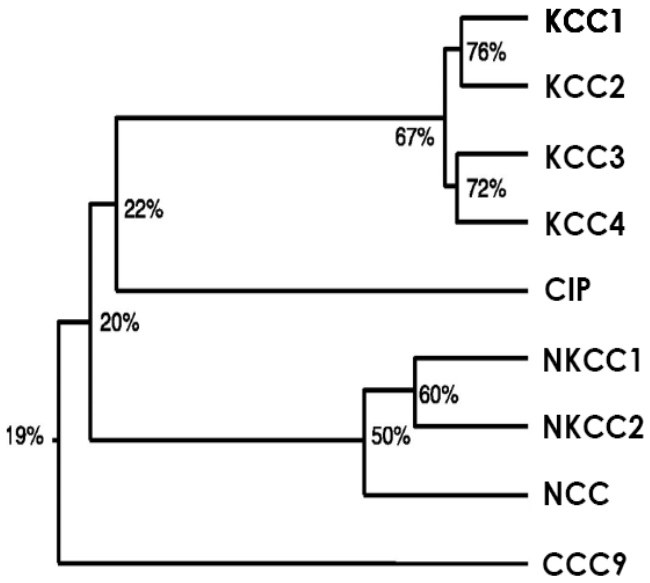

Figure 1.1 Phylogenetic tree of the cation chloride cotransporter family SLC12. Numbers indicate degree of homology *(adapted with permission from Gamba, 2005).*

The first CCC family member protein to be identified was NCC from the urinary bladder of the winter flounder *Pseudopleuronectes americanus* (Gamba et al., 1993). Next was the first NKCC isoform (NKCC1), which was identified in the rectal gland of the shark, *Squalus acanthias* (Xu et al., 1994). Since then, extensive research has been carried out, using various biological tools, in an attempt to understand the different aspects of these CCC proteins, such as their protein structures, tissue expression patterns, physiological properties, regulatory mechanisms underlying their function, as well as their association to human diseases.

A. The protein topology of CCC family members

Hydropathy analysis has revealed that the CCC family proteins share similar topology. NCC, NKCCs, and KCCs all contain twelve transmembrane (TM) regions with both intracellular amino and carboxyl termini flanking each side (Fig. 1.2). The *N*-liked glycosylation sites of the Na^+-coupled Cl^- cotransporters (NCC and NKCCs) were predicted to be in the large extracellular loop between TM6-7, whereas those of the K^+-coupled Cl^- cotransporters (KCC1-4) and CIP were predicted to be in the large extracellular loop between TM5-6. The CCC9 protein is the most distinct member in regards to its molecular structure, in that it is much shorter, contains only eleven TMs, and its glycosylation sites are located in the extracellular carboxyl terminus.

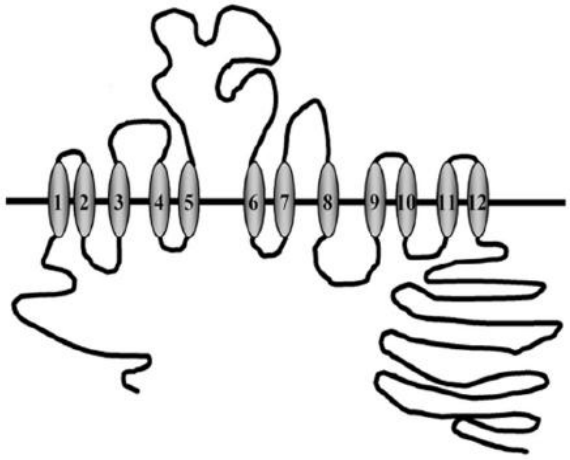

Figure 1.2 Predicted protein topology for NCC, NKCC1/2, and KCC1-4.

B. CCC family protein tissue distribution, physiological roles, and association with pathological conditions

In general, the major physiological roles of the CCC family member proteins are to maintain cell volume, and ion and fluid homeostasis in response to changes in various conditions, such as changes in cell volume, ion concentrations, osmolarity, and pH. One of the major functional differences between the Na^+-coupled Cl^- cotransporters and the K^+-coupled Cl^- cotransporters is the direction the Cl^- ion is transported. That is, NCC and NKCCs uptake Cl^- whereas KCCs extrude Cl^-. Since the influx and efflux of Cl^- is obligatorily accompanied by the absorption and loss of H_2O, respectively, the CCC family member proteins are key players in the process of regulatory volume increase

(RVI) or decrease (RVD). NCC and NKCCs are activated upon cell shrinkage, whereas KCCs are activated upon cell swelling.

In mammals the nine CCC family member proteins exhibit different expression patterns. Some are ubiquitously distributed but display preferential expression in certain tissues, whereas others are expressed in a tissue-specific manner. The expression pattern of each CCC member protein endows them particular physiological contributions to the specific tissues where they are expressed.

1. Na^+-coupled Cl^- cotransporters

1.1 Isoform NCC and NKCC2

NCC and NKCC2 are kidney-specific. NCC is specifically expressed on the apical membrane of the distal convoluted tubule (Costanzo, 1985; Ellison et al., 1987), and NKCC2 is specifically expressed on the apical membrane of the thick ascending limb (TAL) (Greger et al., 1983). NCC and NKCC2 play key roles in salt re-absorption in the kidney. Mutations in the genes of these proteins, which result in excessive salt lost, have been shown to be associated with renal disorders. For instance, loss-of-function mutations in the NCC gene cause Gitelman's syndrome, which is a rare inherited salt-wasting disorder (Karolyi et al., 1998). Loss-of-function mutations in the NKCC2 gene cause Bartter's syndrome, which is characterized by low K^+ levels, decreased acidity of blood, and normal to low blood pressure (Simon et al., 1996).

1.2 Isoform NKCC1

NKCC1 is ubiquitously expressed in both epithelial and non-epithelial cells (Russell, 2000). In epithelial tissue, such as trachea (Musch et al., 1989; Liedtke, 1992) and *Necturus* gall bladder (Larson and Spring, 1983), the specific basolateral membrane location of NKCC1 controls uptake of Cl^- that will be secreted at the apical side. In non-epithelial cells, such as erythrocytes, the main function of NKCC1 is to regulate cell volume in the process of RVI since it can be activated upon cell shrinkage (Lytle, 1997; Hubner et al., 2001). In the nervous system, NKCC1 is expressed on the apical membrane of choroids plexus, in oligodendrocytes, and in neurons of both the central nervous system (CNS) and the peripheral nervous system (PNS) (Plotkin et al., 1997). NKCC1 is

considered to be one of the major players in controlling Cl^- homeostasis in the nervous system (Payne et al., 2003). NKCC1 is expressed in sensory neurons of both dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons (Kanaka et al., 2001; Alvarez-Leefmans et al., 2001; Morales-Aza et al., 2004; Toyoda et al., 2005). NKCC1 knockout mice displayed abnormal gait and locomotion as well as defects in the nociceptive perception of thermal stimuli induced by high temperatures. In addition, these phenotypes were associated with a hyperpolarizing shift in the GABA-elicited responses in sensory neurons (Sung et al., 2000). This study suggests that in DRG neurons NKCC1 is sufficient to maintain the high $[Cl^-]_i$ and depolarizing responses of GABAergic neurotransmission, and hence is crucial for the sensory perception mediated by these neurons. The role of NKCC1 in controlling thermal nociception is supported by another study where thermal noxious stimulus was induced using a different protocol, intradermal injection of capsaicin, in NKCC1 knockout mice (Laird et al., 2004). In addition to this, this study has also shown NKCC1 regulates touch-evoked nociception (Laird et al., 2004).

Besides the well studied nociceptive function of NKCC1, a previously unknown physiological function of NKCC1 has just been identified. It was observed that *in vivo* sciatic nerve transection induced an increase in phosphorylated NKCC1 in adult mouse DRG neurons and consequent neurite outgrowth of these axotomized sensory neurons (Pieraut et al., 2007). This effect of NKCC1 seemed to be attributed to an elevation of $[Cl^-]_i$ and a consequent positive shift in E_{GABA} in injured sensory neurons, suggesting a role of NKCC1 in mediating the regeneration of injured adult sensory neurons by facilitating GABAergic depolarizing responses.

In the CNS, NKCC1 is abundantly expressed in the developing brain (Clayton et al., 1998; Hubner et al., 2001a). It is generally believed that the Cl^- accumulating function of NKCC1 contributes to the excitatory action of GABA-mediated postsynaptic responses in immature neurons of the rat brain (Plotkin et al., 1997; Sun and Murali, 1999; Owens and Kriegstein, 2002). To date no mutations in the NKCC1-encoding gene, *SLC12A2*, has been mapped to any human diseases.

2. K^+-coupled Cl^- cotransporters

K^+/Cl^- cotransport activity was first described as a swelling- and *N*-thylmaleimide (NEM)-activated K^+ efflux pathway in low-K^+ sheep and goat erythrocytes (Lauf et al., 1980). So far, most studies on KCCs have been carried out in erythrocytes from many different species (Lauf et al., 2000). The Cl^- transport activity of KCCs has also been observed in other cell types, such as heart cells (Piwnica-Worms et al., 1985), kidney cells (Warnock et al., 1989), astrocytes (Pasantes-Morales et al., 1990; Olson et al., 1995), and neurons (Jarolimek et al., 1999; Payne et al., 2003). The major function of KCCs described in these studies is their role in RVD upon cell swelling. Due to the wide distribution of KCCs and their important roles in maintaining cellular homeostasis, abnormal function of these proteins can result in prominent pathological conditions. For instance, elevated transport activity of KCCs, which results in cell dehydration, has been shown to be a mechanism involved in sickle cell anemia (Lauf et al., 2000).

Erythroid expressed KCCs can be activated under various conditions, such cell swelling, acidification, magnesium (Mg^{2+}) depletion, urea, thiol-alkylating agent NEM, and oxidizing agents such as hydrogen peroxide (H_2O_2) diamide. The activation of KCCs by cell swelling, NEM, or urea in erythrocytes can be abolished by protein phosphatase inhibitors such as vanadate, calyculin A, or okadaic acid, suggesting the involvement of endogenous protein phophatase activity in the regulation of KCC activity in erythrocytes (Adragna et al., 2004).

2.1 Isoform KCC1

KCC1 is ubiquitously expressed, suggesting a “housekeeping” role in maintaining cellular homeostasis. In the adult rat CNS, KCC1 is expressed in both neuronal and non-neuronal cells, and is abundant in choroid plexus epithelial cells (Kanaka et al., 2001). In the embryonic mouse brain, KCC1 mRNA level is generally low and mainly detected in the developing choroid plexus. Its expression in neurons displays a slight up-regulation until birth (Li et al., 2002).

2.2 Isoform KCC2

KCC2 possesses several unique features among the KCCs: 1) it is exclusively expressed in neurons of the CNS (Payne et al., 1996; Payne, 1997); 2) unlike the other KCCs, which are inactive under isotonic conditions, KCC2 is constitutively active under isotonic conditions when expressed in *Xenopus* oocytes and HEK293 cells (Strange et al., 2000; Song et al., 2002); and 3) unlike the other KCCs, which act exclusively as efflux pathways for K^+ and Cl^-, KCC2 has been suggested to be capable of mediating both the exit and entry pathways for Cl^- due to its high affinity for external K^+ (Payne, 1997).

Since KCC2 was first cloned from a rat brain cDNA library, the neuronal restrictive expression pattern of KCC2 was suggestive of a role in mediating the function of Cl^- permeant ion channels via controlling neuronal Cl^- homeostasis (Payne et al., 1996). A number of studies have indeed proven this hypothesis and revealed an essential role of KCC2 in promoting the developmental maturation of fast inhibitory neurotransmission mediated by $GABA_A$ and glycine receptors (both are Cl^- permeant ion channels), as well as the maintenance of synaptic inhibition in the adult. The evidence supporting this notion will be discussed in more detail in section **II ─ Physiological functions of KCC2**.

In the adult CNS, KCC2 protein is expressed in the spinal cord, brainstem, cerebellum, cortex, and hippocampus (Payne et al., 1996). In the embryonic CNS, KCC2 expression exhibits different temporal profiles among different regions. Interestingly, it seems that a "rule of thumb" for the expression pattern of KCC2 is that it is tightly associated with neuronal maturation. That is, KCC2 expression closely parallels the maturation of different regions in the CNS, and even within the same region, KCC2 expression seems to be only detected in differentiated neurons and not in neuronal precursors (Li et al., 2002). For instance, spinal cord motoneurons are among the first neurons to differentiate in the CNS. KCC2 mRNA is observed in developing spinal cord motoneurons of the ventral horn and the medulla as early as E10.5 (mouse), but is undetectable in other regions which develop at a later stage (Li et al., 2002). KCC2 mRNA is undetectable in the isocortex of the telencephalon until postnatal day 0 (P0), when this region develops into the neocortex and hippocampus (Li et al., 2002). In the hippocampus, KCC2 expression undergoes a dramatic increase within the first two

postnatal weeks, when the maturation of hippocampal neurons is ongoing. KCC2 mRNA is not observed in the ventricular and intermediate zones, where neurogenesis and neuronal migration occur, respectively (Li et al., 2002). Immunohistochemistry experiments using TUJ1 (a specific antibody against the class III β-tubulin), a marker for neuronal differentiation, confirmed that within the same brain region KCC2 expression is restricted to the TUJ1 positive cells (Li et al., 2002). At the cellular level, KCC2 protein is localized in both the soma and dendrites of postsynaptic neurons (Williams et al., 1999; Gulyas et al., 2001). It is colocalized with subunits of the $GABA_A$ receptor, with an inhibitory synaptic scaffolding protein gephyrin, and interacts with the cytoskeleton-associated protein 4.1N in dendrites (Williams et al., 1999; Hubner et al., 2001; Li et al., 2007), which together suggests a role for KCC2 in the synaptogenesis of inhibitory synapses. Interestingly, KCC2 has also been observed in the vicinity of excitatory inputs in the rat hippocampus, suggesting KCC2 may act to provide the microenvironment with a Cl^- exit pathway to control Cl^- homeostasis and cell volume in the presence of high levels of neuronal activity, during which a net influx of Na^+ and Cl^- and consequent dendritic swelling are known to occur (Gulyas et al., 2001). Up to date no spontaneous KCC2 mutation has been identified in human diseases (Hebert et al., 2004).

2.3 Isoform KCC3

KCC3 expression is widespread, but is abundant in muscle, brain, spinal cord, kidney, heart, pancreas, and placenta (Mount et al., 1999; Race et al., 1999). Various mutations in the human gene for KCC3, *SLCA6*, have been associated with the neurological disorder known as Anderman's disease, a neuropathological condition featuring agenesis of the corpus callosom (Howard et al., 2002). Disruption of KCC3 function has also been associated with the development of arterial hypertention, suggesting a possible role of KCC3 in vascular smooth muscle cell relaxation (Adragna et al., 2000; Rust et al., 2006). In the embryonic rodent brain, KCC3 mRNA is scarce, but is slightly increased in the cortical plate, hippocampus and olfactory bulb at birth (Li et al., 2002).

2.4 Isoform KCC4

KCC4 is also ubiquitously expressed, but is abundant in heart and kidney cells. In the kidney, KCC4 may play an important role in salt absorption by the distal convoluted tubule, via mediating the extrusion of K^+ and Cl^- ions (Velazquez et al., 2003). KCC4 knockout mice display renal tubular acidosis and deafness, mimicking the symptoms observed in some forms of human hereditary distal tubular acidosis (Boettger et al., 2002).

KCC4 exhibits minimal expression levels in the nervous system, compared to the other KCCs. In the rodent embryonic brain, KCC4 mRNA is detected in the periventricular zone, cranial nerves, and choroid plexus, and is down-regulated perinatally (Li et al., 2002). High levels of KCC4 protein are detected in peripheral nerves and the spinal cord and low levels are detected in the brain (Karadsheh et al., 2004). In adult rodent brain, KCC4 protein expression is only observed on the apical membrane of choroid plexus epithelial cells and in cranial nerves, and is absent in all other brain regions (Karadsheh et al., 2004). The physiological roles of KCC4 expression in neurons are yet unknown. To date no mutations in the KCC4-encoding gene, *SLC12A7*, has been identified to any human diseases.

II. Physiological functions of KCC2

CCC family member proteins play fundamental physiological roles in maintaining Cl^- homeostasis and cell volume in various tissues of different species. In the nervous system, CCCs play a key role in regulating the intracellular Cl^- concentration ($[Cl^-]_i$), which is tightly linked to the properties of neurotransmission mediated by Cl^- permeant ion channels, such as the GABA receptor type A ($GABA_AR$) and glycine receptor (GlyR). KCC2 and NKCC1 are thought to be the two major players in controlling the Cl^- homeostasis in neurons (Payne et al., 2003). KCC2 extrudes Cl^-, whereas NKCC1 uptakes Cl^-. Therefore, the opposing physiological actions of these two Cl^- cotransporters can directly influence the balance of $[Cl^-]_i$ and set $[Cl^-]_i$ above or below its electrochemical potential equilibrium, depending on the relative abundance and activity of KCC2 and NKCC1. Research in the past decade has revealed KCC2 displays various physiological functions in neurons, including A) promoting the formation of synaptic inhibition during

development; B) controlling neuronal excitability under neuropathological conditions in adult CNS; C) mediating Cl^- homeostasis and cell volume during neuronal spiking; D) bi-directionally cotransporting K^+/Cl^-; and E) cotransporting NH_4^+/Cl^-.

A. Role of KCC2 in promoting the maturation of inhibitory neurotransmission during development

During early development KCC2 plays an essential role in establishing inhibitory neurotransmission mediated by GABA and glycine in brain regions such as the hippocampus, cortex, and retina (Rivera et al., 1999; Leitch et al., 2005). In the adult mammalian CNS, fast inhibitory neurotransmission is mediated predominantly by $GABA_AR$ and GlyR, both of which are ligand-gated ion channels that conduct Cl^- (Betz, 1992; Sieghart, 2006). Depending on the $[Cl^-]_i$, however, $GABA_AR$ and GlyR can also produce excitatory postsynaptic responses, as occurs in immature neurons or under certain neuropathological conditions, such as epileptic seizures and chronic pain (Owens and Kriegstein, 2002). During early development of hippocampal, cortical, and retinal neurons in particular, $[Cl^-]_i$ is relatively high. This is attributed to the predominant expression of NKCC1 and minimal expression of KCC2, which therefore increases $[Cl^-]_i$ and keeps it above its electrochemical equilibrium (Delpire et al., 2000). At this stage, binding of GABA or glycine to its receptors opens $Cl^–$ channels and leads to efflux of $Cl^–$ and consequent depolarizing (excitatory) postsynaptic responses. This depolarization can subsequently elicit calcium (Ca^{2+}) influx through the voltage-dependent calcium channels (VDCCs) and N-methyl D-aspartate receptors (NMDARs) (Fukuda et al., 1998; Leinekugel et al., 1997; Ganguly et al., 2001). This Ca^{2+} oscillation induced by GABA- or glycine- mediated depolarizing responses have been shown to play important roles in various cellular events during early development, such as synaptogenesis, gene expression, and long-term potentiation (LTP) induction (Kirsch et al., 1998; Ganguly et al., 2001; Caillard et al., 1999; Ben-Ari, 2002). In particular, the Ca^{2+} oscillation induced by GABA-mediated depolarizing responses has been shown to be involved in the induction of KCC2 expression during early development, which subsequently shuts down the neurotrophic effect of GABA-mediated excitatory responses at this stage, suggesting

that GABA acts as a self-limiting trophic factor during neural maturation most likely through the induction of KCC2 expression (Ganguly et al., 2001).

During the first two postnatal weeks, there is a steep up-regulation of KCC2 protein expression and concomitant down-regulation of NKCC1 expression in cortical and hippocampal neurons, leading to a switch in $[Cl^-]_i$ from above to below its electrochemical potential equilibrium (Plotkin et al., 1997; Clayton et al., 1998; Rivera et al., 1999; Lu et al., 1999; Mikawa et al., 2002; Vardi et al., 2002; Dzhala et al., 2005). As a result, opening of $GABA_A$Rs elicits the influx of Cl^- and consequently hyperpolarizing (inhibitory) responses (Fig. 1.3 and 1.4). This developmental switch in the polarity of GABA-mediated postsynaptic responses from excitatory to inhibitory has been shown to be associated with a negative shift in the reversal potential for GABA (E_{GABA}) (Rivera et al., 1999; Farrant et al., 2007). Consistently, knockdown of KCC2 expression in rat hippocampal pyramidal cells using antisense oligonucleotides resulted in a significantly positive shift in E_{GABA} (Rivera et al., 1999). Reduction of KCC2 expression by neuronal trauma similarly caused a positive shift in E_{GABA} (Farrant et al., 2007; Rivera et al., 2002; Coull et al., 2003).

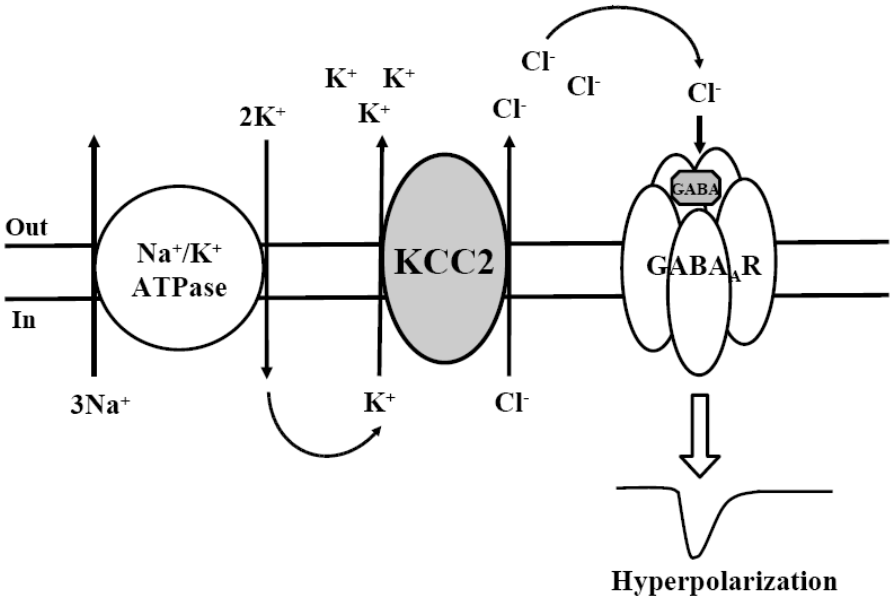

Figure 1.3 Physiological function of KCC2.

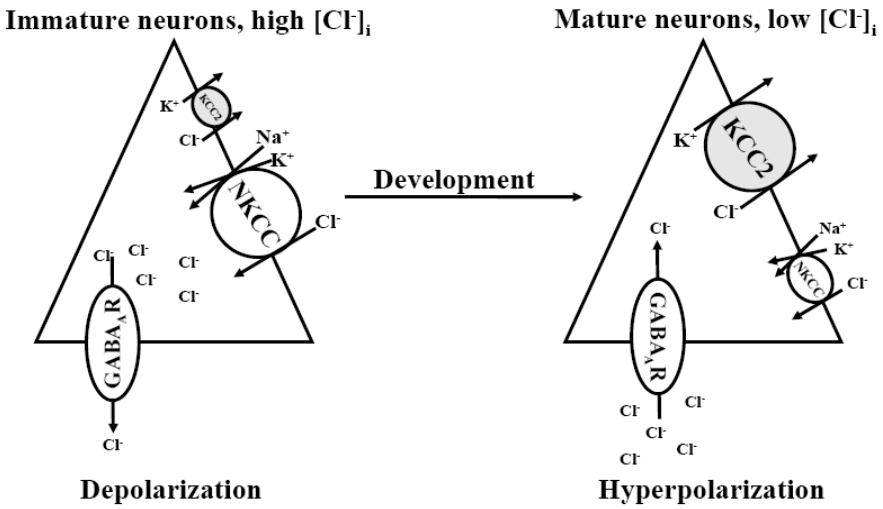

Figure 1.4 KCC2 promotes the developmental transformation of GABAergic neurotransmission from depolarizing to hyperpolarizing (*adapted with permission from Ben-Ari, 2002*)**.**

The essential role of KCC2 during development was proven by studies on KCC2 knockout mice. KCC2 null mice ($KCC2^{-/-}$) exhibit a spastic posture and die immediately after birth due to respiratory failure caused by functional defects in respiratory motorneurons (Hubner et al., 2001). GABAergic and glycinergic responses were observed to be excitatory in E18.5 $KCC2^{-/-}$ mice but inhibitory in the wild-type mice, consistent with the role of KCC2 in switching GABAergic responses from excitatory to inhibitory during development (Hubner et al., 2001). Mice in which more than 90% of KCC2 gene expression is knocked down survive a bit longer than complete KCC2 null mice. However they exhibit continuous generalized seizures and die on average on P17 (Woo et al., 2002). Field potential recordings from the pyramidal neurons of hippocampal subfield CA1, a region known to participate in seizure induction, in KCC2 homozygotes and heterozygotes show multiple population spikes (PS) in contrast to the typical single PS observed in wild-type mice, indicating hyperexcitibility in hippocampal CA1 pyramidal neurons from these KCC2 deficient mice (Woo et al., 2002). The hyperexcitability observed in homozygous hippocampal pyramidal neurons is further increased by blocking the function of $GABA_A$Rs (Woo et al., 2002). To date no spontaneous KCC2 mutation has been identified in human diseases (Hebert et al., 2004).

Given the embryonic lethality of KCC2 knockout mice, mutations in the human KCC2 gene might also be embryonic lethal.

In addition to the crucial impact of KCC2 expression on the functional maturation of GABAergic neurotransmission during development, KCC2 has also been shown to play a role in GABAergic synaptogenesis (Chudotvorova et al., 2005). It was observed that premature expression of KCC2 in immature rat hippocampal neurons resulted in a significant increase in the density of $GABA_A$Rs and functional GABAergic synapses. This effect of KCC2 premature expression was only observed in GABAergic synapses and not in glutamatergic synapses (Chudotvorova et al., 2005), suggesting that KCC2 specifically facilitates GABAergic synaptogenesis. This structural regulation of GABAergic synapses by KCC2 is most likely an important mechanism by which KCC2 promotes the functional maturation of GABAergic neurotransmission during development.

B. Involvement of KCC2 in neuropathological conditions, such as epileptic activity and chronic pain

KCC2 not only is essential during early development but also plays a central role in maintaining synaptic inhibition and controlling neuronal excitability throughout adult life. Deficient expression of KCC2 has been linked to several neuropathological conditions where GABA- or glycine-mediated responses switch from inhibitory back to excitatory (disinhibition), such as epileptic seizures and chronic pain.

It has been shown that the levels of KCC2 mRNA correlate with the susceptibility to sound triggered-seizure (induced by cardiac arrest and resuscitation) in neurons of the inferior colliculus, which is the origin of audiogenic seizures (Reid et al., 2001). KCC2 heterozygote mice survive and do not exhibit obvious phenotypes. However, they showed increased susceptibility to seizures induced by pentylenetetrazole (PTZ), a blocker of the $GABA_A$ receptor (McDonald and Barker, 1977). PTZ treatment also increased resistance to the effect of the anticonvulsant drug, propofol (Woo et al., 2002). This hypersensitivity to seizures in KCC2 heterozygote mice suggest reduction of KCC2 expression may be a mechanism underlying the neuropathology of human epilepsy. Indeed, it has been observed that in hippocampal slice from the epileptic patients GABA-mediated responses

were always depolarizing in neurons lacking KCC2 but hyperpolarizing in the ones expressing KCC2 (Huberfeld et al., 2007). Quantitative RT-PCR analysis of KCC2 expression in the hippocampal subiculum of surgically resected brain specimens from temporal lobe (TL) epilepsy (TLE) patients showed that KCC2 mRNA levels were down-regulated in this region, compared to the hippocampus proper or the TL neocortex specimens (Palma et al., 2006).

Nociception, or more commonly called physical pain, is the unconscious afferent activity produced in the peripheral and central nervous system by mechanical, thermal, or chemical stimuli. It is initiated by nociceptors, which can detect the above noxious stimuli above a certain threshold. Upon stimulation, they sequentially transduce signals to the spinal cord and certain regions in the brain (somatosensory cortex, thalamus and anterior cingulated cortex, for instance), thereby causing the sensation of pain. Therefore, the spinal cord acts as a "relay station" connecting the peripheral sensory neurons and the brain along the nociceptive pathway.

It is known that disinhibition in the dorsal horn of the spinal cord is one of the mechanisms contributing to chronic pain (Woolf et al., 2000). It is also known that down-regulation of KCC2 can lead to disrupted Cl^- extrusion capacity and disinhibition of GABAergic and glycinergic synaptic responses, as discussed above. Therefore, it was reasonable to speculate that down-regulation of KCC2 could also be a mechanism underlying chronic pain. Indeed, several studies have supported this notion. Local blockade or knockdown of KCC2 in lamina I neurons of rat superficial dorsal horn, one of the main spinal nociceptive output pathways, significantly reduced the nociceptive threshold, suggesting that disruption of KCC2 activity in lamina I neurons is sufficient to cause neuropathic pain (Coull et al., 2003). Knockdown of KCC2 in the spinal cord of naïve rats by intrathecal injection of KCC2 antisense oligodeoxynucleotides led to behavioral hypersensitivity similar to the hyperalgesia induced by peripheral inflammation, as assessed by the paw withdrawal response upon noxious thermal stimuli (Zhang et al., 2008). This study suggests KCC2, via controlling the excitibility of spinal cord neurons, is also involved in the induction of inflammatory pain.

C. Potential role of KCC2 in controlling Cl^- homeostasis and cell volume at excitatory synapses during neuronal spiking

In addition to the well-defined functional association of KCC2 with synaptic $GABA_AR$ and GlyR, KCC2 has also been detected in the vicinity of excitatory inputs in the rat hippocampus (Gulyas et al., 2001). That is, during the first two postnatal weeks, an increase of KCC2 expression was observed in the molecular layer of the dentate gyrus, which correlated temporally with the arrival of entorhinal cortical inputs. In hippocampal principal neurons, KCC2 was found to be mainly expressed in dendritic spine, at the origin of spines and, at a much lower level on the somata and dendritic shafts. In interneurons, KCC2 expression is considerably higher in the somata and dendrites (Gulyas et al., 2001). It is known that high levels of excitation can lead to a net influx of Na^+ and Cl^- and consequent dendritic swelling (Hasbani et al., 1998). Therefore, it was proposed that KCC2 expressed in the vicinity of these excitatory inputs may be physically associated with extrasynaptic $GABA_ARs$ and provide the microenvironment with a Cl^- exit pathway to control Cl^- homeostasis and cell volume during high levels of neuronal activity (Gulyas et al., 2001).

D. An "unconventional" function of KCC2 as a bidirectional cotransporter for K^+/Cl^-

As discussed previously, KCC2 exhibits the highest affinity for K^+ (Payne, 1997; Song et al., 2002). Thermodynamic analysis predicts KCC2 can be an ideal buffer system for both extracellular K^+ and intracellular Cl^- (Payne, 1997). That is, depending on the extracellular K^+ concentration ($[K^+]_o$) and $[Cl^-]_i$, KCC2 can act as both a Cl^- extruder and accumulator. Under normal physiological conditions where the $[K^+]_o$ is relatively low, KCC2 extrudes Cl^- and maintains a low $[Cl^-]_i$. However, when the $[K^+]_o$ is raised to a range where KCC2 remains active, the direction of Cl^- transport via KCC2 will switch from outward to inward (Payne, 1997).

The reversibility property of KCC2 K^+/Cl^- cotransport has indeed now been demonstrated by several studies (DeFazio et al., 2000; Jarolimek et al., 1999; Kakazu et al., 2000). In mature (P18-28) rat neocortical pyramidal neurons, both increasing and decreasing $[K^+]_o$ led to changes in $[Cl^-]_i$. That is, raising $[K^+]_o$ increased $[Cl^-]_i$, and

lowering $[K^+]_o$ decreased $[Cl^-]_i$. This was not observed in the immature (P3-6) neurons, where KCC2 expression is minimal at this stage (DeFazio et al., 2000). In cultured rat midbrain neurons, KCC2 helped to maintain low levels of $[Cl^-]_i$ under normal physiological $[K^+]_o$ (2mM). However, when $[K^+]_o$ was elevated to a level similar to that occurring during epileptic form activity (10-12 mM) (Heinemann and Lux, 1977), the Cl^- transport direction of KCC2 switched from outward to inward (Jarolimek et al., 1999). Similar bidirectional K^+/Cl^- cotransport property has also been observed in cultured rat lateral superior olive neurons (Kakazu et al., 2000). It is known one of the major consequences of an acute increase in $[K^+]_o$ is depolarization of the neuronal membranes (Kaila et al., 1997). Although it remains unclear the physiological role for KCC2 to inwardly cotransport K^+/Cl^-, KCC2 may function as a K^+ uptake pathway to remove excess extracellular K^+ during depolarization and therefore serve as a buffer system to maintain $[K^+]_o$ homeostasis.

E. KCC2 as an NH_4^+/Cl^- cotransporter

Other than cotransporting K^+/Cl^-, KCC2 has also been shown to be able to up-take ammonium cation (NH_4^+) in cultured hippocampal and midbrain neurons (Liu et al., 2003). In this study, elevation of extracellular NH_4^+ concentration ($[NH_4^+]_o$) increased $[Cl^-]_i$ and shifted GABA-mediated responses to a more depolarizing level, which was blocked by furosemide at a concentration that specifically inhibits KCC2, suggesting therefore a potential role for KCC2 in mediating NH_4^+/Cl^- co-uptake (Liu et al., 2003). In addition, studies using a mutant Madin-Darby canine kidney (MDCK) cell line (LK-C1) that has been successfully used for studying CCC function, provide direct evidence that KCC2 can indeed uptake NH_4^+ and cotransport Cl^- into cells upon elevation of $[NH_4^+]_o$ (Williams and Payne, 2004). In this regard, KCC2 also acts as a Cl^- entry pathway, with the driving force being derived from high levels of $[NH_4^+]_o$ instead of $[K^+]_o$. Considering that elevation of $[NH_4^+]_o$ can impair the efficacy of inhibitory neurotransmission via various mechanisms, including a rise in $[Cl^-]_i$. (Deisz and Lux, 1982; Irie et al., 1998), these above studies suggest KCC2 may play a role in certain pathological conditions, such as hyperammonemic syndromes. These syndromes are featured by altered neuronal

Cl^- homeostasis and hence impairment of synaptic inhibition in the presence of high levels of $[NH_4^+]_o$.

III. Transcriptional and translational regulations of KCC2

Research in the last two decades, since the initial identification of KCC2 at the molecular level, has shown that the transcription and translation of KCC2 can be regulated by a number of factors, including A) transcription factors, B) GABA, C) neurotrophic factors, D) certain neuropathological conditions, E) neuronal activity, and F) sex hormones.

A. Regulation of KCC2 expression by transcription factors

As discussed above, one of the unique features of KCC2 is its exclusive expression in CNS neurons. The gene regulation for this restricted neuronal expression pattern was initially believed to be attributed to a neuron-restrictive silencer element (NRSE) in intron 1 of the KCC2 gene, which suppresses the transcription of genes in non-neuronal cells (Karadsheh and Delpire, 2001). However, later studies using KCC2 transgenic mice lacking the NRSE of KCC2 gene ($NRSE^{KCC2}$) showed that $NRSE^{KCC2}$ is dispensable for the restricted neuronal expression profile of KCC2 and that a 1.4 kb promoter region upstream of the NRSE-containing intron 1 was sufficient to drive the neuron-specific expression pattern of KCC2 (Uvarov et al., 2005). In addition, it has been shown that KCC2 gene contains multiple transcription start sites and that 10 candidate transcription factor binding sites are highly conserved amongst mammalian KCC2 genes (Uvarov et al., 2005 and 2006). One of these candidate transcription factors, early growth response 4 (Egr4), has been shown to be a key regulator for KCC2 expression during development (Uvarov et al., 2006). In hippocampal and cerebellar neurons, Egr4 protein was found to display similar temporal expression profile to KCC2 during postnatal development (Uvarov et al., 2006). Over-expression of Egr4 strongly induced the expression of KCC2 luciferase reporter constructs containing Egr4 binding site in both neuroblastoma N2a cells, which do not express KCC2 endogenously, and in primary hippocampal cultures (Uvarov et al., 2006). Consistently, RNA interference (RNAi) knock-down of Egr4 and a dominant-negative mutant of Egr4 not only inhibited Egr4-

mediated KCC2 reporter induction in N2a cells but also suppressed endogenous KCC2 expression in cultured hippocampal neurons (Uvarov et al., 2006). These findings together suggest Egr4 plays a crucial role in the developmental up-regulation of the KCC2 gene in cultured hippocampal neurons.

B. Regulation of KCC2 expression by GABA

It is well documented that the expression and function of KCC2 are crucial for the developmental transformation of GABAergic neurotransmission from excitatory to inhibitory (Rivera et al., 1999; Ganguly et al., 2001; DeFazio et al., 2000). However, how GABAergic neurotransmission affects KCC2 gene expression during development remains controversial. Ganguly *et al.* (2001) suggested that in primary mouse hippocampal neurons, GABAergic activity controls the developmental switch of GABAergic transmission from depolarizing to hyperpolarizing by modulating the mRNA levels of KCC2, whose expression is conversely tightly correlated with the switch. Chronic blockade of $GABA_A$ receptors, but not glutamate receptors or overall neuronal activity, not only delayed the switch but also decreased the mRNA levels of KCC2. Therefore, it was proposed in this study that GABA may act as a self-limiting trophic factor to control the polarity of GABAergic neurotransmission during development. By contrast, a study performed by Ludwig et al. (2003), showed that initial KCC2 protein expression was observed before the formation of the first synapse in primary mouse hippocampal neurons. In addition, in both primary hippocampal cultures and organotypic hippocampal slices, GABAergic transmission was not required for the developmental up-regulation of KCC2 during neuronal maturation, nor was glutamatergic transmission or the overall neuronal activity.

C. Regulation of KCC2 expression by BDNF

It is well established that BDNF plays a crucial role in regulating a number of cellular events during early development of the CNS, such as neuronal survival, neuronal precursor cell differentiation, neurite outgrowth, synaptogenesis, and maturation of neuronal function (Huang and Reichardt, 2001). BDNF can bind to at least two receptors at the cell surface. One is the high affinity receptor, tyrosine receptor kinase B (TrkB),

and the other one is the low affinity receptor named p75 neurotrophin receptor (p75NTR) (Rodriguez-Tebar et al., 1990). Using BDNF-overexpressing transgenic mice, it has been shown that BDNF accelerates the formation of GABAergic synapses via enhancing the expression of KCC2, without altering the developmental expression profile of $GABA_A$ receptors (Aguado et al., 2003). In agreement with this, in hippocamal pyramidal neurons from TrkB knockout juvenile mice (P10), the mRNA level of KCC2 is reduced compared to their wild-type littermates, and this is accompanied by a defect in GABAergic synaptogenesis (Carmona et al., 2006).

In contrast to the promoting effect of BDNF on KCC2 expression during early development, in the adult brain BDNF down-regulates the levels of KCC2 mRNA and protein. Exposure of rat organotypic and acute hippocampal slices to exogenous BDNF results in a TrkB-dependent reduction of KCC2 mRNA and protein and consequent impairment of KCC2 Cl^- extrusion activity (Rivera et al., 2002). Epileptic-like activity in rat hippocampal slices causes release of endogenous BDNF, which down-regulates KCC2 mRNA and protein levels and disrupts Cl^- extrusion capacity in a TrkB-dependent manner (Rivera et al., 2004). This effect involves the TrkB downstream signalling pathways mediated by Shc/FRS-2 (src homology 2 domain containing transforming protein/fibroblast growth factor receptor substrate 2) and the PLCγ (phospholipase C γ)-CREB (cAMP response element-binding protein) cascade (Rivera et al., 2004).

These above findings demonstrate that depending on the developmental stages of the brain BDNF has opposing effects on the expression levels of KCC2. Intriguingly, the action of BDNF always requires the presence of TrkB no matter whether BDNF positively or negatively regulate KCC2 expression. One possibility is that different signalling cascades downstream of TrkB may be activated at different developmental stages. In fact, there has been research giving a hint for the molecular mechanisms that may support this hypothesis. Using transgenic mice in which the Shc/FRS-2 or PLCγ signalling pathway was selectively blocked ($TrkB^{SHC/SHC}$ and $TrkB^{PLC/PLC}$), it was shown that both signalling pathways were required for BDNF-TrkB mediated down-regulation of KCC2 (Rivera et al., 2004). Most interestingly, in the absence of PLCγ-mediated signalling pathway, BDNF not only failed to down-regulate KCC2 but also enhanced its protein expression level. These observations suggest that PLCγ-mediated signalling

pathway may be initially silent at early developmental stage and later "switched on" around the time point when KCC2 expression starts increasing. In other words, the switch from Shc pathway acting alone to Shc and PLCγ pathways acting jointly may be a mechanism underlying the developmental up-regulation of KCC2 and the concomitant hyperpolarizing shift in GABA and glycine mediated responses. Dissecting the precise roles for each of these two signalling pathways during neuronal maturation will potentially reveal novel molecular mechanisms mediating the developmental transformation of GABAergic and glycinergic neurotransmission.

D. Regulation of KCC2 expression by epileptic activity, neuronal trauma, and pain

Down-regulation of KCC2-mediated Cl^- extrusion capacity and consequently the disruption of Cl^- homeostasis have been regarded as one of the mechanisms contributing to loss of inhibition mediated by $GABA_AR$ and GlyR. Down-regulation of KCC2, and consequently disinhibition of GABAergic responses and the aberrant increase of neuronal excitability, has been observed in models for neuropathological conditions such as epileptic activities, neuronal trauma and neuropathic pain. Therefore, it was proposed that down-regulation of KCC2 might be an early response to various kinds of neuronal trauma (Rivera et al., 2004).

1. Regulation of KCC2 expression by epileptic activity

A number of studies have shown epileptic activity down-regulated KCC2 expression and consequently impairs Cl^- extrusion capacity. For instance, sustained epileptic activity in acute hippocampal slices induced by extracellular Mg^{2+} depletion (a regime producing spontaneous activity), down-regulates KCC2 mRNA and protein expression in CA1 pyramidal neurons, leading to a reduced capacity for neuronal Cl^- extrusion (Rivera et al., 2004). In rat hippocampal dentate granule cells, status epilepticus (STEP), a pathophysiological condition where the brain is in a state of persistent seizures, induced by the convulsant pilocarpine (a muscarinic alkaloid that acts as a muscarinic receptor agonist in the parasympathetic nervous system), led to a fall of KCC2 protein level and a concomitant positive shift in E_{GABA} lasting for weeks (Pathak et al., 2007). In

subicular pyramidal neurons of patients with temporal lobe epilepsy associated with hippocampal sclerosis, KCC2 mRNA and protein were absent from a subpopulation of these neurons (Huberfeld et al., 2007). These neurons exhibit depolarizing GABAergic neurotransmission, in contrast to the KCC2 positive neurons, which display hyperpolarizing GABAergic activity (Huberfeld et al., 2007). These findings suggest that down-regulation of KCC2 expression by epileptic activity is likely one of the mechanisms underlying the disinhibition of GABA-mediated responses.

2. Regulation of KCC2 expression by neuronal trauma

A line of research has shown KCC2 is down-regulated after neuronal trauma generated by various experimental models, which leads to an accumulation of $[Cl^-]_i$, a shift in GABAergic or glycinergic responses towards a depolarizing level, and consequently disinhibition (Coull et al., 2003; Nabekura et al., 2002; Jin et al., 2005; Bonislawski et al., 2007). For instance, peripheral nerve injury has been shown to lead to a trans-synaptic reduction in the expression of KCC2 in lamina 1 neurons of the superficial dorsal horn, impaired Cl^- extrusion capacity, and consequently excitatory GABAergic and glycinergic responses (Coull et al., 2003). In a rat facial-nerve-transection model, axotomy in facial motoneurons caused reduction of KCC2 mRNA, which was accompanied by an elevation of the resting $[Ca^{2+}]_i$, induction of $GABA_A$ and NMDA receptor-mediated Ca^{2+} oscillations, as well as the transformation of GABAergic neurotransmission from hyperpolarizing back to depolarizing (Toyoda et al., 2002). Neuronal cultures isolated from the dorsal motoneurons of the vagus after *in vivo* axotomy showed significant reduction of KCC2 mRNA (Nabekura et al., 2002). KCC2 mRNA and protein were down-regulated in layer V pyramidal neurons of chronically injured neocortex (Jin et al., 2005). When traumatic brain injury (TBI) was generated by fluid percussion injury (FPI) in mice, there was a significant reduction of KCC2 mRNA and protein in dentate gyrus, which led to elevation of $[Cl^-]_i$ and depolarizing GABAergic responses (Bonislawski et al., 2007). All these findings suggest that down-regulation of KCC2 may be a common mechanism underlying the disinhibition of GABAergic or glycinergic responses upon generation of neuronal trauma in different neuronal cell types.

3. Regulation of KCC2 expression by neuropathic and inflammatory pain

It is regarded that a loss of inhibition in the primary sensory and dorsal horn neurons of the spinal cord is an important mechanism underlying pain (Woolf et al., 2000). Superficial dorsal horn is one of the main spinal nociceptive output pathways. In a neuropathic pain model, peripheral nerve injury was induced in rat lamina I neurons of the superficial dorsal horn by chronically constricting the sciatic nerve led to down-regulation of KCC2 protein level, impaired Cl^- extrusion capacity, and depolarizing switch of GABAergic and glycinergic responses (Coull et al., 2003). KCC2 has also been shown to be regulated by inflammatory pain. Peripheral inflammation induced by complete Freund's adjuvant injection in rat spinal cord caused a significant reduction of KCC2 expression but had no effect on NKCC1 (Zhang et al., 2008). In addition, the reduction of KCC2 protein expression was most profound two weeks after the peak of hyperalgesia, suggesting a role for KCC2 in the maintenance of chronic pain (Zhang et al., 2008). These findings suggest that KCC2 expression can be regulated by both neuropathic and inflammatory pain.

E. Regulation of KCC2 expression by LTP

As discussed above, KCC2 expression is down-regulated by high levels of neuronal activity occurring under certain pathological conditions, such as epileptic activity, neuronal trauma, and neuropathic pains. Research in recent years has just begun to reveal the contribution of synaptic plasticity to KCC2 expression. In rat acute hippocampal slices, LTP expression induced by a theta-burst stimulation (TBS) protocol was observed to lead to a substantial reduction of KCC2 protein level specifically in the CA1 region in an NMDAR-dependent manner (Wang et al., 2006). In contrast, induction of long-term depression (LTD) had no effect on KCC2 protein level (Wang et al., 2006). In agreement with this, exposure of hippocampal slices to the anesthetic drug propofol, which has been found to exert its anesthetic effect in hippocampal neurons by enhancing GABA-mediated inhibitory responses (Hara et al., 1993 and 1994; Patton et al., 2001; Wang et al., 2006), not only impaired LTP expression but also blocked LTP-induced down-regulation of KCC2 protein expression level (Wang et al., 2006). These findings together suggest that LTP negatively regulate the expression of KCC2 and that KCC2

may be involved in mediating LTP expression. The latter study also suggests maintenance of KCC2 expression during LTP induction may be a common mechanism by which anesthetics exert their antinociceptive effect.

F. Regulation of KCC2 expression by sex hormones

The substantia nigra pars reticulata (SNR) has been shown to be involved in the control of seizures (Iadarola and Gale, 1982) and movement (DiChiara et al., 1977; De Sarro et al., 1984). A line of research has suggested that SNR is sexually biphormic, in regards to the expression profile of hormone receptors (Ravizza et al., 2002), dopamine transporter mRNA levels (Rivest et al., 1995), GABA turnover and content, and $GABA_AR$ composition (Manev and Pericic, 1987; Flugge et al., 1986; Canonaco et al., 1996; Facciolo et al., 2000) between females and males. Of particular relevance, females and males display different responses to $GABA_A$ receptor agonists, such as muscimol, in SNR-mediated seizure control (Veliskova and Moshe, 2001).

Interestingly, there is evidence demonstrating that KCC2 mRNA levels also follow a sexually bimorphic feature in SNR neurons. Male P15 rat SNR neurons have less KCC2 mRNA and respond to muscimol with depolarization compared to SNR neurons from the female rats of the same age (Galanopoulou et al., 2003). The depolarizing response evoked by muscimol in male SNR neurons raised $[Ca^{2+}]_i$, but not in females (Galanopoulou et al., 2003)**.** The levels of KCC2 mRNA can be differentially modulated by sex hormones in P15 rat SNR neurons. That is, while testosterone and dihydrotestosterone up-regulate KCC2 mRNA in both sexes, estradiol down-regulate KCC2 mRNA only in males but not in females (Galanopoulou and Moshe, 2003). Moreover, in male P15 SNR, the antagonists of L-type voltage-sensitive calcium channels or $GABA_AR$ (nifedipine and bicuculline, respectively) not only decrease KCC2 mRNA but also prevent the down-regulation of KCC2 mRNA by estradiol, suggesting a functional interaction of sex hormones and GABAergic signalling in controlling the transcription of KCC2 in SNR neurons. These studies together have suggested an interesting regulatory role for sex hormones in the regulation of KCC2 expression.

IV. Post-translation regulation of KCC2 by kinase and phosphatase activities

It is well established that up-regulation of KCC2 mRNA and protein levels during development is essential for the transformation of GABAergic and glycinergic neurotransmission from excitatory to inhibitory in the forebrain regions, the hippocampus and neocortex, through an increased Cl^- extrusion capacity and consequently decreased $[Cl^-]_i$ (Rivera et al., 1999; Ganguly et al., 2001; DeFazio et al., 2000). Although a body of evidence has shown that various factors, such as BDNF, neuronal activity, epileptic activity, neuronal trauma, and neuropathic pain (section **III**) can modulate Cl^- extrusion capacity by altering KCC2 mRNA and protein levels, increasing studies also suggest that post-translational mechanisms can regulate KCC2 function, such as phosphorylation/dephosphorylation and membrane trafficking. In this section I will in particular discuss the roles of kinase/phosphatase activities in the functional expression of KCC2.

A. Short-term regulation of KCC2 transport activity by kinase and phosphatase activities

Coordination of the Cl^- entry and exit pathways mediated by the two branches of the CCC family proteins, NCC/NKCCs and KCCs, is essential for the precise control of Cl^- homeostasis within cells. Extensive studies have been carried out in an attempt to understand the molecular mechanisms underlying the coordinated transport of Cl^- across the cell membrane. It is now known that the activity of CCCs can be regulated by kinase and phosphatase activities. In general, dephosphorylation activates KCCs and inhibits NKCCs, whereas phosphorylation has the opposite effect (Adragna et al., 2006; Flatman, 2002). Compared to the other CCC family proteins, far less is known about the roles of phosphorylation/dephosphorylation in the regulation of KCC2 activity. Recent studies have just begun to provide insight into this question and shown that KCC2 function can be rapidly regulated by intracellular phosphorylation/dephosphorylation cascades, independent of changes in the levels of KCC2 gene expression.

In primary hippocampal cultures, treatment with the pan kinase inhibitor, staurosporine, led to robust activation of KCC2 activity within 5 minutes (Khirug et al., 2005), strongly suggesting cellular phosphorylation events were occurring to alter the

function of KCC2. A big question was which kinases or phosphatases are specifically involved in the regulation of KCC2 function. This question has been addressed by a number of other studies using different biological systems, which show that tyrosine kinases/phosphatases, protein kinase C (PKC)**,** creatine kinase, and with no lysine (K) (WNK) kinases are involved in the regulation of KCC2 activity.

1. Regulation of KCC2 by tyrosine kinase and phosphatase activities

It was observed that in cultured immature hippocampal neurons, which initially expressed an inactive form of KCC2, KCC2 was activated by the co-activation of two protein tyrosine kinases (PTK), insulin-like growth factor 1 (IGF-1) receptor and c-Src (Kelsch et al., 2001). In mature neurons, which initially expressed an active form of KCC2, KCC2 was inactivated by treatment with membrane permeable tyrosine kinase inhibitors lavendustin A and genistein (Kelsch et al., 2001), suggesting that the activity of endogenous tyrosine kinases may be involved in regulating KCC2 function. In acute rat hippocampal slices, activation of TrkB, by Mg^{2+} depletion (0 Mg^{2+})-induced epileptic activity shortened the half life of KCC2 at the plasma membrane from 20 to 10 minutes. Longer period (1- 3 hours) of 0 Mg^{2+} exposure led to a reduction of KCC2 mRNA and protein levels, and consequently impairment of Cl^- extrusion capacity (Rivera et al., 2004). It was unknown whether the effects of IGF-1, c-Src, or 0 Mg^{2+}-induced epileptic activity on KCC2 expression or activity were correlated to changes in the phosphorylation state of KCC2.

Direct evidence for the regulation of the tyrosine phosphorylation state of KCC2 emerged from studies using various neuronal stressors. Exposure of neurons to H_2O_2 has been used as a model of oxidative stress and neuronal damange (Whittemore et al., 1995). Oxidative stress produces free radicals and peroxides, consequently causing cell death and tissue injury. Oxidative stress is involved in several neurodegenerative diseases, such as Parkinson's disease, Alzheimer's diseases, and Amyotrophic Lateral Sclerosis. In primary hippocampal cultures, oxidative stress induced by H_2O_2 and epileptic activities induced by 0 Mg^{2+} or BDNF treatment all rapidly decreased the tyrosine phosphorylation of KCC2, which occurred before the reduction of the overall levels of KCC2 mRNA and protein (Wake et al., 2007). The reduction of tyrosine phosphorylated KCC2 by these

treatments was accompanied by an impairment of Cl^- extrusion capacity (Wake et al., 2007). In addition, tyrosine phosphatase inhibitors, sodium orthovanadate (Na_3VO_4), phenylarsine oxide (PAO) and sodium molybdate (Na_2MoO_4), not only increased the basal levels of tyrosine phosphorylated KCC2, but also prevented the loss of tyrosine phosphorylated KCC2 induced by H_2O_2 (Wake et al., 2007). H_2O_2 also rapidly reduced the amount of KCC2 at the cell surface, a change that was also reversed by Na_3VO_4 (Wake et al., 2007). All these observations suggest the tyrosine phosphorylation state of KCC2 seems to play a more important role than its mRNA or protein levels in the early changes of KCC2 activity under the conditions characterized. Although no direct evidence for the correlation between KCC2 tyrosine phosphorylation state and cell surface expression level was provided in this study, it strongly suggests a positive regulatory role for the former in the control of the latter.

2. Regulation of KCC2 by PKC

Neuronal trauma, such as that induced by nerve injury, has been shown to result in an elevation of $[Ca^{2+}]_i$, down-regulation of KCC2 expression and function, and a switch of GABAergic transmission from hyperpolarizing to depolarizing (Nabekura et al., 2002; Toyoda et al., 2003). Modification of neuronal activity by coincident pre- and post-synaptic spiking caused a similar outcome via a Ca^{2+}-dependent mechanism (Woodin et al., 2003). Repetitive postsynaptic spiking activity led to a rapid and sustained down-regulation of KCC2 activity also via a Ca^{2+}-dependent manner, which was abolished by a pan kinase inhibitor, staurosporine (Fiumelli et al., 2005). Although elevation of $[Ca^{2+}]_i$ is known to be able to activate a number of Ca^{2+}-dependent kinases and phosphatases in neurons, such as protein kinase C (PKC), protein kinase A (PKA), Ca^{2+}/calmodulin kinase (CaMK), or phosphatase calcineurin, this effect seemed to specifically require the activity of PKC, because inhibiting the activity of PKA, CaMK, and calcineurin had no effect (Fiumelli et al., 2005). A study performed by Lee et al. (2007) has well described a mechanism underlying the regulation of KCC2 by PKC. It was shown in this study that serine 940 (Ser^{940}) within the C-terminus of KCC2 is the major direct target for PKC-mediated phosphorylation. Phosphorylation of Ser^{940} increased the cell surface stability of KCC2 via slowing down its internalization from the plasma membrane, leading to

enhanced transport activity of KCC2 (Lee et al., 2007). The contrasting effect of PKC on KCC2 transport activity from these two studies suggests that alternative regulatory mechanisms, such as whether KCC2 is the direct target for PKC-dependent phosphorylation, may be involved under different conditions.

3. Regulation of KCC2 activity by creatine kinase

Using yeast two-hybrid screening, Inoue et al. (2004) identified the brain-type creatine kinase (CKB), which is an ATP-generating enzyme and highly expressed in neuronal cells, as a binding partner of KCC2. Further studies show that the transport activity of KCC2 in primary cortical neurons can be significantly disrupted by inhibiting CKB activity, highlighting the functional significance of CKB in regulating the function of KCC2 in neurons (Inoue et al., 2005). These findings suggest the activity of endogenous CKB is necessary for the function of KCC2 in cortical neurons. However, it remains elusive whether the effect of CKB on KCC2 transport activity requires the binding of CKB to KCC2 and whether the phosphorylation state of KCC2 can be altered by CKB.

4. Regulation of KCC2 by WNK kinases

As discussed above, one major physiological function of CCC family member proteins is to maintain cell volume via controlling Cl^- homeostasis. Therefore, the activity of these cotransporters is sensitive to factors that can cause changes in the cell volume. KCCs are activated by cell swelling and inhibited by cell shrinkage, whereas NKCCs and NCC are activated by cell shrinkage and inhibited by cell swelling (Lang et al., 1998). A number of studies have been performed in an attempt to identify the molecular mechanisms underlying the cell osmolarity/volume control of CCC activity. Most of the work was done in erythrocytes, kidney cells, or *Xenopus* oocytes. Several kinases that are involved in the cell volume maintenance function of CCCs have been identified, such as myosin light-chain kinase (MLCK) (Klein et al., 1995; Di Ciano-Oliveira et al., 2005), Jun N-terminal kinase (JNK) (Klein et al., 1999), Rho kinase (Di Ciano-Oliveira et al., 2003), and with no lysine (K) (WNK) kinases (Kahle et al., 2006).

Out of the above kinases, the WNK family kinases are particularly involved in the regulation of KCC2 activity. WNK is a family of serine/threonine kinases lacking a conserved lysine residue in the catalytic domain (Veríssimo and Jordan, 2001). This family of kinases is composed of four member proteins, WNK1-WNK4. They play an important role in controlling electrolyte homeostasis through regulation of the activity of a number of ion channels, and in particular the CCC family member proteins (Kahle et al., 2006). For instance, WNK1 and WNK4 inhibit NCC activity by reducing its cell surface expression, whereas WNK3 enhances NCC activity (Yang et al., 2003; Rinehart et al., 2005). WNK3 has the opposite effect on the activity of NKCC1/2 and KCCs in heterologous *Xenopus* oocytes. That is, WNK3 activates NKCC1/2 by increasing the phosphorylation of two threonine residues necessary for their transport activities (Kahle et al., 2005; Rinehart et al., 2005). In contrast, WNK3 inhibits the Cl^- extrusion capacity of KCC1/2 (Kahle et al., 2005). Interestingly, it was observed that WNK3 is co-expressed with NKCC1 and KCC2 in $GABA_AR$ expressing neurons of some brain regions, such as hippocampus, cerebellum, and cerebral cortex (Kahle et al., 2005). Moreover, in hippocampal and cerebellar neurons, WNK3 mRNA shows extremely similar temporal expression pattern to that of KCC2 during development (Kahle et al., 2005). Therefore, WNK3 may reciprocally regulate the Cl^- entry and exit pathways by controlling the transport activity of NKCC1 and KCC2 respectively in neurons, thereby controlling the polarity of GABAergic neurotransmission during neuronal maturation and under certain neuropathological conditions where loss of GABAergic inhibition occurs.

Similar to WNK3, WNK4 has also been shown to be a negative regulator of KCC transport activity in heterologous *Xenopus* oocytes. WNK4 inhibits cell swelling-induced activation of KCC1, KCC3, and KCC4 (Garzón-Muvdi et al., 2007). Consistently, a catalytically inactive mutant of WNK4 (WNK4-D318A) fails to inhibit the Cl^- transport activity of KCC4 (Garzón-Muvdi et al., 2007). Moreover, in the presence of WNK4-D318A, the Cl^- transport activity of KCC2 and KCC3 is massively enhanced under isotonic conditions (Garzón-Muvdi et al., 2007), suggesting that WNK4 may intrinsically suppress the activity of KCC2 and KCC3 under isotonic conditions. This effect is enhanced by co-expression of a catalytically inactive mutant of Ste20-related proline-alanine-rich kinase (SPAK), which is a binding partner for WNK4 and has been shown to

be a positive regulator for NKCC1 activity (Dowd and Forbush 2003; Piechotta et al., 2003; Gagnon et al., 2006; Garzón-Muvdi et al., 2007). In agreement with this, co-expression of WNK4 and SPAK inhibits KCC2 activity under both isotonic and hypotonic conditions in heterologous *Xenopus* oocytes (Gagnon et al., 2006). Together, these findings suggest that the interaction of SPAK with WNK may ensure the maximal inhibition of KCC2 activity *in vivo*.

5. Regulation of KCC2 by PP1/PP2A

In regards to cell volume-mediated activation of CCC family member proteins, KCC2 is unique in that it can constitutively cotransport K^+/Cl^- under isotonic conditions (Payne, 1997; Strange et al., 2000). A 15 amino acids fragment within the C-terminus of KCC2 has been identified to be both necessary and sufficient for KCC2 constitutive K^+/Cl^- cotransport activity (Mercado et al., 2006). Interestingly, while swelling-induced activity of KCC2 can be abolished by calyculin A, a potent inhibitor of the serine/threonine protein phosphatases PP1 and PP2A, the constitutive transport activity of KCC2 is completely insensitive to calyculin A treatment, suggesting that these two scenarios of K^+/Cl^- cotransport are mediated by different mechanisms (Mercado et al., 2006).

Together, it is evident that cellular phosphorylation/dephosphorylation events play a central role in the short-term regulation of KCC2 transport activity. The coordination of these signalling cascades will ensure that KCC2 responds to different physiological/neuropathological conditions rapidly and precisely, which may serve as an important mechanism controlling the synaptic plasticity of GABA- and glycine-mediated inhibitory neurotransmission in the CNS.

B. Regulation of KCC2 phosphorylation during brain development

In contrast to the large number of reports on the roles of kinase/phosphatase activities in the short-term regulation of KCC2 expression and function discussed above, little is known about the contributions of kinase/phosphatase activities to the functional expression of KCC2 during development. It was observed in lysates obtained from the

cortex of P3-30 mice that the increase of tyrosine-phosphorylated KCC2 during development seemed to be proportional to the increase of the total KCC2 protein expression (Stein et al., 2004). Moreover, tyrosine phosphorylated KCC2 was already present before the switch of GABAergic transmission occurred (Stein et al., 2004). Therefore, it was suggested that the tyrosine-phosphorylation state of KCC2 was less important than the transcriptional up-regulation of KCC2 in the maturation of GABAergic transmission (Stein et al., 2004). In contrast, studies on the cochlear nucleus suggest that neither total KCC2 protein expression nor its plasma membrane insertion appears to contribute to the functional activation of KCC2. Rather, the phosphorylation state of KCC2 seems to be tightly associated with the maturation of inhibitory neurotransmission in these neurons (Vale et al., 2005). These studies suggest the phosphorylation state of KCC2 may play distinct roles in different regions of the CNS or even different sub-types of neurons within the same region, during the maturation of fast inhibitory neurotransmission mediated by GABA and glycine.

V. Post-translational regulation of KCC2 via endocytic trafficking and oligomerization

Several other post-translational mechanisms, besides phospho-modifications, have been shown to regulate KCC2 activity and cellular localization, such as endocytic trafficking and oligomerization.

A. Clathrin-mediated endocytosis

Endocytosis is a process by which cells uptake extracellular substances. It confers the cells' capability of maintaining intracellular homeostasis, taking up nutrients, transducing a variety of extracellular signals, and protecting from infectious material. There are three classical endocytic pathways: macropinocytosis, caveolar endocytosis, and clathrin-mediated endocytosis.

Macropinocytosis is a means by which cells uptake extracellular fluid by invaginating of the cell membrane. Caveolae are flask-shape pits in the membrane that consist of the protein caveolin-1 and are enriched in cholesterol and glycolipids. Caveole-dependent endocytosis mediates that internalization of viruses,

glycosylphosphatidylinositol (GPI)-anchored proteins, endothelin, and growth hormone, etc (Nichols and Lippincott-Schwartz, 2001; Pelkmans and Helenius, 2002; Duncan et al., 2002). Clathrin-mediated endocytosis (CME) is by far the best understood endocytic pathway. It is the predominant endocytic pathway for the selective uptake of plasma membrane proteins or phospholipids in eukaryotic cells. CME is sub-categorized into two forms: constitutive endocytosis and regulated endocytosis. The former occurs under resting conditions, whereas the latter requires a trigger by specific signals.

CME is achieved by the formation of clathrin-coated vesicles (CCVs). The components of CCVs not only serve to structurally shape the transport machinery but also carry the sorting signals by selectively packaging the plasma membrane proteins or phospholipids to be internalized (cargos). The major structural components of CCVs include the coat protein clathrin, the adaptor protein 2 (AP-2) complex, as well as a variety of accessory proteins facilitating the formation and budding processes.

The formation of CCVs is initiated by the recruitment of the AP-2 complex to the docking site on the plasma membrane via binding to phosphatidylinositol 4, 5-bisphosphate (PIP2). Once associated with the plasma membrane, AP-2 on the one hand interacts with the cytoplasmic sorting signals in the cargo molecules; on the other hand, AP-2 recruits clathrin to the plasma membrane and stimulates its assembly. The assembly unit of clathrin, the triskelion, is composed of three heavy chains and three light chains. These triskelions form lattices of hexagons and pentagons around AP-2, forming the outer layer of the CCVs and leading to a curvature structure that invaginates the plasma membrane called clathrin-coated pit (CCP). The scission of the CCPs is achieved by the action of motor GTPase, dynamin (Pearse, 1987; Schmid et al. 1998). After endocytosis, cargo molecules pass through a series of endosomal compartments. They are first delivered to the early endosomes and sorting endosomes, where the decision for the destination of the endocytosed molecules is to be made. They subsequently are either sorted to the recycling pathway, through recycling endosomes, to get back to the cell surface, or destined to the late endosomes/lysosomes for degradation. The AP-2 complex contains the recognization sites for a variety of endocytic/sorting motifs present within the cytosolic portions of the cargo molecules, such as the tyrosine-based YxxΦ (where x represents any amino acid and Φ is a bulky hydrophobic amino acid residue) and di-

leucine based motifs (Bonifacino and Traub, 2003). Therefore, AP-2 is a key component of the CME machinery determining the specificity of the cargo molecules at the initial stage of endocytosis, as well as in some cases their sorting pathways following endocytosis.

B. Regulation of KCC2 via endocytic trafficking

In the field of neuroscience, there is a large body of evidence suggesting endocytic trafficking is an important mechanism by which the function of many proteins that regulate neurotransmission, such as $GABA_AR$, can be modulated (Esteban, 2003; Michels and Moss, 2007). Importantly, the endocytic trafficking dynamics of these ion channels in addition enable the efficacy of neurotransmission to be modulated in a rapid manner in response to various environmental conditions, independent of changes in gene and protein expression, events that occur on much longer time scales. Therefore, endocytic trafficking of these ion channels is recognized as an important mechanism by which synaptic plasticity can be controlled. As mentioned above, KCC2 plays an essential role in regulating the polarities of GABAergic and glycinergic neurotransmission, via mediating neuronal Cl^- homeostasis. The endocytic trafficking of KCC2, which can alter its cell surface expression level and hence Cl^- transport capacity, may therefore indirectly influence the properties of GABA- or glycine-mediated responses and inhibitory synaptic plasticity. However, the properties and molecular mechanisms controlling KCC2 endocytic trafficking have only begun to be elucidated.

In auditory brainstem lateral superior olive (LSO) neurons the level of KCC2 protein was found to be constant throughout the period of glycinergic neurotransmission transformation from depolarizing to hyperpolarizing, which is contrary to that observed in forebrain regions (Balakrishnan et al., 2003). However, there was an increase of cell surface insertion of KCC2 during this period (Balakrishnan et al., 2003). Since expression of the Cl^- accumulator NKCC1 in the LSO neurons was not detected during early development, when glycinergic transmission was depolarizing, it was suggested that up-regulation of KCC2 plasma membrane insertion might contribute to the maturation of glycinergic neurotransmission in these neurons. Similarly, intracellular translocation of KCC2 from the cytosol to the plasma membrane was also observed in ganglion cells of

ferret retina during the period when the transition of GABAergic neurotransmission from depolarizing to hyperpolarizing occurs (Zhang et al., 2006). This, together with down-regulation of the NKCC1 protein, is proposed to be responsible for the maturation of GABAergic transmission in these neurons (Zhang et al., 2006).

Although above studies suggest a role for KCC2 trafficking in the maturation of GABAergic and glycinergic inhibitory neurotransmission during development, the mechanisms behind remain elusive. A study performed by Rivera et al. (2004) provides strong evidence for the cellular mechanisms contributing to the regulation of KCC2 membrane trafficking by neuronal activity. In this study, it was observed that in acute rat hippocampal slices KCC2 displayed a high rate of turnover between the neuronal plasma membrane and intracellular pools. The endocytosis of KCC2 was significantly accelerated by epileptic-like activity induced by 0 Mg^{2+}, and this effect was dependent on the activity of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, a subtype of the glutamate receptors (Rivera et al., 2004). This study suggests a novel mechanism by which the synaptic plasticity of GABA-mediated inhibitory neurotransmission can be controlled, in brief, by altering the rate of endocytosis of the functionally linked K^+/Cl^- cotransporter KCC2. A similar mechanism may also apply to the rapid shift of the polarity of GABAergic and glycinergic responses under other conditions, such as neuronal spiking (Woodin et al., 2003; Fiumelli et al., 2005) and changes in cellular kinase/phosphatase activities (Kelsch et al., 2001; Khirug et al., 2005; Wake et al., 2007; Lee et al., 2007). In fact, the activity of endogenous phosphatases and PKC has been suggested to regulate KCC2 membrane trafficking via altering the phosphorylation state of KCC2 itself (Wake et al., 2007; Lee et al., 2007).

Understanding the mechanisms controlling the membrane trafficking of KCC2 will undoubtedly reveal novel pathways whereby inhibitory synaptic plasticity can be modulated.

C. Regulation of KCC2 via oligomerization

Topological analysis suggests that all CCCs contain both intracellular N- and C-termini, with twelve transmembrane spanning segments in between (Gamba, 2005). In contrast to the well-understood primary structures of the CCCs, far less is known about

the quaternary structures of these proteins. In the past several years, a number of studies have been carried out in an attempt to better understand the quaternary state of CCCs as well as to map the regions involved in the regulation of their quaternary assembly.

Using various approaches, such as yeast two-hybrid, chemical cross-linking, GST-fusion protein pull-down, ultra-centrifugation, and co-immunoprecipitation, it has been shown CCCs exist as oligomers (including dimers) in cells. For instance, it has been shown the structural and functional unit of NCC at the plasma membrane is a homo-dimer when expressed in *Xenopus laevis* oocytes (de Jong et al., 2003). In rat parotid plasma membranes, the dominant structural unit of NKCC1 is a homo-dimer (Moore-Hoon et al., 2000). Similar preference for dimeric formation was also observed with the NKCC2 protein when expressed in *Xenopus laevis* oocytes (Starremans et al., 2003). Mapping studies have provided evidence that the N-terminus of NKCC1 is not required for its homo-dimerization (Moore-Hoon et al., 2000; Parvin et al., 2007), whereas the C-terminus plays a key role in this process (Simard et al., 2004; Parvin et al., 2007). In addition, two regions within the proximal and distal portions of the NKCC1 C-terminus, respectively, were suggested to be involved in regulating NKCC1 homo-dimerization (Simard et al., 2004).

For the K^+-coupled Cl^- cotransporter KCCs, the proposal that they form homo- or hetero-oligomers emerged from the observation that when expressed in the heterologous *Xenopus laevis* oocytes an N-terminus truncation of KCC1 displayed dominant negative inhibition for the transport activity of wild type KCC1 and KCC3 and, with lower potency, KCC4 and KCC2 (Casula et al., 2001). In the nervous system, a correlation between the oligomeric state and the functional expression of KCC2 was first proposed in the cochlear nucleus neurons (Vale et al., 2005). In these neurons, it was observed that the protein levels of KCC2 in the KCC2-inactive immature neurons were comparable to those in the KCC2-active mature neurons, and so was the cellular distribution pattern of KCC2 (Vale et al., 2005). It was there suggested in this study that changes in the quaternary assembly state of KCC2 might be one of the mechanisms underlying the activation of KCC2 during the maturation of cochlear nucleus neurons. Direct evidence supporting the existence of oligomeric KCC2 in naïve tissues came from the observations in rat brain stem lateral superior olive (LSO) neurons (Blaesse et al., 2006). It was observed that in

mature LSO neurons, in which KCC2 was active, KCC2 existed as a mixture of dimers, trimers, and tetramers. However, in immature neurons, in which KCC2 was inactive, monomeric KCC2 predominated, although the maturation state and the plasma membrane expression levels of KCC2 protein were both comparable to those observed in the mature neurons (Blaesse et al., 2006). Although no direct evidence was provided in this study regarding the contribution of the oligomeric state of KCC2 to its physiological function, the synchronicity between the up-regulation of the proportion of oligomeric KCC2 and the activation of KCC2 function suggested KCC2 oligomerization may be a novel mechanism regulating the maturation of GABAergic neurotransmission in brain stem LSO neurons.

In heterologous *Xenopus laevis* oocytes, it has been shown KCCs can form not only homo-dimers but also hetero-dimers between the different isoforms (Simard et al., 2007). Moreover, KCC4 can also form a hetero-dimer with the more distally related NKCC1 (Simard et al., 2007). Although the homo- and hetero-oligomeric formation of the endogenous proteins needs to be further characterized in naïve cells, this study potentially reveals a novel mechanism by which CCC member proteins control the coordination of the Cl^- exit and entry pathways via regulated physical interaction between different isoforms.

In general, oligomerization seems to be a common feature for the CCC member proteins. This is perhaps not surprising considering a large number of ion channels and transporters exist as oligomers, such as the Na^+/K^+ ATPase (Taniguchi et al., 2001), the H^+/K^+ ATPase (Sachs, 1995), the Na^+/H^+ exchanger (Fafournoux et al., 1994), the epithelial sodium channel (Canessa et al., 1994), the aquaporins (Agre et al., 1998), and the Na^+/Cl^--coupled neurotransmitter transporters (Sitte and Freissmuth, 2003).

So far, most of the research concerning the quaternary properties of the CCCs and the mapping studies resolving the dimerization regions have largely been performed on the Na^+-coupled Cl^- cotransporters, including NCC and NKCCs. In comparison, few studies have been carried out on KCC2. Specifically, what is the molecular determinant(s) regulating the oligomerization of KCC2 and what is the correlation between the oligomeric state of KCC2 and its Cl^- extrusion capacity remain elusive. Therefore, understanding these issues will potentially provide more insight into the mechanisms

controlling the properties of GABA- and glycine- mediated synaptic responses in the CNS both during development and in the adult.

VI. Summary

The electroneutral cation Cl^- cotransporter proteins compose a family of solute carriers that are essential for maintaining cellular homeostasis. The neuron-specific isoform of the K^+/Cl^- cotransporter, KCC2, is one of the major proteins maintaining neuronal $[Cl^-]_i$ homeostasis in adult CNS neurons. KCC2 plays an essential role in mediating the maturation of GABA and glycine dependent fast inhibitory neurotransmission during development, and controlling neuronal excitibility in adult brain via maintaining a low $[Cl^-]_i$. In addition to gene regulation, accumulating studies have suggested post-translational mechanisms, such as phosphorylation/dephosphorylation modifications, membrane trafficking, and oligomerization, may also play a crucial role in the control of KCC2 function. A number of factors, such as cellular kinase and phosphatase activities, neuronal activity, and oxdative stress, have been identified to regulate KCC2 phosphorylation state, cell surface expression, membrane trafficking, or transport activity. Together, the coordination of different regulatory mechanisms ensures the function of KCC2 is precisely controlled, whereby the efficacy of GABA and glycine mediated synaptic inhibition can be well maintained.

RESEARCH RATIONAL AND OBJECTIVES

Post-translational regulations of KCC2

Whereas KCC2 gene expression is essential for the control of KCC2 function, the post-translational mechanisms regulating KCC2 were unknown when I began my PhD studies. However, there was evidence showing that KCC2 transport activity in hippocampal neurons could be rapidly altered by insulin, c-Src and BDNF dependent signalling pathways and neuronal activity induced by coincident pre and postsynaptic spiking (Kelsch et al., 2001; Wardle and Poo, 2003; Woodin et al., 2003). The effects observed in these studies, those occurring in a manner of 10~20 minutes, strongly suggested that post-translational mechanisms were involved in the control of KCC2 function. As mechanisms controlling the cell surface expression of many transporters and their endocytosis have been shown to have a profound and dynamic effect on their transport activity (Melikian et al., 1999; Ortiz, 2006), we postulated that the number of KCC2 molecules at the cell surface might also be subject to modulation. In addition, as protein endocytosis requires an interaction with the endocytic machinery through specific endocytic motifs in the cargo proteins, and various motifs have been mapped to their intracellular termini, we hypothesized that endocytic motif(s) should exist in KCC2 if it was indeed mediated by the endocytic pathway.

Studies on the NCC and NKCC proteins showed that they all existed as dimers at the cell surface, suggesting that quaternary assembly might be a common feature for the CCC family member proteins (Brunet et al., 2005; de Jong et al., 2003; Moore-Hoon et al., 2000; Starremans et al., 2003). In addition, regions in the C-termini of NKCC1/2 have now been indicated to mediate their dimerization (Brunet et al., 2005; Simard et al., 2004; Parvin et al., 2007). However, the oligomeric state of KCC2 or any other KCC proteins had not been reported when I started with my research project. Therefore, we reasoned that similar to NCC and NKCCs dimerization might also be a post-translational mechanism regulating KCC2, and if so specific molecular regions should be present to mediate KCC2 dimerization. I therefore set out to test the above hypotheses, which would potentially deepen our insight into the molecular mechanisms governing KCC2 function

and hence the means by which GABA and glycine mediated inhibitory neurotransmission may be controlled.

As such, the principle objective of my work described in this thesis was to characterize the cellular and molecular mechanisms regulating KCC2 cell surface expression and identify the molecular motifs mediating KCC2 endocytosis and dimerization. The specific objectives of the research presented here were:

1) To characterize the cellular and molecular mechanisms underlying KCC2 constitutive endocytosis and identify the molecular motif(s) mediating this process (**Chapter 2**).

2) To characterize the dimeric state of KCC2 and identify the molecular region(s) mediating its dimerization (**Chapter 3**).

3) To examine the contribution of developmental stages, neurotrophic/growth factor dependent signalling pathways, and kinase/phosphatase activities to the cell surface expression of KCC2 in neurons (**Chapter 4**).

PREFACE TO CHAPTER 2

KCC2 is a neuron-specific potassium-chloride cotransporter which belongs to the cation chloride cotransporter superfamily. It is now well established that gene regulation is an important mechanism governing KCC2 expression and function. In comparison, far less is known about the post-translational mechanisms regulating KCC2. Endocytosis is an important mechanism by which the cell surface expression and transport activity of many transporters can be controlled (Melikian and Buckley, 1999; Ortiz, 2006). In addition, the interaction of certain molecular motifs in these proteins with the endocytic machinery has been shown to be required for their endocytosis (Al-Hasani et al., 2002; Holton et al., 2005; Schmidt et al., 2006). Therefore, we reasoned that endocytosis might also be a post-translational mechanism regulating KCC2 and if so, certain molecular motif(s) in KCC2 should be present to mediate this process.

The specific aims of the present study were to identify the cellular mechanisms and molecular motif controlling KCC2 constitutive endocytosis.

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Identification of a Novel Di-Leucine Motif Mediating K^+/Cl^- Cotransporter KCC2 Constitutive Endocytosis

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I. ABSTRACT

The neuron-specific potassium-chloride cotransporter 2 (KCC2) plays a crucial role, by controlling chloride extrusion, in the development and maintenance of inhibitory neurotransmission. Although it is now well established that activity-dependent mechanisms can down regulate KCC2 gene expression, the role of post-translational mechanisms in controlling KCC2 expression, specifically at the cell surface, are poorly understood. We therefore set out to identify the mechanisms and motifs regulating KCC2 endocytosis, one important pathway that may control KCC2 membrane expression. Using a fluorescence-based assay, we show KCC2 when expressed in HEK293 cells is constitutively internalized via a dynamin- and clathrin-dependent pathway. Consistent with this, we demonstrate KCC2 from adult mouse brain associates in vivo with the clathrin-binding adaptor protein-2 (AP-2) complex. Using an endocytosis reporter system, we identify the presence of an autonomous endocytosis motif in the carboxyl cytoplasmic terminus of KCC2. By site-directed mutagenesis we define this novel KCC2 endocytic motif as a non-canonical di-leucine motif, $_{657}$LLXXEE$_{662}$. Finally by mutating this motif in the context of full-length KCC2 we demonstrate that this novel KCC2 endocytic motif is essential for the constitutive internalization of KCC2 and for binding to the AP-2 complex. Subsequent sequence analysis reveals this motif is highly conserved between the closely related K^+/Cl^- family members that mediate chloride efflux, but absent from the more distant related cotransporters controlling chloride influx. In conclusion, our results indicate constitutive internalization of KCC2 is clathrin-mediated and dependent on the binding of AP-2 to this novel endocytic motif. Furthermore, this process appears to be an evolutionarily conserved mechanism amongst functionally homologous cotransporters.

II. INTRODUCTION

Fast inhibitory neurotransmission is mediated by two classes of ligand gated Cl^- channels, the γ-amino-butyric acid type-A receptor ($GABA_AR$) and the glycine receptor (GlyR) (Betz, 1992; Sieghart, 2006). In each case Cl^- influx is triggered upon channel opening resulting in hyperpolarization of the postsynaptic membrane. This in turn, leads to a reduction in the likelihood of further neurotransmitter release (Betz, 1992; Sieghart, 2006). In the majority of neurons, Cl^- influx and fast hyperpolarizing inhibition are critically dependent on low intracellular chloride concentrations ($[Cl^-]_i$). The neuron-specific K^+-Cl^- cotransporter, KCC2, has now been identified as an essential protein in establishing and maintaining this low $[Cl^-]_i$, by controlling Cl^- extrusion (Rivera et al., 1999).

During central nervous system (CNS) development, KCC2 gene expression is upregulated and underlies the transition of GABA and glycine responses from the immature depolarizing to the hyperpolarizing responses found in adults (Rivera et al., 1999). This transition occurs as the overall developmental increase in KCC2 activity leads to a reduction in $[Cl^-]_i$, which at resting membrane potentials promotes Cl^- influx (hyperpolarization) rather than efflux upon ion channel opening (Rivera et al., 1999). Further, in mature neurons a reduction in KCC2 gene expression, via antisense oligonucleotide suppression, leads to an increase in $[Cl^-]_i$ and a shift in $GABA_AR$ responses to immature depolarizing (Rivera et al., 1999). These observations have demonstrated that dynamic regulation of KCC2 gene expression can alter the direction of $GABA_AR$ and GlyR signaling. Consistent with this and its essential role in inhibition, KCC2 knockout mice die shortly after birth (Hubner CA, 2001). In addition, a loss of KCC2 expression is recognized as a contributing factor in the pathological conditions of chronic pain (Coull et al., 2003), nerve injury (Nabekura et al., 2002) and epilepsy (Woo et al., 2002; Huberfeld et al., 2007).

Besides the above-mentioned regulation of KCC2 gene expression, short-term modulation of the KCC2 protein has also been demonstrated. Several kinase activities can modulate KCC2 Cl^- transport activity in both immature (Inoue et al., 2006; Kahle et al., 2005; Kelsch et al., 2001; Khirug et al., 2005) and mature (Fiumelli et al., 2005)

hippocampal neurons. The precise molecular mechanisms involved however, have yet to be eluded. In addition a rapid loss of KCC2 cell surface expression has been demonstrated under conditions of increased interictal activity (Rivera et al., 2004) and oxidative stress (Wake et al., 2007), indicating that regulation of KCC2 membrane trafficking may be a crucial mechanism by which KCC2 function can be controlled.

KCC2 is a 12 transmembrane protein with both amino and carboxyl intracellular termini and belongs to the cation-chloride cotransporter (CCC) superfamily, which consists of one Na^+-Cl^- cotransporter (NCC), two Na^+-K^+-Cl^- cotransporters (NKCCs) and four K^+-Cl^- cotransporters (KCCs). Different CCCs exert opposite Cl^- transport activities, with NCC and NKCCs taking up Cl^-, whereas KCCs extrude Cl^- (Payne et al., 2003). The functional unit of CCCs is most likely to be a dimer, as homo- and hetero-dimerization have now been demonstrated for NKCC (Moore-Hoon and Turner, 2000), NCC (de Jong et al., 2003) and KCC family proteins (Blaesse et al., 2006). The molecular mechanisms regulating the membrane trafficking of any CCC family member however are presently unknown, albeit they play essential roles in controlling chloride homeostasis in multiple tissues.

The cellular mechanisms controlling the cell surface expression of many transporters and their membrane internalization, in particular, have been shown to have a profound and dynamic effect on overall transporter activity (Melikian and Buckley, 1999; Ortiz, 2006). As one requirement for protein internalization is an interaction with the cellular endocytic machinery, the identification of the molecular motifs governing these interactions has in several cases revealed pivotal regulatory domains within these proteins (Al-Hasani et al., 2002; Holton et al., 2005; Schmidt et al., 2006). How KCC2 membrane expression is controlled, specifically the cellular mechanism and the molecular motifs contributing to its membrane internalization are presently unknown. Therefore in the present study we set out to investigate the mechanisms controlling the membrane internalization of KCC2. Here we report our findings, using an array of endocytosis reporter systems and site-directed mutagenesis, of the cellular mechanisms and the molecular motif within KCC2 directing its constitutive internalization from the plasma membrane.

III. MATERIALS AND METHODS

Antibodies

The following antibodies were used, mouse monoclonal anti-HA (HA.11, Covance, Berkeley, CA, USA), rabbit polyclonal anti-KCC2 (Upstate, Lake Placid, NY, USA), mouse monoclonal anti-IL2 receptor alpha (Tac; kindly provided by Julie Donaldson, NIH, Bethesda, MD, USA), mouse monoclonal anti-α-adaptin clone 8 for immunoblotting (BD Biosciences, Mississauga, ON Canada) and anti-α-adaptin clone AP.6 for immunoprecipitation (Affinity BioReagents, Golden CO USA). Donkey anti-mouse and anti-rabbit antibodies conjugated to Alexa-488 and Alexa-546 were from Invitrogen (Carlsbad, CA, USA), while those conjugated to peroxide from Jackson Labs (Bar Harbor, Maine, USA).

Plasmids and constructs

The full length murine KCC2 cDNA was cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) from RNA of mouse forebrain, using the specific pimers forward 5´-ATAGGATCCGCCACCATGCTCAACAACCTGACGGAC-3´) and reverse (5´-TATTCTAGATCAGGAGTAGATGGTGATGACC-3´). A triple tandem copy of the influenza virus haemagglutin (HA) peptide (YPYDVPDYA) was inserted into the unique *NotI* restriction site in the second predicted extracellular loop of KCC2, as described for the NHE-3 transporter (D'Souza et al., 1998), to generate HA-KCC2. The LL657,658AA mutation was introduced by site-directed mutagenesis, using the Quick-exchange mutagenesis kit (Stratagene, La Jolla, CA, USA). The Tac backbone construct was kindly provided by Julie Donaldson (NIH). To generate the Tac chimeric deletion and mutated constructs we amplified parts of the carboxyl cytoplasmic tails of KCC2 by PCR, using the forward primer (5´-ATATCTAGACGGGGGGCGGAG-3´) and the reverse primers (see Table 2.1). These were then cloned in-frame into an *XbaI* site inserted at the 3' end of the Tac cDNA. Tac-C(LL657,658AA) and Tac-C(Y555A) were generated by site-directed mutagenesis from Tac-C. HA-tagged transferin receptor (HA-TfR, Belouzard and Rouillé, 2006) was kindly provided by Yves Rouille (Institut Pasteur de Lille, Lille Cedex, France) and the HA-TfR-KCC2 amino cytoplasmic tail chimaera

generated by overlapping PCR ligation so that KCC2 (amino acids 2-102) was fused to HA-TfR (amino acids 60-760). All sequences were confirmed by the McGill and Genome Quebec innovation center. The green fluorescent (GFP) dominant negative (DN) dynamin-2 K44A construct is from Marc McNiven (Mayo Clinic College of Medicine, Rochester, MA, USA), the GFP-DN-EPS15 (lacking amino acids 95-295) and the GFP-Rab11 construct were provided by Robert Lodge (NIH, Bethesda, MD, USA), the GFP-DN-Caveolin was provided by Matthew Mulvey (University of Utah, Salt Lake City, UT, USA) and the GlyR-α1 construct is from R. Harvey (School of Pharmacy, London UK).

Cell culture and Transfections

Human Embryonic Kidney 293 cells (HEK293) were maintained at 37 °C in DMEM (Invitrogen), 5% CO2 supplemented with 10% FBS, 100 U/ml penicillin/ streptomycin and 2 mM L-glutamine. Prior to transfection, cells were plated onto poly-L-lysine-coated plates or glass coverslips. Transfections were carried out with Lipofectamine 2000™ (Invitrogen). Cells were assayed 18-20 hours after transfection. For electrophysiological experiments, cells were transiently transfected with either wild-type or HA-tagged KCC2, GlyR-α1 and pEGFP-N (Clontech, Mountain View, CA, USA) at a ratio of 10:3:1, using the calcium phosphate method (Wong et al., 2006). After 8-10 hours, the cells were washed twice with phosphate buffer saline^{++} (PBS^{++} contains 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and maintained in fresh medium. Electrophysiological recordings were performed 24-48 hours later.

Immunoprecipitation

Whole mouse forebrain lysates were prepared from adult mice (50% C57bl6/ 50% ICR). All procedures with animals were performed in accordance with the "Canadian Council on Animal Care" guidelines for the use of animals in research. Animals were rapidly decapitated and the brain homogenized in immunoprecipitation (IP) buffer (10 mM HEPES pH7.4, 25 mM NaCl, 1% Triton-X-100, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml pepstatin) using a glass-Teflon homogenizer for 20 strokes. Homogenates were cleared by centrifugation at 14,000 g 30 minutes at 4 ºC. Protein concentration was determined by BCA assay (Biorad, Hercules, CA, USA). 2 mg

of lysate was used for each immunoprecipitation. Antigen-antibody complexes were immunoprecipitated with 2 mg anti-KCC2, anti-α-adaptin, or pre-immune rabbit or mouse IgG bound to Protein A or G-Sepharose (Amersham, UK) at 4 ºC for 2 h, washed three times in IP buffer and eluted in Laemmli buffer at 95 ºC for 5 minutes. Proteins were separated on 8% SDS-PAGE gels, followed by Western blotting as described previously (Heir et al., 2006) with antibodies to KCC2 and α-adaptin.

For the IPs performed from HEK293 cells, 3 X 10^6 cells were used for each condition. 18-20 hours after transfection cells were placed on ice, washed with chilled PBS, and lysed in IP buffer (10 mM HEPES pH7.4, 25 mM NaCl, 1% Triton-X-100, 1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml pepstatin). Solubilized cell lysates were cleared by centrifugation at 14,000 g 15 minutes at 4 ºC. Antigen-antibody complexes were immunoprecipitated with 2 mg anti-HA or pre-immune mouse control IgG bound to Protein G-Sepharose (Amersham, UK) at 4 ºC for 2 h, washed three times in IP buffer and eluted in Laemmli buffer at 95 ºC for 5 minutes. Proteins were separated on 8% SDS-PAGE gels, followed by Western blotting as described previously (Heir et al., 2006) with antibodies to HA and α-adaptin.

Fluorescence-based endocytosis assay

Transiently transfected cells were incubated in conditioned medium containing anti-HA or anti-Tac for 30 minutes on ice. Cells were washed extensively and incubated at 37 ºC for 15 or 30 minutes, fixed in 4% paraformaldehyde and then incubated in blocking buffer (PBS, 10% horse serum, and 0.5% bovine serum albumin-BSA) with Alexa 546-conjugated anti-mouse secondary antibody (Invitrogen), to label cell surface anti-Tac- or anti-HA-bound proteins. Cells were subsequently permeabilized in blocking buffer plus 0.1% Triton-X-100 and incubated with Alexa 488-conjugated anti-mouse secondary antibody (Invitrogen) to label internalized anti-Tac or anti-HA-bound proteins. After a final wash, cells were mounted on glass slides with GelTol Aqueous Mounting Medium (Thermo Scientific, Waltham, MA, USA). For cells in which a GFP-tagged protein was cotransfected following endocytosis any membrane bound antibodies were removed by incubation with citrate stripping buffer (50 mM Sodium Citrate pH 4.6, 280 mM Sucrose, 0.01 mM Deferoxamine Mesylate). Cells were then fixed, permeabilized

and labeled with Alexa 546-conjugated anti-mouse secondary antibodies as described above. Confocal images were collected with a 63x objective on a Zeiss LSM 510 microscope. Endocytosis was quantified as the proportion of cells demonstrating more than 10 internal labeled puncta from 30 cells on a per experiment basis (N=3).

Biotinylation endocytosis assay

Transiently transfected cells pre-incubated 1 hour with leupeptin (100 mg/ml) were placed on ice, washed twice with PBS^{++}, incubated 20 minutes with 1 mg/ml Sulfo-NHS-SS-biotin-PBS^{++}, washed three times with PBS^{++} containing 0.1% BSA, then twice with PBS^{++} alone and incubated at 37°C for either 30 or 60 minutes in the continuous presence of 100 μg/ml leupeptin, unless otherwise indicated. Subsequently, cells were placed on ice washed twice with PBS^{++} and the remaining cell surface bound biotin stripped by two washes in stripping buffer (50 mM glutathione in 75 mM NaCl, 10 mM EDTA, 1% BSA, and 0.075 M NaOH). Cells were then washed two times in PBS^{++} and lysed in lysis buffer (20 mM Tris-HCl pH7.6, 50 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.1% SDS, 1 mM Na_3VO_4, 50 mM NaF, and 10 mM $Na_4P_2O_7$, 1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml pepstatin) for 1 hour at 4 °C. Nuclei and insoluble material were removed by centrifugation at 14,000 g, 15 minutes, 4 °C. Biotinylated proteins were purified on NeutrAvidin-coated beads (Pierce) at 4 °C for 2 h, washed three times with lysis buffer and eluted in Laemmli buffer at 95 °C 5 minutes. Proteins were separated on 8% SDS-PAGE gels, followed by Western blotting with the KCC2 antibody. Intensities of immunoreactive bands were quantified by densitometry analysis of exposed films using a Biorad GS-800 densitometer. Background intensity readings were subjected from all readings and results were expressed as a percentage of the total surface labeling for each clone.

Electrophysiological recordings

Whole-cell Gramicidin-perforated patch recordings were performed on transfected cells using thin-walled borosilicate glass pipettes (4-6 MW) coated with dental wax. Cells were voltage-clamped at -60 mV and recordings started 10 minutes after obtaining stable electrical access to the cell. All recordings were performed using an Axopatch 200B

amplifier (Axon Instruments Inc., Foster City, CA, USA), and series resistances (30-40 MW) were compensated by 75-80% in all experiments. Current records were filtered at 10 kHz and digitized at 50-100 kHz. The reference electrode was connected to the bath via an agar bridge of 3M KCl. Data acquisition was performed using pClamp9 software (Axon Instruments Inc., Foster City, CA, USA), and illustrated using Origin 7 (OriginLab Corp., Northampton, MA, USA). All experiments were performed at room temperature.

External solutions contained (mM): 150 NaCl, 5 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 2% phenol red, with the osmotic pressure adjusted to 290 mOsm using sucrose. Stock solutions of Gramicidin were prepared in DMSO (50 mg/ml) and diluted into the internal solution to obtain a final concentration of 5 mg/ml just before the start of the experiment. The internal solution contained (mM): 115 NaCl, 10 NaF, 5 HEPES, 5 Na_4BAPTA, 0.5 $CaCl_2$, 1 $MgCl_2$, 10 Na_2ATP. pH was adjusted to 7.3 with 5N NaOH, and the osmotic pressure adjusted to match the external solution. Internal solution was kept on ice throughout the experiment.

IV. RESULTS

Endogenous neuronal KCC2 interacts with the clathrin-mediated endocytic machinery

Clathrin-mediated endocytosis (CME) is a prominent mechanism by which plasma membrane proteins are internalized. It involves the recruitment of membrane proteins (cargo) by the adaptor protein-2 (AP-2), to clathrin-coated pits, which are subsequently pinched from the plasma membrane to form internalized endocytic vesicles (Bonifacino and Traub, 2003). To examine whether neuronal KCC2 interacts with the CME machinery, we tested whether the AP-2 complex is bound endogenously to neuronal KCC2 by co-immunoprecipitation. For this we prepared whole cell lysates from adult mouse brain, immunoprecipitated (IP) KCC2 with a rabbit polyclonal antibody to KCC2 and used pre-immune rabbit IgG for control immunoprecipitates (Fig. 2.1A, top). In a reciprocal immunoprecipitation we used a mouse monoclonal antibody to the α-adaptin subunit of the AP-2 complex and pre-immune mouse IgG was used as the control (Fig. 2.1A, bottom). All immunoprecipitates were then analyzed by Western blot (IB) with antibodies to KCC2 and α-adaptin. We found α-adaptin was present in the immunoprecipitates isolated with the anti-KCC2 antibody, but not in the control pre-immune IgG immunoprecipitates (Fig. 2.1A). Also that KCC2 was present in the immunoprecipitates isolated with the anti-α-adaptin antibody, but not the control mouse IgG. This demonstrates endogenous neuronal KCC2 is present in a complex with the CME machinery and suggests KCC2 membrane internalization may be controlled by the CME-pathway.

Generation and functional characterization of a HA-tagged version of KCC2

To begin to identify the molecular mechanisms controlling KCC2 endocytosis, we needed to establish an assay in which we could test the CME dependence of KCC2 endocytosis and map the motif(s) within KCC2 controlling its endocytosis. For this, we generated an exofacial hemagglutinin (HA)-tagged KCC2, in which a triple HA tag was inserted into the second predicted extracellular loop of KCC2 (Fig. 2.1B). This construct would enable us to visually detect the endocytosis of KCC2 using a fluorescence-based

antibody uptake assay (see below). As the CME cellular machinery including AP-2 is conserved between different cell types, we chose to use human embryonic kidney 293 (HEK293) cells for our analysis. The advantage of using this cellular system compared to neurons, which endogenously express KCC2, is their ease of manipulation. Furthermore HEK293 cells are used routinely to map CME dependent endocytosis motifs in a wide range of neuronal proteins (Holton et al., 2005; Grampp et al., 2007; Kittler et al., 2000).

First of all, we ensured that insertion of the triple HA epitope tag in the second extracellular loop of KCC2 did not affect the maturation or cell surface expression of KCC2 (data not shown). To also make certain the HA tag did not disrupt transporter activity we examined the transport activity of HA-KCC2 in HEK293 cells. Using the Gramidicin perforated-patch recording technique to avoid artefactual changes in intracellular chloride concentration (Kyrozis and Reichling, 1995), we measured the reversal potential (E_{Gly}) of homomeric human α1 glycine receptors as a means to estimate $[Cl^-]_i$ and thus KCC2 transport activity. Voltage ramps from -60 to +60 mV (500 millisecond duration) were given before and during bath application of 1 mM Glycine and subtracted off-line to measure E_{Gly}. In perforated-patch recordings from HEK293 cells ($n \geq 3$), co-expression of KCC2 resulted in a negative shift of E_{Gly} from -8.3 ± 1.8 mV to -47 ± 4.4 mV (Fig. 2.1C,D), in agreement with a previous report (Inoue et al., 2006). A similar shift of E_{gly} to -45 ± 2.7 mV was also observed when HA-KCC2 was expressed (Fig. 2.1C,D), confirming that the HA tag does not affect the ability of KCC2 to transport Cl^- ions. Therefore, the HA-tagged version of the KCC2 construct was used as a substitute for wild-type KCC2 for all subsequent studies.

HA-KCC2 is internalized by clathrin-mediated endocytosis in HEK293 cells

To follow HA-KCC2 endocytosis in HEK293 cells we used a fluorescence-based endocytosis protocol, as outlined in Fig. 2.2A, in which HA-KCC2 internalization is visualized via HA.11 monoclonal antibody uptake. After 15 minutes of anti-HA uptake rapid constitutive internalization of HA-KCC2 was visualized (Fig. 2.2B, middle panel), with anti-HA labeling localized predominantly to discrete puncta underlying the plasma membrane. After 30 minutes, however, the majority of the internal anti-HA labelling localized to a compact perinuclear compartment (Fig. 2.2B, right-hand panel). No internal

anti-HA labeling was detected at time zero (Fig. 2.2B, left-hand panel), confirming the cells remain intact during the antibody labeling procedure. These results demonstrate HA-KCC2 undergoes constitutive endocytosis in HEK293 cells.

To confirm HA-KCC2 endocytosis is CME dependent, as suggested by our finding that neuronal KCC2 interacts with the clathrin adaptor AP-2 (Fig. 2.1A), we analyzed the endocytosis of HA-KCC2 in HEK293 cells in the presence of two dominant-negative (DN) mutants of CME. These are (i) the GTPase Dynamin-2 mutant lysine (K) 44 to alanine (A), which lacks the GTP hydrolysis activity required for the scission of clathrin-coated pits from the plasma membrane (Damke et al., 1994) and (ii) the epidermal growth factor receptor pathway substrate 15 mutant (Eps15Δ95/295) that lacks the second and third Eps15-Homology (EH) domains of Eps15, which inhibits AP-2 docking to the plasma membrane (Benmerah et al., 1999). HEK293 cells were transfected with HA-KCC2 and either GFP, GFP-Dynamin-2 (K44A) or GFP-Eps15(Δ95/295). HA-KCC2 endocytosis was then analyzed by the antibody uptake assay, except in this experiment only permeabilized anti-HA labeling was detected with Alexa-546 labeling, after the removal of surface bound anti-HA. The proportion of GFP transfected cells showing internalized anti-HA labeling at 15 and 30 minutes of internalization was then quantified as described in materials and methods. HA-KCC2 internalization in HA-KCC2 and GFP vector cotransfected cells was detected in 74.17±1.2% and 86.11±4.5% of transfected cells at 15 and 30 minutes respectively (Fig. 2.2C). While in contrast, cotransfection with either GFP-Dynamin-2 (K44A) or GFP-Eps15(Δ95/295) resulted in a reduction in HA-KCC2 internalization, with internalization detectable only in 1.1±1.1% and 4.4±1.1% of cells at 15 minutes and 2.2±1.9% and 4.4±1.1% of cells at 30 minutes respectively (Fig. 2.2C). As Dynamin-2 is also required for the alternate internalization pathway, the caveolin-dependent pathway (Yao et al., 2005), we examined whether the DN-Caveolin-GFP mutant (Pelkmans et al., 2001) altered HA-KCC2 endocytosis. We found 72.22±1.4% and 82.22±1.4% of cells exhibited internalization at 15 and 30 minutes respectively, which was comparable to HA-KCC2 alone transfected cells and indicates GFP-DN-Caveolin does not affect HA-KCC2 endocytosis of (Fig. 2.2C). Therefore together these results demonstrate that the constitutive endocytosis of HA-KCC2 is CME dependent.

The perinuclear localization of internalized HA-KCC2 (Fig. 2.2B) is reminiscent of the localization of internalized proteins, such as the transferrin receptor, to the endocytic recycling compartment (Martys et al., 1995), suggesting therefore that internalized HA-KCC2 may be trafficked via the endocytic-recycling pathway. To examine this, we first wanted to establish that internalized HA-KCC2 is not targeted for lysosomal degradation. To do this, we compared the stability of newly internalized HA-KCC2 in cells treated in the absence or presence of the lysosomal inhibitor, leupeptin. HEK293 cells transfected with HA-KCC2 were pre-treated with leupeptin (where indicated) and surface labeled with the cleavable form of biotin, Sulfo-NHS-SS-biotin, at 4 ºC to arrest membrane trafficking. After biotin labeling, cells were incubated at 37 ºC for 60 minutes (again with leupeptin where indicated) to allow endocytosis and intracellular trafficking to occur. Biotinylated proteins remaining at the cell surface were then cleaved (+ strip, Fig. 2.2D) or not (-strip, Fig 2.2D) with membrane impermeant reduced glutathione and remaining biotinylated proteins isolated by streptavidin column purification. Subsequently surface expressed (-strip, Fig. 2.2D) and internalized HA-KCC2 (+strip, Fig. 2.2D), +/- leupeptin treatment was detected and quantified by Western blotting (anti-HA). This analysis showed that the amount of internalized HA-KCC2 after 60 minutes is unaltered by leupeptin treatment (Fig. 2.2D), indicating endocytosed HA-KCC2 is not targeted for lysosomal degradation in HEK293 cells. Having established this, we next examined whether the compact perinuclear localization of internalized HA-KCC2 at 30 minutes (Fig. 2.2B) is representative of localization to the endosomal-recycling compartment. For this, we co-transfected HA-KCC2 with GFP tagged-rab11, an established marker for recycling endosomes (Ullrich et al., 1996). We found internalized HA-KCC2 detected by the HA.11 uptake assay colocalizes with the GFP-rab11 labeled endocytic-recycling compartment after 30 minutes (Fig. 2.2E). Taken together these results demonstrate constitutively internalized KCC2 is trafficked via the endosomal-recycling pathway and not targeted for lysosomal degradation.

The KCC2 carboxy tail contains constitutive endocytosis signals

Sequence specific motifs directing the endocytosis of different classes of transporters are in many instances located within the intracellular amino (N) or carboxy

(C) termini of these proteins (Al-Hasani et al., 2002; Holton et al., 2005; Schmidt et al., 2006). Therefore we next examined whether either the intracellular N- or C-terminus of KCC2 contains a functional endocytic signal(s). For this, we fused the KCC2 N and C termini respectively to the endocytosis deficient proteins, the intracellular domain deletion (Δ3-59) of the transferrin receptor (TfRΔ3-59, HA-tagged, Fig. 2.3A) and the interleukin 2 receptor α-chain (Tac, Fig. 2.4A) (Holton et al., 2005; Belouzard and Rouille, 2006) and assessed their endocytic abilities. We chose these two approaches: to fuse KCC2 N- and C- terminus to the N-terminus of the TfR deletion (Δ3-59) and the C-terminus of the Tac protein respectively, in order to maintain the natural orientation of each of the KCC2 domains. Fusion of a viable autonomous endocytic motif is known to rescue the endocytosis deficiency of these two constructs (Holton et al., 2005; Belouzard and Rouille, 2006; Tan et al., 1998). First using the fluorescence-based endocytosis assay with anti-HA.11, we found wild-type HA-TfR efficiently endocytosed (Fig. 2.3B), with HA-TfR internalization detected in 94.4±1.1% and 100% cells after 15 and 30 minutes respectively (Fig. 2.3C). However, in contrast HA-TfRΔ3-59/KCC2N, in which the TfR N-terminus (amino acids 3-59) is replaced with the KCC2 N-terminus (amino acids 2-102), was not able to internalize efficiently in our assay (Fig. 2.3B). HA-TfRΔ3-59/KCC2N internalization was detected in only 7.8±1.1% and 12.2±1.1% of cells after 15 and 30 minutes respectively (Fig. 2.3C). We confirmed this was not due to a defect in the total or cell surface expression of the HA-TfRΔ3-59/KCC2N protein (data not shown), suggesting therefore that the KCC2 N-tail does not contain an endocytic motif.

Next we examined whether the KCC2 C-tail contains a functional endocytic motif, we assayed the internalization efficiency of Tac and Tac-C in which the KCC2 C-terminus (amino acids 635-1115) is fused in frame to the C-terminus of Tac (Fig. 2.4A). To follow their internalization we used the fluorescence-based endocytosis assay in combination with an anti-Tac antibody. From this analysis we found Tac, as previously reported (Tan et al., 1998), did not internalize from the plasma membrane after either 15 or 30 minutes of incubation at 37 ºC (Fig. 2.4B,C). However in sharp contrast Tac-C, efficiently internalized at both 15 and 30 minutes (Fig. 2.4B). Internalization of Tac-C was detected in 96.7±1.9% and 100% of cells after 15 and 30 minutes respectively,

compared to zero cells for Tac (Fig. 2.4C). Taken together these results demonstrate the KCC2 C-terminus, but not the N-terminus contains a constitutive endocytic motif(s).

KCC2 C-terminus amino acids 651-662 encompasses a discrete endocytic motif

In order to map the region of the C-tail containing the putative endocytic motif, we generated a series of progressive carboxyl truncations of Tac-C (Tac-CD1-6, Fig. 2.5A), and assessed each truncated protein for its ability to internalize. Prior to performing the endocytosis assays, each construct was confirmed for production of the correct length protein and to be expressed at the cell surface (data not shown). Using the fluorescence based endocytosis assay with anti-Tac, we found the Tac constructs, Tac-CD1-Tac-CD5 (Fig. 2.5A), which correspond to the progressive carboxyl deletion of KCC2 residues 663-1115, all internalized as efficiently as Tac-C (see Fig. 2.5C), suggesting the endocytic motif(s) lies within the first 28 amino acids of the KCC2 C-tail. In contrast when we compared the internalization ability of Tac-CD5 to a Tac-CD6 construct, in which a further deletion of residues 651-662 was made (Fig. 2.5A), we found Tac-CD6 did not internalize (Fig. 2.5B). Tac-CD6 internalization was detected only in 3.3±4.1% cells compared to 100% cells for Tac-CD5 after 30 minutes (Fig. 2.5C). This data indicates the 12 amino acids present in the CD5 but absent in the CD6 fragment of KCC2 C-tail, which correspond to amino acids 651-662 of the mature KCC2 protein, contain an endocytic motif(s).

Identification of a novel di-leucine endocytic motif

Within these 12 amino acids unique to the CD5 construct (Fig. 2.6A) we observed that there is a classical tyrosine (Y)-based endocytic sequence $_{655}$YALL$_{658}$, which conforms to the consensus YxxΦ (where -x- represents any amino acid and Φ is a bulky hydrophobic amino acid). These YxxΦ motifs have been shown to mediate clathrin dependent endocytosis through binding of the clathrin AP-2 complex (Bonifacino and Traub, 2003). In addition, within this sequence there is a di-leucine $_{657}$LL$_{658}$ that could also constitute an endocytic clathrin AP-2 complex binding motif (Bonifacino and Traub, 2003). Di-leucine motifs though are usually preceded by an acidic residue within the consensus, (D/E)XXXL(L/I), where D=aspartic acid, E=glutamic acid and I=isoleucine

(Bonifacino and Traub, 2003). In KCC2, this acidic residue is replaced by an alanine $_{653}$**A**RYALL$_{658}$. However we noticed that there is a di-glutamic acid sequence located after the di-leucine $_{657}$LLRL**EE**$_{662}$. Hence, we postulated this di-leucine sequence might alternatively represent the potential endocytic motif. Therefore to test whether the tyrosine or the di-leucine motif mediate the endocytosis of Tac-CD5, we used alanine substitution mutagenesis to generate Tac-CD5(Y655A) and Tac-CD5(LL657,658AA) (Fig. 2.6A), knocking out each of the potential motifs independently. We then transfected these constructs into HEK293 cells and compared their endocytosis to wild type Tac-CD5, using the fluorescence-based endocytosis assay. We found Tac-CD5(Y655A) internalized similarly to wild-type Tac-CD5 (Fig. 2.6B), with internalization of Tac-CD5(Y655A) detected in 95.6±2.1% of cells after 30 minutes compared to 100% cells for wild type Tac-CD5 (Fig. 2.6C). In contrast, Tac-CD5(LL657,658AA) internalized very inefficiently (Fig. 2.6B), with internalization detected in only 2.5±2.8% (n=3, $p<0.01$) of cells after 30 minutes (Fig. 2.6C). Similar endocytosis rates for these Tac-CD5 mutants were also observed at the 15 minute internalization time point (data not shown). These results indicate the di-leucine residues ($_{657}$LL$_{658}$) and not tyrosine-655 are important for the membrane internalization potential of Tac-CD5.

The importance and role of each of the leucine residues for the internalization of Tac-CD5 was next tested, by generating individual leucine to alanine substitute mutants (L657A and L658A, Fig. 2.6A) and internalization evaluated as described above. We found that the L657A substitution had a mild effect on Tac-CD5 endocytosis, with Tac-CD5(L657A) internalization detected in 67.8±8.7% (n=3, $p<0.05$) cells after 30 minutes compared to 100% cells to wild type Tac-CD5 (Fig. 2.6C). In contrast the L658A substitution had a bigger effect on endocytosis, with Tac-CD5(L658A) internalization detected only in 13.3±1.9% (n=3, $p<0.01$) cells after 30 minutes (Fig. 2.6C). These results indicate both leucine residues are required to mediate efficient endocytosis of Tac-CD5, but that of the two; the second leucine (L_{658}) is the most important residue.

The di-leucine $_{657}$LL$_{658}$ as mentioned above is not present within a classical acidic-di-leucine endocytic motif consensus, but acidic residues are found after the di-leucine sequence. As previous studies have indicated the importance of the amino acid residues surrounding the di-leucine for the potency of the endocytic signal (Bonifacino

and Traub, 2003), we decided to examine the role of the residues surrounding the $_{657}$LL$_{658}$ motif. Specifically we tested the role of L_{660} and the two glutamic acid residues $_{661}$EE$_{662}$ following the di-leucine residues. Again we generated alanine substitution mutants in the context of the Tac-CD5 construct. Tac-CD5(L660A) and Tac-CD5(EE661,662AA) (Fig. 6A) were tested for their ability to internalize in our endocytosis assay. We found Tac-CD5(L660A) internalized as efficiently as wild type Tac-CD5 (Fig. 2.6C), with internalization detected in 100% cells after 30 minutes. In contrast we found Tac-CD5(EE661,662AA) internalization was affected, with internalization of Tac-CD5(EE661,662AA) detected only in 83.3±4.2% (n=3, $p<0.05$) after 30 minutes (Fig. 2.6C). This result suggests acidic residues $_{661}$EE$_{662}$ form part of the endocytic motif, but perhaps play a minor role in Tac-CD5 internalization compared to the di-leucine residues, $_{657}$LL$_{658}$. Taken altogether the results of the alanine mutagenesis experiments demonstrate there is an endocytosis motif in the region encompassing amino acids 657-662 of KCC2, which is a novel acidic di-leucine endocytic motif, $_{657}$LLXXEE$_{662}$.

The di-leucine residues $_{657}$LL$_{658}$ comprise the only endocytosis motif in the KCC2 C-terminus

Our findings so far strongly suggest the identified di-leucine motif is the only endocytic motif within the full-length KCC2 C-terminus. To test and confirm this, we generated the Tac-C(LL657,658AA) mutant by site-directed mutagenesis and assessed its ability to endocytose using the fluorescence-based endocytosis assay, with the anti-Tac antibody. As controls for this assay, we used Tac-C and also generated a Tac-C(Y665A) mutant, which according to our results with Tac-CD5(Y665A) (Fig. 2.6), should internalize as efficiently as Tac-C. We found, as predicted that Tac-C(Y665A) internalized similarly to wild type Tac-C (Fig. 2.7A), with internalization of Tac-C(Y665A) and wild type Tac-C detected in 90±3.3% cells and 95.6±2.2% after 15 minutes and 95.6±2.2% and 100% of cells after 30 minutes, respectively (Fig. 2.7B). In contrast, Tac-C(LL657,658AA) endocytosed poorly (Fig. 2.7A), with Tac-C(LL657,658AA) internalization detectable only in 3.3±1.9% and 6.7±3.8% of cells after 15 and 30 minutes respectively. These results demonstrate the di-leucine motif is the only

functional endocytosis motif within the context of the full length KCC2 C-tail and is both necessary and sufficient for the endocytosis-targeting function of the KCC2 C-tail.

The di-leucine motif is essential for KCC2 constitutive endocytosis

Finally, we examined whether the di-leucine motif is required for the constitutive endocytosis of full length KCC2. For this, the HA-KCC2(LL657,658AA) mutant was generated by site-directed mutagenesis and the endocytosis of this mutant and wild type HA-KCC2 in transiently transfected HEK293 cells were quantified using the surface biotinylation endocytosis assay. The advantage of using this quantitative assay versus the fluorescent approach was that we could determine whether internalization is blocked or if only the rate of endocytosis is altered. The endocytosis assay was performed essentially as described (Fig. 2.2D) on HEK293 cells transfected with either HA-KCC2 or HA-KCC2(LL657,658AA). Following the endocytosis assay, biotinylated HA-KCC2 variants both surface expressed (-strip, Fig. 2.8A) and internalized (+strip, Fig. 2.8A) were detected by streptavidin purification followed by Western blot detection with a KCC2 antibody. Western blots were subsequently quantified by densitometry. We found both wild type HA-KCC2 and HA-KCC2(LL657,658AA) expressed efficiently at the cell surface (0', -strip, Fig. 2.8A) and that glutathione stripping at 0 minutes removed the majority of remaining surface biotinylated HA-KCC2 and HA-KCC2(LL657,658AA) (0', + strip, Fig. 2.8A). Residual glutathione-resistant biotinylated protein was then considered as background and subtracted from the signal derived from further incubation time points. After 30 minutes incubation at 37°C (endocytosis), we detected an increased amount of biotinylated HA-KCC2 protected from glutathione stripping (30', + strip, left panel Fig. 2.8A), which represents the amount of internalized HA-KCC2 and equates to 5±1.1% of surface expressed wild type HA-KCC2 (Fig. 2.8B). In contrast, we observed no increase in the amount of biotinylated HA-KCC2(LL657,658AA) protected from glutathione stripping after 30 minutes (30', + strip, right panel Fig. 2.8A), indicating HA-KCC2(LL657,658AA) did not internalize (Fig. 2.8B). Hence we demonstrate that the LL657,658AA mutation is sufficient to block HA-KCC2 internalization, thus indicating that $_{657}LL_{658}$, is essential for the constitutive endocytosis of HA-KCC2.

In Fig. 2.1 we have shown that endogenous neuronal KCC2 is associated with the CME machinery through binding to the CME pathway AP-2 complex, suggesting therefore that KCC2 endocytosis may be mediated via the binding of this adaptor complex. We therefore tested whether $_{657}LL_{658}$, which is essential for the constitutive endocytosis of KCC2 is the binding domain for the AP-2 complex. To examine this, we tested the binding abilities of HA-KCC2 and HA-KCC2(LL657,658AA) to the α-adaptin subunit of the AP-2 by co-immunoprecipitation. Immunoprecipitated complexes were prepared from whole cell lysates of HEK293 cells transiently transfected with HA-KCC2 or HA-KCC2(LL657,658AA), using a mouse monoclonal antibody to HA or pre-immune mouse IgG as the control. These immunoprecipitates were then Western blotted with antibodies to HA and α-adaptin. We found α-adaptin was present in the anti-HA immunoprecipitates isolated from HA-KCC2 expressing cells but not HA-KCC2(LL657,658AA) expressing cells or in the control immunoprecipitates (Fig. 2.8C). This confirms that transiently expressed HA-KCC2 interacts with the AP-2 complex of HEK293 cells and that the LL657,658AA mutation is sufficient to block the binding of α-adaptin and the AP-2 complex to KCC2. Taken together with the Tac-CD5 (Fig. 2.6) and Tac-C (Fig. 2.7) data, these results demonstrate that we have identified a di-leucine based endocytosis motif that is essential for the CME dependent constitutive endocytosis of KCC2 via binding of the AP-2 complex.

V. DISCUSSION

KCC2 is essential in the vast majority of CNS neurons for the development and maintenance of inhibitory neurotransmission (Rivera et al., 1999; Hubner et al., 2001; Coull et al., 2003; Nabekura et al., 2002; Woo et al., 2002). In addition loss of KCC2 expression and function is associated with several neuropathological conditions including chronic pain (Coull et al., 2003), nerve injury (Nabekura et al., 2002), and epilepsy (Woo et al., 2002). Given this seemingly crucial role for KCC2 in the mature nervous system, it was surprising how little was known of the cellular mechanisms controlling KCC2 stability and function, in particular how KCC2 surface expression is controlled. In this study we examined the cellular mechanisms controlling the endocytosis of KCC2. We show that (i) constitutive endocytosis of KCC2 is controlled by the clathrin mediated endocytic pathway, (ii) endogenous neuronal KCC2 associates with the clathrin binding adaptor protein-2 complex, (iii) constitutively internalized KCC2 is sorted to the endosomal recycling pathway, (iv) KCC2 carboxy tail contains an autonomous endocytic signal and (v) this endocytic signal is a novel acidic di-leucine motif $_{657}$LLXXEE$_{662}$, which we show is both necessary and sufficient to mediate the binding of KCC2 to the AP-2 complex and the constitutive endocytosis of KCC2. Together our results reveal that the CME pathway and a discrete endocytic motif in the KCC2 carboxyl tail mediate the constitutive endocytosis of KCC2.

Our molecular mapping study is the first to report an endocytic motif in any CCC family protein. By generating a functional HA-tagged version of KCC2 (HA-KCC2, Fig. 2.1B-D), we were able to demonstrate that KCC2 constitutive endocytosis is CME pathway dependent (Fig. 2.2C). Whether other CCC family proteins are endocytosed also in a clathrin-dependent manner is yet to be determined. CME dependent endocytosis is largely controlled through the binding of clathrin adaptor complexes and our results indicate KCC2 binds *in vivo* to the clathrin binding AP-2 complex (Fig. 2.1A). In this study we initially identified a key 12 amino acid region $_{651}$SAARYALLRLEE$_{662}$ within the KCC2 carboxyl tail proximal to the last transmembrane domain (Fig. 2.5), encoding an autonomous endocytic signal. Using alanine substitution mutagenesis we then demonstrated that the di-leucine residues ($_{657}$LL$_{658}$) within this region are central to this

endocytosis motif (Figs. 2.6-7). Finally we demonstrated that these residues are essential for the constitutive endocytosis of HA-KCC2 (Fig. 2.8A-B) and the binding of KCC2 to the AP-2 complex (Figs. 2.8C). Interestingly, we found that L_{658} plays a major role within the endocytosis motif whereas L_{657} plays a more minor role in conveying the endocytosis-targeting function of this di-leucine motif (Fig. 2.6). In addition we have shown that although these di-leucine residues are not present within a classical acidic di-leucine endocytic motif, (E/D)XXXL(L/I) (Bonifacino and Traub, 2003), two glutamic acid residues downstream $_{657}$LLRLEE$_{662}$ are required for the full function of this endocytic motif (Fig. 2.6). This confirms previous findings that the composition of the surrounding amino acids can impact the function of an endocytosis motif (Bonifacino and Traub, 2003). An additional interesting finding from this mapping study was that although KCC2 has a potentially good tyrosine based endocytosis motif ($_{655}$YALL$_{658}$) within this region, it is apparently not utilized (Fig. 2.6). This was surprising and it will be of future interest to determine why this tyrosine motif is not used in KCC2 endocytosis, or if it plays an alternative targeting role for KCC2.

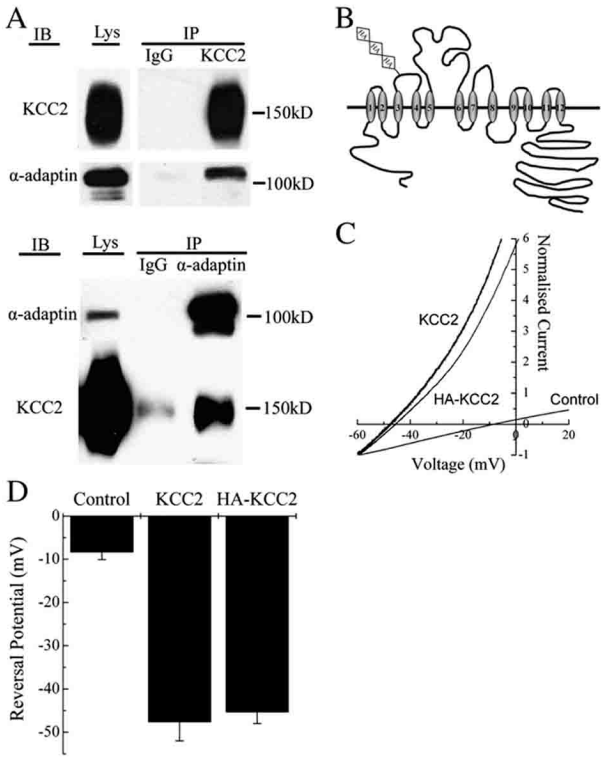
Sequence alignment of the region of the mouse KCC2 carboxyl tail containing the endocytic motif, with the same region of homologous murine cation-chloride co-transporters (Fig. 2.8D), reveals this novel KCC2 endocytic motif is highly conserved between related KCC family members. This suggests the mechanisms controlling the constitutive endocytosis of KCC family members may be conserved. Moreover this endocytic motif is not found in the analogous region of the more distally related NKCC1, NKCC2 or NCC proteins (Fig. 2.8D). Given the opposing physiological functions of KCC proteins versus NKCC and NCC proteins, in mediating either chloride efflux or influx respectively, the presence or absence of this endocytic motif provides further indication of how these related protein families may be differentially regulated. A role for specific regions of the KCC2 carboxyl terminus in controlling transporter activity has been reported (Bergeron et al., 2006; Mercado et al., 2006), but how these regions mediate this function is as yet unknown. It is postulated that either acquired conformations or specific protein-protein interactions are involved. In this regard, it is interesting that we have mapped the KCC2 constitutive endocytosis motif to the C-tail of KCC2. Recent reports also indicate that KCC2 and other CCC family members encode

the capacity to both homo and hetero-oligomerize (Moore-Hoon and Turner, 2000; de Jong et al., 2003; Blaesse et al., 2006; Simard et al., 2007). The regions within these proteins mediating this function are beginning to be elucidated and an important role for the carboxyl tail yet again is indicated (Simard et al., 2007). How both homo- and hetero-oligomerization impact on the function of our defined endocytic motif, will need to be addressed. For example does dimerization affect the availability of the endocytic motif? The mapping of the precise regions of the carboxyl tail required to form dimers will provide a starting point to address this question.

The endocytic motif identified in this study regulates the constitutive endocytosis of KCC2 (Fig. 2.2). Both seizure activity (BDNF dependent signaling) and oxidative stress (hydrogen peroxide induced) have been shown to induce a rapid reduction in KCC2 surface expression by enhancing KCC2 internalization in hippocampal neurons (Rivera et al., 2004; Wake et al., 2007). This suggests the additional existence of regulatory mechanisms that can enhance KCC2 constitutive endocytosis. This BDNF and oxidative stress dependent regulation of KCC2 surface expression (Rivera et al., 2004; Wake et al., 2007) is associated with a reduction in tyrosine phosphorylation of KCC2 (Wake et al., 2007), suggesting that tyrosine phosphorylation or de-phosphorylation may be a means by which KCC2 endocytosis could be regulated. It will therefore be of interest to examine whether stress dependent regulation of KCC2 endocytosis utilizes the same endocytic motif we have mapped in this study. Also whether the tyrosine phosphorylation state of KCC2 controls the function of this endocytic motif, perhaps by altering its availability and its interaction with the endocytic machinery. A recent report has also indicated the additional existence of a protein kinase-C dependent inhibition of KCC2 endocytosis, involving the phosphorylation of a site in the KCC2 C-terminus distal to the novel endocytic motif reported here (Lee et al., 2007). It will be interesting to examine whether this phosphorylation event also impacts the function of the novel KCC2 endocytosis motif reported in this study. Finally, we have shown that constitutively endocytosed KCC2 is targeted to the recycling endosomal pathway (Fig. 2.2). It will therefore also be of future interest to determine whether BDNF-signaling and oxidative stress also target KCC2 internalization to the recycling-endosomal pathway or alternatively for degradation.

Given the essential role of KCC2 in regulating the strength of inhibitory neurotransmission, membrane trafficking of KCC2 represents an optimal mechanism by which inhibition may be indirectly regulated. Our results defining the motif and mechanism by which the constitutive endocytosis of KCC2 is controlled, are an initial step in understanding the molecular determinants by which KCC2 transporter membrane trafficking may be regulated. Finally given the conservation of this motif amongst K^+/Cl^- family proteins, our studies are also applicable to examining how endocytosis is controlled in related KCC proteins and its impact on their function of controlling cellular chloride homeostasis in the many different tissues of the body in which these transporters are expressed.

Figure 2.1. Endogenous neuronal KCC2 and the clathrin adaptor protein-2 (AP-2) complex interact. (A) Mouse whole brain lysate (Lys) was immunoprecipitated (IP) with antibodies to KCC2 and control pre-immune rabbit IgG (top) or the AP-2 complex α-adaptin subunit and control mouse IgG (bottom), and Western blotted (IB) with antibodies to KCC2 or α-adaptin. (B) Schematic of HA epitope-tagged KCC2. Three tandem copies of an influenza virus HA peptide (YPYDVPDYA) were inserted into the second extracellular loop of mouse KCC2. (C) Functional characterization of HA epitope-tagged KCC2. Current-voltage (IV) plots in the absence (control) and presence of either KCC2 or HA-KCC2. Note the negative shift in E_{Gly} observed when either KCC2 or the HA-KCC2 construct are co-expressed with human α1 glycine receptor subunits. (D) Summary bar graph showing the effect of both KCC2 and HA-KCC2 on E_{Gly}. Data are mean ± s.e., of at least 3 experiments in all conditions.


A
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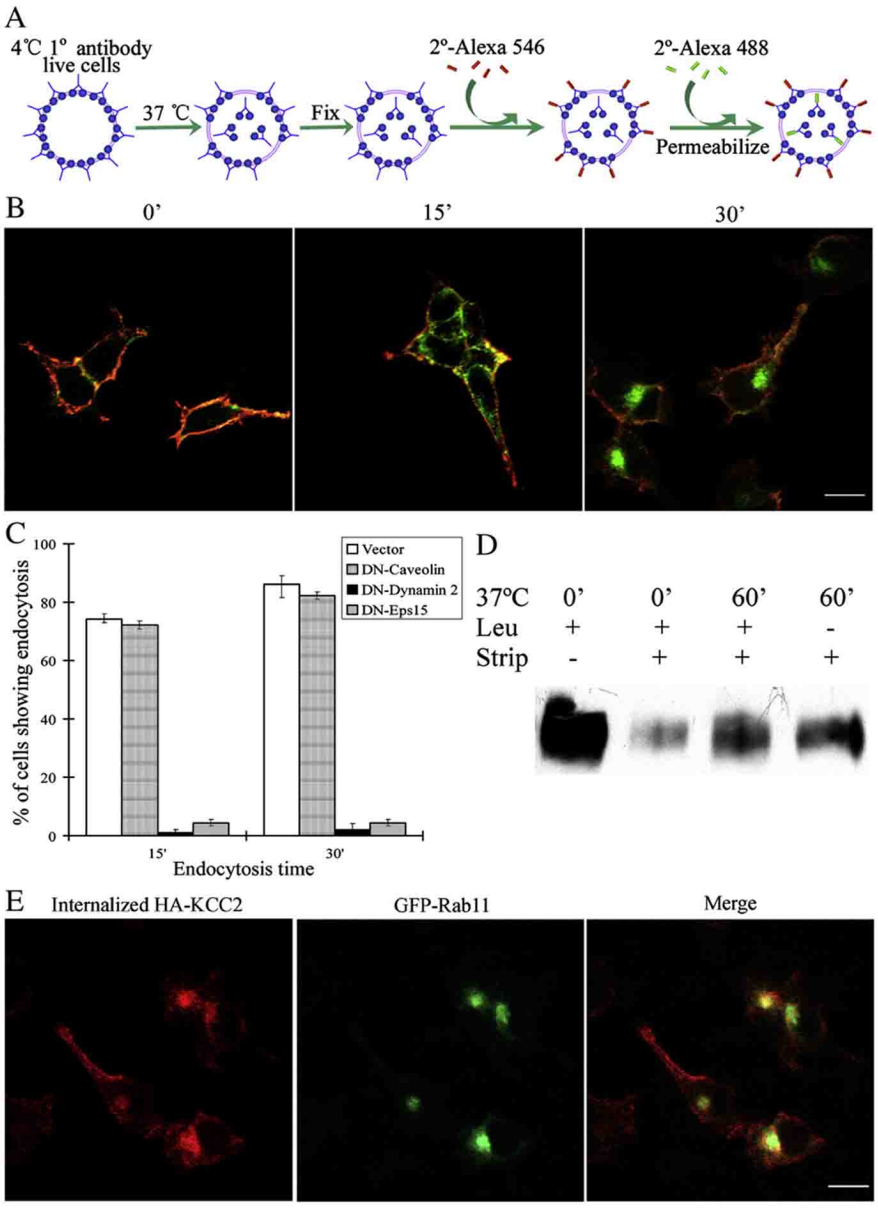
α-adaptin
100kD
KCC2
150kD
B
C
KCC2
HA-KCC2
Control
Normalised Current
Voltage (mV)
D
Control
KCC2
HA-KCC2
Reversal Potential (mV)

Figure 2.2. KCC2 is internalized by clathrin-mediated endocytosis and targeted to the endosomal-recycling pathway. (A) Schema illustrating the fluorescence-based endocytosis assay, for details see materials and methods. (B) Endocytosis of HA-KCC2. HEK293 cells transfected with HA-KCC2 were assayed for anti-HA internalization using a monoclonal antibody to HA, as depicted in (A). Shown are representative confocal images of surface anti-HA (red) and internalized anti-HA (green) at 0, 15 and 30 minutes. (C) Dominant-negative mutants of the clathrin-mediated endocytic pathway block HA-KCC2 endocytosis. HEK293 cells transiently expressing HA-KCC2 and either GFP vector (control, white bars), GFP-DN-Caveolin (checked bars), GFP-Dynamin-2 (K44A) (black bars), or GFP-Eps15(Δ95/295) (grey bars) were assayed as described in (B) and HA-KCC2 endocytosis at 15 and 30 minutes quantified as described in materials and methods. Note, only permeabilized anti-HA labelling was performed with Alexa-546 secondary antibodies. Data are expressed as the percentage of cells that internalized anti-HA and presented as mean ± s.e. (n=3). (D) Internalized HA-KCC2 is not targeted for lysosomal degradation. Cell surface biotinylation was performed using the non-permeable cleavable biotinylation reagent Sulfo-NHS-SS-Biotin (1 mg/ml), on HA-KCC2 transfected cells incubated +/- leupeptin (Leu, 100 μg/ml) to inhibit lysosomal degradation and HA-KCC2 internalization assayed as described in materials and methods. A typical immunoblot is shown detecting from left to right, total initial surface HA-KCC2 (0', +Leu, -strip), strip control (0', +Leu, +strip), HA-KCC2 internalized plus leupeptin (60', +Leu, +strip) and HA-KCC2 internalized no leupeptin (60', -Leu, +strip), using an anti-KCC2 antibody. (E) Internalized HA-KCC2 co-localizes with the recycling endosomal marker, Rab11. HEK293 cells transiently expressing HA-KCC2 and GFP tagged Rab11 were labelled with anti-HA for 30 minutes at 37 ºC, then remaining surface bound anti-HA stripped as described in materials and methods and internalized anti-HA detected with Alexa-546 secondary antibodies. Shown are representative images (n=3) of internalized anti-HA (red) GFP-Rab11 (green) and the merge (yellow) illustrating co-localization of the proteins. Scale bar: 10 μm.


A
4℃ 1° antibody
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B
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% of cells showing endocytosis
100
80
60
40
20
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Vector
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DN-Eps15
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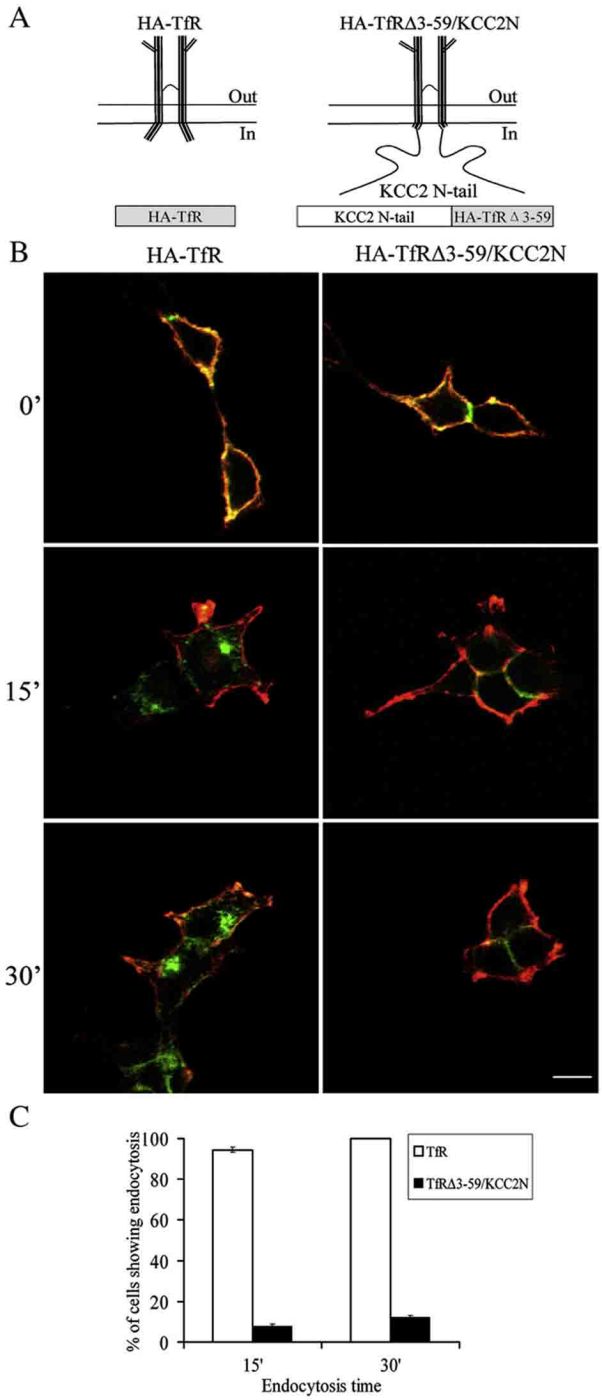
37°C 0' 0' 60' 60'
Leu + + + -
Strip - + + +
E
Internalized HA-KCC2
GFP-Rab11
Merge

Figure 2.3. The KCC2 amino (N) terminus does not mediate endocytosis. (A) Schematic illustration of HA-tagged Transferrin receptor (HA-TfR, left) and HA-TfR(Δ3-59) KCC2 N-tail chimera (HA-TfRΔ3-59/KCC2N, right). The N-tail (amino acids 3-95) of TfR was replaced with KCC2 N-tail (amino acids 2-102) to produce HA-TfRΔ3-59/KCC2N. (B) Endocytosis of HA-TfR and HA-TfRΔ3-59/KCC2N. HEK293 cells transfected with HA-TfR or HA-TfRΔ3-59/KCC2N were assayed for anti-HA internalization as depicted in Fig. 2A. Shown are representative confocal images of surface anti-HA (red) and internalized anti-HA (green) at 0, 15, and 30 minutes. Note internalized HA-TfR (green) was detected at 15 and 30 minutes, with no corresponding internalized HA-TfRΔ3-59/KCC2N at these time points, indicating the lack of endocytic signals in the KCC2 N-tail. Scale bar: 10 μm. (C) Quantification of HA-TfR (white bars) and HA-TfRΔ3-59/KCC2N (black bars) endocytosis at 15 and 30 minutes. Data are expressed as the percentage of cells that internalized anti-HA and presented as mean ± s.e (n=3).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HA-TfR
HA-TfRΔ3-59/KCC2N
0'
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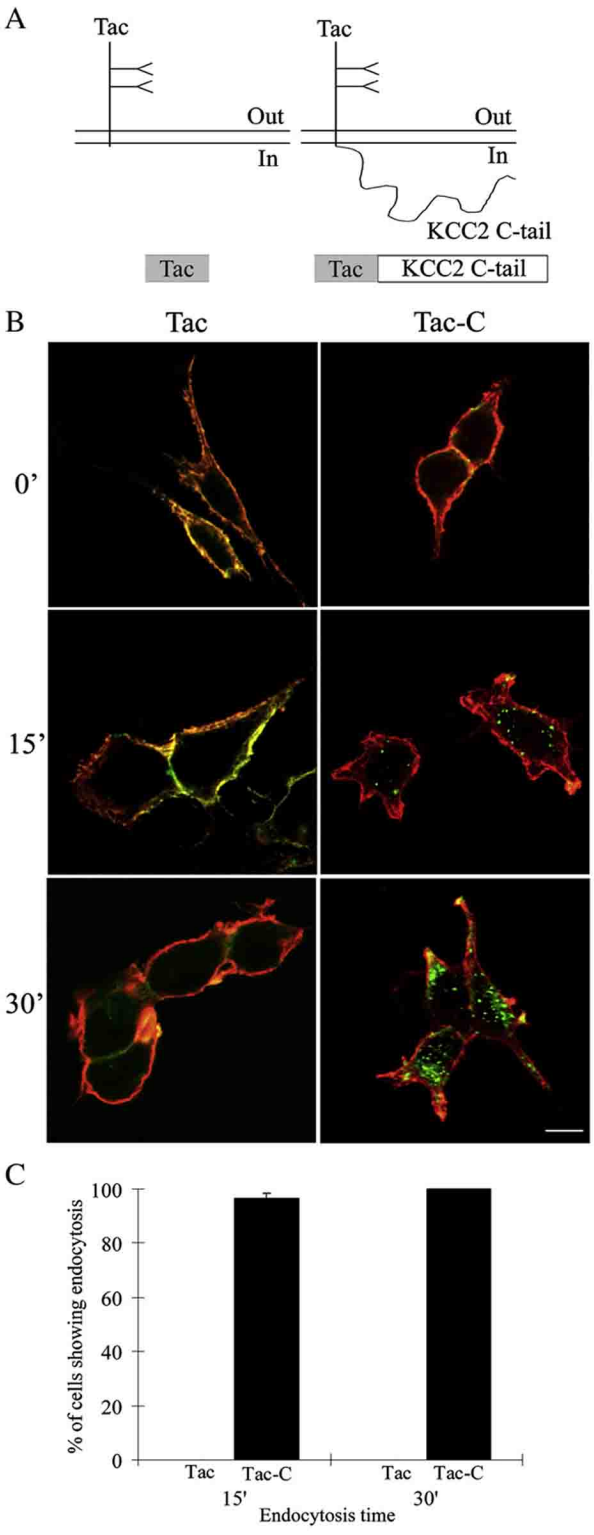
% of cells showing endocytosis
100
80
60
40
20
0
15'
30'
Endocytosis time
TfR
TfRΔ3-59/KCC2N

Figure 2.4. KCC2 carboxyl (C) terminus is sufficient to target Tac for endocytosis. (A) Schematic illustration of the interleukin receptor 2 alpha subunit intracellular truncation mutant (Tac, left) and the Tac/ KCC2 C-tail chimera (Tac-C, right). The KCC2 C-tail (amino acids 635-1115) was fused to the C-terminus of Tac to produce Tac-C. (B) Endocytosis of Tac and Tac-C. HEK293 cells transfected with Tac or Tac-C were assayed for internalization using a monoclonal antibody to Tac as depicted in Fig. 2A. Shown are representative confocal images of surface anti-Tac (red) and internalized anti-Tac (green) at 0, 15 and 30 minutes. Note internalized Tac-C (green) was detected at 15 and 30 minutes, while Tac did not internalize at these time points, indicating the KCC2 C-tail contains an autonomous endocytic signal. Scale bar: 10 μm. (C) Quantification of Tac (white bars) and Tac-C (black bars) endocytosis at 15 and 30 minutes. Data are expressed as the percentage of cells that internalized anti-Tac and presented as mean ± s.e (n=3).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Tac
Out
In
Tac
Out
In
KCC2 C-tail
Tac
Tac
KCC2 C-tail
B
Tac
Tac-C
0'
15'
30'
C
% of cells showing endocytosis
100
80
60
40
20
0
Tac
Tac-C
Tac
Tac-C
15'
30'
Endocytosis time

Figure 2.5. KCC2 C-terminus residues 651-662 contain an endocytic motif. (A) Schematic illustration of the Tac-C carboxyl truncation constructs, Tac-CD1-CD6. The KCC2 C-tail amino acids contained in each construct are shown. (B) Endocytosis of Tac-CD5 and Tac-CD6. HEK293 cells transfected with Tac-CD5 (left) or Tac-CD6 (right) were assayed for anti-Tac internalization as depicted in Fig. 2A. Shown are representative confocal images of surface anti-Tac (red) and internalized anti-Tac (green) at 0, 15 and 30 minutes. Note internalized Tac-CD5 (green) was detected at 15 and 30 minutes, while no internalized Tac-CD6 was detected at any time point. Scale bar: 10 μm. (C) Quantification of Tac-C carboxyl truncation constructs endocytosis at 30 minutes. Data are expressed as the percentage of cells that internalized anti-Tac and presented as mean ± s.e (n=3). Data from (B) and (C) indicate the endocytic motif is located within amino acids 651-662 of the mature KCC2 protein.

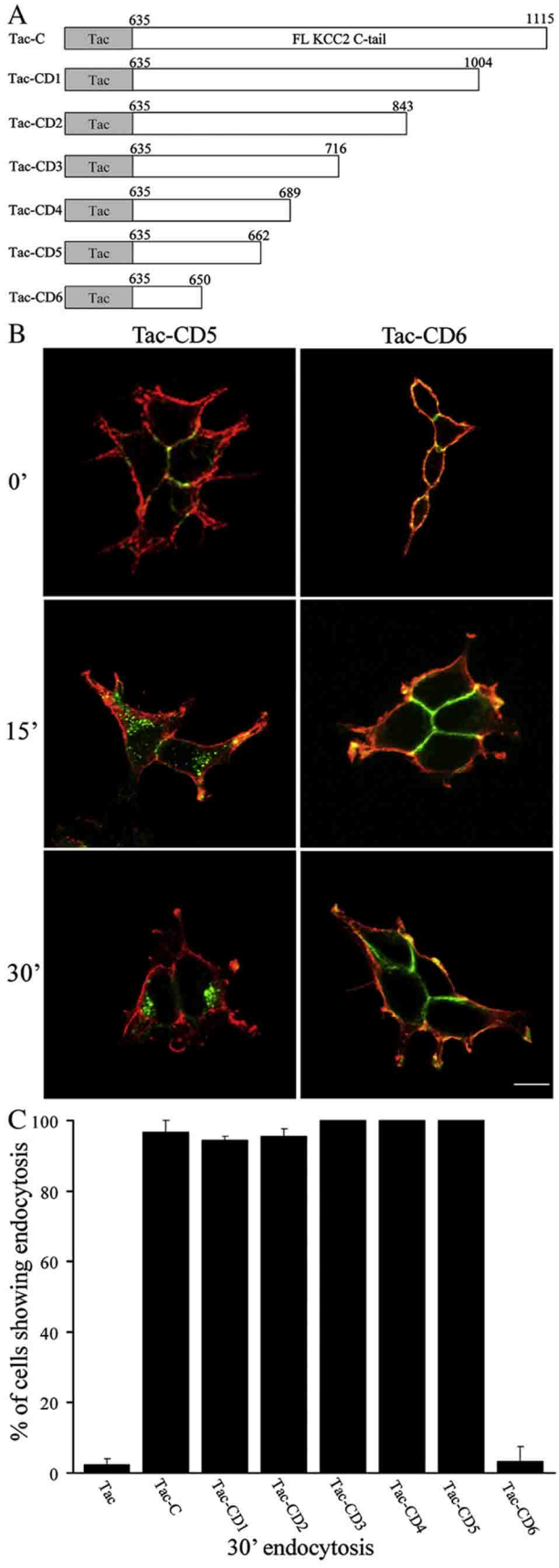
A
Tac-C
Tac
635
1115
FL KCC2 C-tail
Tac-CD1
1004
Tac-CD2
843
Tac-CD3
716
Tac-CD4
689
Tac-CD5
662
Tac-CD6
650
B
Tac-CD5
Tac-CD6
0'
15'
30'
C
% of cells showing endocytosis
100
80
60
40
20
0
Tac
Tac-C
Tac-CD1
Tac-CD2
Tac-CD3
Tac-CD4
Tac-CD5
Tac-CD6
30' endocytosis

Figure 2.6. Identification of a di-leucine based endocytic motif within KCC2 C-terminus amino acids 651-662. (A) *Top,* Alignment of the KCC2 amino acid sequence in the CD5 (635-662) and CD6 (635-650) regions of KCC2. Note the amino acids containing the potential YXXΦ and LL endocytic motifs are underlined. *Bottom*, alignment of the different Tac-CD5 point mutation mutants. The alanine-substituted amino acid residues are underlined. (B) Endocytosis of Tac-CD5, Tac-CD5(Y655A), and Tac-CD5(LL657, 658AA). HEK293 cells transfected with Tac-CD5(WT) wild-type (left), Tac-CD5(Y655A) (middle), or Tac-CD5(LL657, 658AA) (right) were assayed for anti-Tac internalization as depicted in Fig. 2A. Shown are representative confocal images of surface anti-Tac (red) and internalized anti-Tac (green) at 0 and 30 minutes. Note no internalization of Tac-CD5(LL657, 658AA) was detected at 30 minutes, while Tac-CD5 and Tac-CD5(Y655A) both internalized. Scale bar: 10 μm. (C) Quantification of Tac-CD5 wild type and the CD5 mutant endocytosis at 30 minutes. Data are expressed as the percentage of cells that internalized anti-Tac and presented as mean ± s.e (n=3). Significant differences from control (wild type Tac-CD5) were found (*$P<0.05$, **$P<0.01$) using the Student's *t*-Test, two tails.

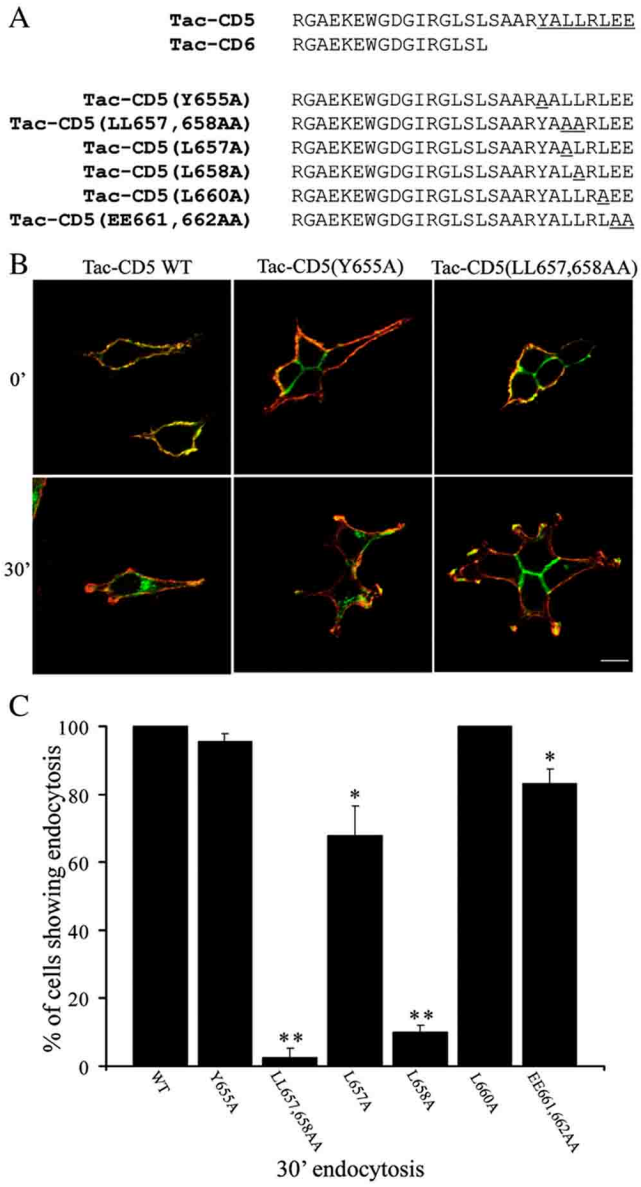
A
Tac-CD5 RGAEKEWGDGIRGLSLSAARYALLRLEE
Tac-CD6 RGAEKEWGDGIRGLSL
Tac-CD5(Y655A) RGAEKEWGDGIRGLSLSAARAALLRLEE
Tac-CD5(LL657,658AA) RGAEKEWGDGIRGLSLSAARYAARLEE
Tac-CD5(L657A) RGAEKEWGDGIRGLSLSAARYAALRLEE
Tac-CD5(L658A) RGAEKEWGDGIRGLSLSAARYALARLEE
Tac-CD5(L660A) RGAEKEWGDGIRGLSLSAARYALLRAEE
Tac-CD5(EE661,662AA) RGAEKEWGDGIRGLSLSAARYALLRLAA
B
Tac-CD5 WT
Tac-CD5(Y655A)
Tac-CD5(LL657,658AA)
0'
30'
C
% of cells showing endocytosis
100
80
60
40
20
0
*
**
WT
Y655A
LL657,658AA
L657A
L658A
L660A
EE661,662AA
30' endocytosis

Figure 2.7. Di-leucine residues $_{657}$LL$_{658}$ are necessary for Tac-C endocytosis. (A) Endocytosis of Tac-C, Tac-C(Y655A), and Tac-C(LL657, 658AA). HEK293 cells transfected with Tac-C (WT, left), Tac-C(Y655A) (middle), or Tac-C(LL657, 658AA) (right) were assayed for anti-Tac internalization as depicted in Fig. 2A. Shown are representative confocal images of surface anti-Tac (red) and internalized anti-Tac (green) at 30 minutes. Note no internalization of Tac-C(LL657, 658AA) was detected, while Tac-C and Tac-C (Y655A) both internalized. Scale bar: 10 μm. (B) Quantification of Tac-C wild type (white bars), Tac-C (Y655A) (black bars) and Tac-C(LL657, 658AA) (grey bars) endocytosis at 15 and 30 minutes. Data are expressed as the percentage of cells that internalized anti-Tac (n=3) and presented as mean ± s.e.

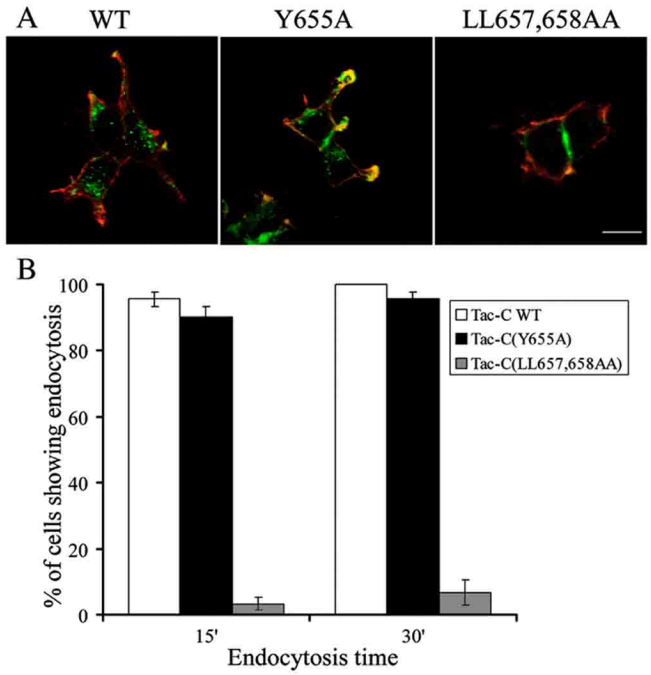
A
WT
Y655A
LL657,658AA
B
% of cells showing endocytosis
100
80
60
40
20
0
15'
30'
Endocytosis time
Tac-C WT
Tac-C(Y655A)
Tac-C(LL657,658AA)

Figure 2.8. Di-leucine $_{657}$LL$_{658}$ is essential for the constitutive endocytosis of HA-KCC2. (A) Comparison of the endocytosis rates of HA-KCC2 and HA-KCC2(LL657, 658AA). Cell surface biotinylation was performed using the non-permeable cleavable biotinylation reagent Sulfo-NHS-SS-Biotin (1 mg/ml) on HA-KCC2 and HA-KCC2(LL657, 658AA) transfected cells and internalization detected as described in materials and methods. Typical immunoblots are shown detecting from left to right, total initial surface (0', -strip), strip control (0', +strip) and internalization for 30 minutes (30', +strip) for HA-KCC2 (*left*) and HA-KCC2 (LL657,658AA) (*right*), using an anti-KCC2 antibody. (B) Quantification of HA-KCC2 and HA-KCC2 (LL657, 658AA) endocytosis at 30 minutes. Data are expressed as the percentage of each internalized KCC2 variant at 30 minutes and presented as mean ± s.e. (n=3). The difference in internalization between HA-KCC2 and HA-KCC2(LL657,658AA) was found to be statistically significant (**P<0.01), using the Student's *t*-test, two tails. (C) Comparison of binding of the AP-2 complex α-adaptin subunit to HA-KCC2 and HA-KCC2(LL657, 658AA). Whole cell lysates from HA-KCC2 and HA-KCC2(LL657, 658AA) transiently expressing HEK293 cells were immunoprecipitated (IP) with antibodies to HA or pre-immune mouse IgG, and Western blotted (IB) with antibodies to HA or α-adaptin. α-adaptin was co-immunoprecipitated with HA-KCC2 but not with HA-KCC2(LL657,658AA). (D) Amino acid sequence alignment of the first 28 residues of the mouse KCC2 carboxyl terminus with the orthologous region in other mouse cation-chloride cotransporters. The location of this region is marked by dashes on a schematic representation of the structure of a cation-chloride cotransporter. Black bars illustrate transmembrane domains. Note, the novel di-leucine endocytic motif identified in KCC2 is conserved in KCC1, KCC3 and KCC4 (shown shaded), but it is not found in NKCC1, NKCC2 or NCC.

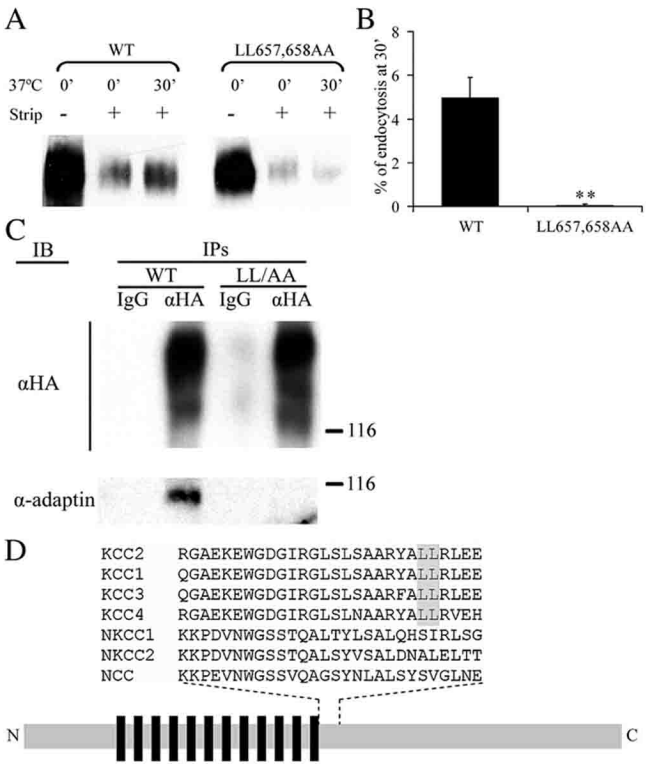
A
WT
LL657,658AA
37°C 0' 0' 30' 0' 0' 30'
Strip - + + - + +
B
% of endocytosis at 30'
8
6
4
2
0
**
WT
LL657,658AA
C
IB
IPs
WT
LL/AA
IgG αHA IgG αHA
αHA
116
α-adaptin
116
D
KCC2 RGAEKEWGDGIRGLSLSAARYALLRLEE
KCC1 QGAEKEWGDGIRGLSLSAARYALLRLEE
KCC3 QGAEKEWGDGIRGLSLSAARFALLRLEE
KCC4 RGAEKEWGDGIRGLSLNAARYALLRVEH
NKCC1 KKPDVNWGSSTQALTYLSALQHSIRLSG
NKCC2 KKPDVNWGSSTQALSYVSALDNALELTT
NCC KKPEVNWGSSVQAGSYNLALSYSVGLNE
N
C

Table 2.1.

Oligonucleotides used to generate the different Tac-KCC2 carboxyl tail constructs.

Tac-C	ATATCTAGATAATCAGGAGTAGATGGT
Tac-CD1	TATTCTAGATTAATGCACCTCTGGGTCTGTCTCC
Tac-CD2	TATTCTAGATTAGTGGTGTCGCAGCAGGAAGG
Tac-CD3	TACTCTAGATTACAGAAAGGTGCCCTCAAGGACG
Tac-CD4	TCCTCTAGATTACGGATGCACCACGTTCTGATCC
Tac-CD5	TCTTCTAGATTATTCCTCCAGGCGCAAGAGAGC
Tac-CD6	AAGTCTAGATTAGAGAGACAGGCCTCGGATTCC
Tac-CD5 (Y655A)	ATATCTAGATTATTCCTCCAGGCGCAAGAGAGCAG CGCGTGC
Tac-CD5(LL657,658AA)	AATTTCTAGATTATTCCTCCAGGCGCGCGGCAGCA TAGCG
Tac-CD5(L657A)	ATTCTAGATTATTCCTCCAGGCGCAAGGCAGCATA GCG
Tac-CD5(L658A)	ATTCTAGATTATTCCTCCAGGCGCGCGAGAGCATA GCG
Tac-CD5(L660A)	CTATCTAGATTATTCCTCCGCGCGCAAGAGAGC
Tac-CD5(EE661,662AA)	ATTTCTAGATGCCGCCAGGCGCAAGAGAGCATA

The oligonucleotides are written 5' to 3' and the *XbaI* restriction site is underlined.

PREFACE TO CHAPTER 3

The neuron-specific K^+/Cl^- cotransporter KCC2 belongs to the electroneutral cation chloride cotransporter superfamily, which includes one Na^+/Cl^- cotransporter (NCC), two $Na^+/K^+/2Cl^-$ cotransporters (NKCC1/2), and four K^+/Cl^- cotransporters (KCC1-4). Previous studies on NCC and NKCCs have shown they form dimers in cells (de Jong et al., 2003; Moore-Hoon and Turner, 2000; Starremans et al., 2003). Molecular mapping analysis on NKCC1/2 has revealed the regions mediating their self-interaction or homo-dimerization (Simard et al., 2004; Parvin et al., 2007; Brunet et al., 2005). In contrast to these Na^+-coupled Cl^- cotransporters, the molecular mechanisms mediating the quaternary assembly of the K^+-coupled Cl^- cotransporters (including KCC2) were far less well understood. KCC2 oligomerization has been observed in certain type of neurons and heterologous human embryonic kidney 293 cells (Blaesse et al., 2006), and the C-terminus of KCC2 has been suggested to encode the ability to mediate its self-interaction (Simard et al., 2007). However, the molecular mechanisms, in particular the precise region(s), which control KCC2 dimerization remained elusive.

The specific aims of the present study were to identify the molecular regions and potential structural basis mediating KCC2 dimerization.

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Identification of Regions Mediating K^+/Cl^- Cotransporter KCC2 Dimerization

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Keywords: KCC2, Chloride transporter, Dimerization

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I. ABSTRACT

The neuron-specific potassium-chloride cotransporter 2 (KCC2) plays a crucial role, by controlling chloride extrusion, in the maturation and maintenance of fast inhibitory neurotransmission. Although it is well established that various mechanisms can regulate KCC2 gene expression, little is known about the post-translational mechanisms regulating KCC2, specifically its quaternary assembly. Although the importance of KCC2 C-terminus in its self-interaction has been previously indicated, the specific dimerization region(s) have not been mapped. We therefore set out to identify the molecular region(s) mediating KCC2 dimerization, one mechanism that may have an important impact on KCC2 function. Using a combination of experimental approaches, we show that when expressed in human embryonic kidney 293 cells KCC2 C-terminus, but not N-terminus, encodes the capacity to mediate KCC2 dimerization. Further mapping of the interaction domain(s) reveals the existence of two regions in the proximal and central KCC2 C-terminus that mediate KCC2 dimerization, amino acids 651-662 and 782-843 (mouse) respectively. We show these two regions form reciprocal interactions between two KCC2 molecules. Secondary structural analysis predicts these two fragments contain α-helices and may participate in helix-helix interactions. Finally, we find mutating the $_{657}$LL$_{658}$ residues to alanines is sufficient to abolish KCC2 dimerization *in vivo*, suggesting an essential role for these two residues in the control of KCC2 dimerization.

II. INTRODUCTION

In the adult mammalian central nervous system (CNS), fast inhibitory neurotransmission is predominantly mediated by two types of ligand gated chloride (Cl^-) channels, the γ-amino-butyric acid type-A receptor ($GABA_AR$) and the glycine receptor (GlyR) (Betz, 1992; Sieghart, 2006). Ligand binding to these receptors leads to ion channel opening and influx of Cl^-, which consequently hyperpolarizes the postsynaptic neuronal membrane (Betz, 1992; Sieghart, 2006). The Cl^- influx induced by activation of these Cl^- ion channels is critically dependent on a low intracellular Cl^- concentration ($[Cl^-]_i$). The neuron-specific K^+/Cl^- cotransporter, KCC2, has now been proven to be an essential protein in establishing and maintaining this low $[Cl^-]_i$ in neurons, by controlling Cl^- extrusion (Rivera et al., 1999).

Studies using antisense oligonucleotides to inhibit KCC2 expression in rat hippocampal pyramidal neurons have shown that KCC2 gene expression is essential for the transformation of GABAergic and glycinergic neurotransmission from depolarizing to hyperpolarizing (Rivera et al., 1999). Consistent with the essential role of KCC2 during neuronal maturation, KCC2 knockout mice die shortly after birth due to respiratory failure caused by functional defects in respiratory motoneurons (Hubner et al., 2001). In addition, accumulating evidence suggests post-translational mechanisms, such as regulated membrane protein trafficking, phosphorylation/dephosphorylation modification and oligomerization of KCC2, may also play an important role in mediating the developmental maturation of GABA- and glycine- mediated fast inhibitory synaptic responses, independently of KCC2 gene expression (Rivera et al., 2004; Kelsch et al., 2001; Khirug et al., 2005; Wake et al., 2007; Lee et al., 2007; Zhao et al., 2008).

KCC2 belongs to the cation-chloride cotransporter (CCC) superfamily, which includes one Na^+/Cl^- cotransporter (NCC), two $Na^+/K^+/Cl^-$ cotransporters (NKCCs) and four K^+/Cl^- cotransporters (KCCs) (Hebert et al., 2003). Functionally, CCCs are branched into two categories. One branch includes the Na^+-coupled Cl^- cotransporters, NCC and NKCCs, which up-take Cl^-, and the other one includes the K^+-coupled Cl^- cotransporters, KCCs, which extrude Cl^- (Payne et al., 2003). Hydropathy studies have revealed that CCCs display similar primary structure (Gamba et al., 2004). Specifically, KCC2

contains twelve transmembrane (TM) regions with both intracellular amino and carboxyl termini flanking each side (Payne et al., 1996, Fig. 3.2A,). Although the primary structure of all CCC member proteins has been well studied, little is known about their quaternary assembly.

Emerging evidence has suggested that CCCs exist as homo- and hetero-dimers. NCC, NKCC1, and NKCC2 have been shown to preferentially form dimers in cells (de Jong et al., 2003; Moore-Hoon and Turner, 2000; Starremans et al., 2003). Molecular mapping studies on NKCC1/2 have revealed the regions within their C-termini that mediate their self-interaction or homo-dimerization (Simard et al., 2004; Parvin et al., 2007; Brunet et al., 2005). In contrast to the Na^+- coupled Cl^- cotransporters, far less is known about the oligomeric state of the K^+- coupled Cl^- cotransporters, KCCs, and the regulatory mechanisms involved. Studies on KCC proteins have shown that they can also form homo- and hetero-dimers when heterologously expressed in *Xenopus laevis* oocytes, and that KCC4 can even form hetero-dimers with NKCC1 (Simard et al., 2007).

In regards to KCC2, dimer or higher order oligomer formation has been observed in both heterologous human embryonic kidney (HEK) 293 cells and brain stem lateral superior olive (LSO) neurons (Blaesse et al., 2006). Interestingly, the oligomeric state of KCC2 in LSO neurons during development has suggested a potential functional role for KCC2 oligomerization (Blaesse et al., 2006). That is, inactive monomeric KCC2 predominated in immature neurons, whereas in mature neurons active KCC2 existed as a mixture of dimers, trimers, and tetramers (Blaesse et al., 2006). Given a comparable level of protein maturation and plasma membrane expression of KCC2 between these two developmental stages in LSO neurons, this study suggests in certain types of neurons the oligomeric state of KCC2 may contribute to its functional activation during development (Blaesse et al., 2006). However, to date the molecular regions mediating KCC2 dimerization are essentially unknown, and the physiological roles for KCC2 quaternary assembly remain unclear. A previous study utilizing a yeast two-hybrid mapping strategy has suggested the KCC2 C-terminus encodes the ability to self-interact, but did not examine whether this region mediates KCC2 dimerization (Simard et al., 2007).

In the present study, we show using a combination of approaches, the regions within the KCC2 protein that mediate its dimerization. We have identified two key

regions in KCC2 C-terminus, amino acids 651-662 and 782-843 (mouse) respectively, which mediate KCC2 dimerization via forming reciprocal interactions between two KCC2 molecules. In addition, we have shown the LL657,658AA mutation is sufficient to abolish KCC2 dimerization *in vivo*.

III. MATERIALS AND METHODS

Antibodies

The following antibodies were used, mouse monoclonal anti-HA (HA.11, Covance, Berkeley, CA, USA), mouse monoclonal anti-c-Myc (generated in the lab), rabbit polyclonal anti-KCC2 (Upstate, Lake Placid, NY, USA), rabbit polyclonal anti-IL2 receptor alpha subunit (Tac; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Plasmids and constructs

The full length murine KCC2 cDNA, HA-KCC2, HA-TfRΔN-KN, Tac-KC, Tac-CD2-6, Tac-CD5(Y655A), and Tac-CD5(LL657,658AA) constructs were generated as previously reported (Zhao et al., 2008). The Tac backbone construct was kindly provided by Julie Donaldson (NIH). HA-tagged transferrin receptor (HA-TfR, Belouzard and Rouille, 2006) was kindly provided by Yves Rouille (Institut Pasteur de Lille, Lille Cedex, France). To generate Myc-KCC2, a c-Myc peptide (MEQKLISEEDL) was inserted into the unique *NotI* restriction site in the second predicted extracellular loop of KCC2 by PCR, using the forward primer (5'-ATAGGATCCGCCACCATGCTCAACAACCTGACGGAC-3') and the reverse primer (5'-TATGCGGCCGCCAGATCCTCTTCTGAGATGAGTTTTTGTTCCTCCCCACTGGCATCT-3'). This was then cloned in-frame into the *BamHI* and *NotI* sites of the untagged KCC2 construct. The Y655A and LL657, 658AA mutations were introduced into c-Myc-KCC2 by site-directed mutagenesis, using the Quick-exchange mutagenesis kit (Stratagene, La Jolla, CA, USA). To generate Tac-CD7 and Tac-CDfC constructs, we amplified parts of KCC2 carboxyl terminus by PCR, using the primers listed in Table 3.1. These were then cloned in-frame into an *XbaI* site inserted at the 3' end of the Tac cDNA. To generate GST fusion proteins containing KCC2 C-terminal deletion and mutated fragments, we amplified parts of KCC2 carboxyl terminus by PCR, using the forward primer (5´-ATAGGATCCCGGGGGGCGGAGAAGGAGT-3´) and the reverse primers listed in Table 3.1. These were then cloned in-frame into *BamHI* and *EcoRI* sites of the

pGEX-4T1 vector (Clontech, Mountain View, CA, USA). All sequences were confirmed by the McGill and Genome Quebec innovation center.

Cell culture and transfections

Human Embryonic Kidney 293 cells (HEK293) were maintained at 37 °C in DMEM (Invitrogen, Burlington, ON, Canada), 5% CO2 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and 2mM L-glutamine. Transfections were carried out with Lipofectamine 2000™ (Invitrogen). Cells were assayed 18-20 h after transfection.

Immunoprecipitation

18-20 hours post transfection, HEK293 cells were lysed in immunoprecipitation (IP) buffer (50 mM Tris·HCl pH8.0, 150 mM NaCl, 1% Triton-X-100, 0.1% SDS, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for 1 h at 4 ºC. Lysates were cleared by centrifugation at 14,000 rpm 15 min at 4 ºC. Supernatant was pre- cleared with 40 μl of Protein G-Sepharose and 1 μg of non-immune mouse IgG (Amersham, UK) 30 min at 4 ºC, and the sepharose beads were then removed by centrifugation at 6,500 rpm 2 min at 4 ºC. The pre-cleared lysates were subsequently used for immunoprecipitation. Antigen-antibody complexes were immunoprecipitated with 2 μg of anti-HA antibody, HA.11, bound to 25 μl of Protein G-Sepharose at 4 ºC for 2 h. The immunoprecipitates were then washed three times in IP buffer, eluted in Laemmli buffer at 95 ºC for 5 min, and separated on 8% SDS-PAGE gels. Bound proteins were detected by Western blotting as described previously (Heir et al., 2006) with appropriate antibodies.

GST fusion protein preparation

Regions of KCC2 C-terminus or their mutants were cloned into the pGEX-4T1 vector, as described above. *Escherichia coli* BL21 cells expressing each of these constructs were grown overnight in LB medium containing the selection antibiotics ampicillin (1 μg/ml). The overnight cultures were then diluted to 1/20 and grown for another 2-2.5 h at 37 °C until OD_{600} reaches 0.8-1.0. Cells were next induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Sigma) for 3 h at 37 °C and pelleted

at 4 °C. Cell pellets were washed in phosphate buffered saline (PBS) once, lysed in lysis buffer (50 mM Tris·HCl pH7.6, 1% Triton-X-100, 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml pepstatin], and sonicated for 4 X 15 sec at 6 microns. Solubilized cell lysates were cleared by centrifugation at 13,500 rpm for 15 min, and the supernatant was incubated with glutathione-Sepharose (Sigma) beads at 4 °C for 1 h. Beads were then washed three times in washing buffer (50 mM Tris·HCl pH8.0, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin), and finally suspended in washing buffer containing 25% glycerol. The production of the fusion proteins was subsequently analyzed by SDS-PAGE gels and Coomassie staining.

GST pull-down assay

HEK 293 cells expressing the Tac chimeric proteins were lysed in pull-down (PD) buffer [50 mM Tris·HCl pH8.0, 150 mM NaCl, 0.5% Triton-X-100, 1 mM sodium orthovanadate (Na_3VO_4), 50 mM sodium fluoride (NaF), 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin] for 1 h at 4 °C. Lysates were cleared by centrifugation at 14,000 rpm 15 min at 4 ºC. Supernatants were pre-cleared with glutathione-Sepharose and GST on beads for 30 min at 4 ºC. The pre-cleared lysates were then incubated with 15 μg of GST or GST fusion proteins on beads for 2 h at 4 ºC. Beads were then washed twice in PD buffer, once in pull-down buffer containing 300 mM NaCl, and finally once in PD buffer containing 50 mM NaCl. Bound protein complexes were eluted in Laemmli buffer at 95 ºC for 5 min, separated on 9% SDS-PAGE gels, and detected by Western blotting as described previously (Heir et al., 2006) with a specific antibodies to Tac.

IV. RESULTS

KCC2 dimerizes *in vivo*

To detect KCC2 dimerization, we employed a co-immunoprecipitation approach and used two differently tagged versions of KCC2, hemagglutinin (HA)- and c-Myc-tagged KCC2. Different than the KCC2 constructs used in other studies, where a tag was added to the end of C- or N-terminus (Blaesse et al., 2006; Simard et al., 2007), the HA or c-Myc tag in our constructs is inserted into the second predicted extracellular loop of KCC2 (Zhao et al., 2008). Generation of such KCC2 constructs precludes the likelihood of disrupting the native conformations of KCC2 cytoplasmic termini and hence their potential ability to mediate KCC2 dimerization. We have shown previously that insertion of the HA tag at this site does not interfere with the function of KCC2 (Zhao et al., 2008). We first examined whether extracellularly tagged HA- and c-Myc-KCC2 interacted *in vivo* by co-immunoprecipitation. HEK293 cells were transiently transfected with HA-KCC2 or c-Myc-KCC2 alone for control, or co-transfected with HA-KCC2 and c-Myc-KCC2. Cell lysates were either immunoprecipitated with a mouse monoclonal anti-c-Myc (αMyc) or anti-HA (αHA) antibody, and the immunoprecipitates were then immnoblotted for c-Myc and HA. As shown in Fig. 3.1, HA-KCC2 and c-Myc-KCC2 were specifically co-immunoprecipitated only in the co-transfected cell lysates and not in the control, suggesting that the extracellularly tagged KCC2 proteins dimerize *in vivo* in our assay system.

KCC2 C-terminus mediates dimerization

The C-termini were shown to be required for the self-interaction/dimerization of NKCC1/2, and their self-interaction/dimerization fragments have been mapped in this region (Moore-Hoon et al., 2000; Simard et al., 2004; Parvin et al., 2007; Brunet et al., 2005). Similar to NKCCs, the C-termini of all the KCCs have also been shown to be required for their self-interaction, whereas the N-termini are dispensable (Simard et al., 2007). However, to date the role of the KCC2 cytoplasmic termini in its dimerization remains unclear, and the defined regions mediating KCC2 dimerization have not been mapped.

Therefore, we set out to examine whether either the intracellular N- or C-terminus of KCC2 contains regions mediating the dimerization of KCC2. For this, we took advantage of two chimeric proteins, HA-TfRΔN-KN and Tac-KC, where KCC2 N- or C-terminus was fused to the N-terminus deletion of the transferrin receptor (TfRΔN, HA-tagged) or C-terminus deletion of the interleukin 2 receptor α-chain (Tac), as reported previously (Zhao et al., 2008, Fig. 3.2B and C). We assessed the interacting abilities of these chimeric proteins with the full-length KCC2 protein by co-immunoprecipitation. For the N-terminus, HEK293 cells were co-transfected with KCC2 (un-tagged) and HA-TfRΔN-KN. For controls, cells were either untransfected (UT), or transfected with HA-TfRΔN, HA-TfRΔN-KN, or KCC2 alone, or co-transfected with KCC2 (un-tagged) and HA-TfRΔN. Lysates were immunoprecipitated with αHA and the immunoprecipitates were then immunoblotted with either αHA or a rabbit polyclonal anti-KCC2 antibody (αKCC2, Upstate, USA), which was raised against a sequence within the distal portion of KCC2 C-terminus. As shown in Fig. 3.3A, KCC2 was not observed in the control αHA immunoprecipitates as expected, nor was it observed in the immunoprecipitates from the lysates co-expressing KCC2 and HA-TfRΔN-KN, suggesting that KCC2 N-terminus does not contain regions mediating KCC2 dimerization.

We next examined whether KCC2 C-terminus contains dimerization region(s). For this, HEK293 cells were co-transfected with c-Myc-KCC2 and Tac-KC. For controls, cells were either untransfected (UT), or transfected with Tac or Tac-KC alone, or co-transfected with c-Myc-KCC2 and Tac. Cell lysates were immunoprecipitated with αMyc, and the immunoprecipitates were then immunoblotted with either αMyc or a rabbit polyclonal anti-Tac antibody (αTac). As shown in Fig. 3.3B, Tac-KC was specifically observed in the immunoprecipitates from c-Myc-KCC2 and Tac-KC co-expressing cells, suggesting the KCC2 C-terminus endows KCC2 the ability to dimerize. Together, the above results suggest KCC2 C-terminus, but not N-terminus, encodes the molecular information to mediate KCC2 dimerization.

Identification of a proximal dimerization region in the KCC2 C-terminus

Previous reports have shown that KCC2 undergoes both constitutive and regulated endocytosis (Rivera et al., 2004; Lee et al., 2007; Wake et al., 2007; Zhao et al., 2008).

Given that dimerization impacts the endocytosis of a wide range of plasma membrane proteins (Milligan, 2008; Wang et al., 2005; Heldin, 1995), we postulated there might also be a link between KCC2 dimerization and endocytosis. If this was the case, we expected that the endocytic motif containing region in KCC2 might also be involved in the control of its dimerization. We have previously shown the KCC2 C-terminal fragment, amino acids (a.a.) 651-662 (fragment A, Fig. 3.4A), contains an adaptor protein 2 (AP-2) binding motif that mediates KCC2 constitutive endocytosis (Zhao et al., 2008). Therefore, we examined whether fragment A played a role in the dimerization-mediating function of KCC2 C-terminus. For this, we employed a glutathione-S-transferase (GST) pull-down assay, using KCC2 CD5 (which includes fragment A) or CD6 (which excludes fragment A) fragment as the bait (Fig. 3.4A), and tested their interaction with the KCC2 C-terminus using the chimeric protein, Tac-KC (Fig. 3.4A and B). Cells were transfected with Tac-KC, and the cell lysates were incubated with GST (control), GST-CD5, or GST-CD6 immobilized on glutathione-Sepharose beads. The bound proteins were detected by immunoblotting for Tac. As shown in Fig. 3.4B, Tac-KC is specifically bound to GST-CD5, but not to GST or GST-CD6, suggesting that fragment A may be a locus where KCC2 dimerization occurs.

Identification of a second dimerization region in the central KCC2 C-terminus

We next examined whether fragment A interacted with KCC2 C-terminus through binding to itself or to a distinct region. Cell lysates from either untransfected, or Tac, Tac-KC, Tac-CD5, or Tac-CD6 transfected cells were incubated with GST-CD5, and bound proteins were then detected by immunoblotting for Tac. As shown in Fig. 3.4C, GST-CD5 bound Tac-KC, but not Tac-CD5, Tac-CD6, or Tac, therefore excluding the possibility that fragment A self-interacts (Fig. 3.4D). Thus, this result suggests fragment A interacts with KCC2 C-terminus through binding to at least one other distinct region.

In order to identify the region(s) in KCC2 C-terminus that interacts with fragment A, we tested the ability of GST-CD5 to interact with a series of progressive carboxyl truncations of Tac-KC (Tac-CD2-4, Fig. 3.4A and 3.5A). As shown in Fig. 3.5A, GST-CD5 bound Tac-KC and Tac-CD2, but not Tac-CD3 or Tac-CD4, Fig. 3.5A), suggesting the region between a.a. 717-843 binds fragment A. To further delineate the fragment A-

interacting region, we generated Tac-CD7, which contains a.a. 636-781 (Fig. 3.4A), and then assessed its interaction with the CD5 fragment. As shown in Fig. 3.5B, GST-CD5 specifically bound Tac-CD2 as expected, but not Tac-CD7. Further mapping of the fragment A interacting region within fragment B was not successful due to insolubility of the Tac constructs. Together, these results delineate a.a. 782-843 (fragment B, Fig. 3.4A) as the minimal identified region that interacts with fragment A. However, this result did not exclude the possibility of an additional fragment A-binding site between the end of fragment B and the end of the KCC2 C-terminus, a.a. 844-1115 (fragment C, Fig. 3.4A). To test this, we generated a Tac chimera containing fragment C (Tac-CDfC, Fig. 3.4A), and examined its ability to interact with fragment A. As shown in Fig. 3.5C, no binding between these two fragments was observed, therefore excluding the existence of an additional site within KCC2 C-terminus, besides fragment B, that interacts with fragment A. Together, our results so far have revealed two distinct interacting regions within KCC2, fragments A and B in the proximal and central C-terminus respectively, that may mediate KCC2 dimerization. In addition, we show that fragment A does not self-interact but interacts with fragment B. However, whether fragment B self-interacts remained unknown at this stage of our analysis (Fig. 3.5D).

The LL657,658AA mutation abolishes KCC2 dimerization *in vivo*

Within fragment A, we have previously shown $_{657}LL_{658}$ is essential for the constitutive endocytosis of KCC2 (Zhao et al., 2008). Within the same region, there is also a good candidate tyrosine (Y)-based endocytic sequence $_{655}YALL_{658}$ (Fig. 3.6A), which conforms to the classical endocytic motif, YxxΦ (where "x" represents any amino acid and "Φ" is a bulky hydrophobic amino acid). Although we did not detect any effect of Y_{655} on KCC2 constitutive endocytosis, we previously postulated it may have alternative targeting functions for KCC2 (Zhao et al., 2008). The ability of fragment A to mediate both KCC2 dimerization (Fig. 3.4B) and constitutive endocytosis implicated a potential link between these two processes. If this was the case, then we expected that the endocytic motif in KCC2 may also have a function in mediating its dimerization. Therefore, we decided to examine the effect of both Y_{655} and $_{657}LL_{658}$ on the interaction of fragment A with KCC2 C-terminus. For this, we employed a site-directed mutagenesis

approach to specifically mutate Y_{655} and $_{657}LL_{658}$ to alanine (A) in the context of GST-CD5, generating GST-CD5(Y655A) and GST-CD5(LL657,658AA) respectively (Fig. 3.6A), and assessed the interaction of these mutants with Tac-KC. As shown in Fig. 3.6B, both mutations completely abolished the interaction of fragment A with Tac-KC, suggesting that Y_{655} and $_{657}LL_{658}$ are both required for fragment A to interact with fragment B.

To determine whether these residues are also crucial for KCC2 dimerization *in vivo*, we introduced the Y655A or LL657,658AA mutation into each of HA-KCC2 and c-Myc-KCC2, generating the HA-KCC2 (Y655A), HA-KCC2 (LL657,658AA), c-Myc-KCC2 (Y655A), and c-Myc-KCC2 (LL657,658AA) constructs, and then assessed the interaction between each of these HA-KCC2 variants with its corresponding c-Myc-KCC2 variant by co-immunoprecipitation. For this, each c-Myc-KCC2 variant was transfected alone (control), or co-transfected with its HA-KCC2 counterpart in HEK293 cells. Cell lysates were immunoprecipitated with αHA, and the immunoprecipitates were then immunoblotted for HA and c-Myc. As expected, the wild-type c-Myc-KCC2 and HA-KCC2 were specifically co-immunoprecipitated (Fig. 3.6C and D). We did not detect any effect of Y655A on their interaction (Fig. 3.6C), suggesting that Y_{655} is not important for the full-length KCC2 dimerization under our conditions examined. In contrast, the LL657,658AA mutation completely abolished the interaction between c-Myc- and HA-KCC2 (Fig. 3.6D), strongly suggesting the $_{657}LL_{658}$ residues play an essential role in mediating KCC2 dimerization *in vivo*. In addition, this result also suggests fragment B does not interact with itself but rather with fragment A, because otherwise disrupting fragment A by the LL657,658AA mutation would have been insufficient to block KCC2 dimerization (Fig. 3.6E). In addition, HA-KCC2(LL657,658AA) dimerized normally with the wild-type Myc-KCC2 (data not shown), suggesting the blockade of KCC2 dimerization requires the LL657,658AA mutation to be present in both KCC2 molecules.

V. DISCUSSION

KCC2 has been established as an essential protein in the vast majority of CNS neurons for the development and maintenance of inhibitory neurotransmission mediated by $GABA_A$ and glycine receptors (Rivera et al., 1999; Hubner et al., 2001; Coull et al., 2003; Nabekura et al., 2002; Woo et al., 2002). While gene expression is undoubtedly essential for KCC2 function, accumulating evidence suggests post-translational mechanisms may also play a crucial role in controlling KCC2 function (Rivera et al., 2004; Wake et al., 2007; Lee et al., 2007; Blaesse et al., 2006). KCC2 has been shown to form oligomers in neurons and heterologous HEK293 cells, and it is suggested that the oligomeric state of KCC2 may potentially play a role in its functional activation during the development of certain types of neurons (Blaesse et al., 2006). The molecular mechanisms, in particular the molecular determinants, that mediate KCC2 quaternary assembly however remain elusive. In this study, we mapped the regions that mediate KCC2 dimerization. We show that (i) KCC2 C-terminus, but not N-terminus, mediates KCC2 dimerization, (ii) two regions, namely fragment A (a.a. 651-662) and fragment B (a.a. 782-843) in the proximal and central KCC2 C-terminus respectively, are the key regions mediating KCC2 dimerization, (iii) these two fragments can form reciprocal interactions between two KCC2 molecules, and (iv) the LL657,658AA mutation is sufficient to abolish KCC2 dimerization *in vivo*, most likely through the interference of a potential α-helical interaction between fragments A and B.

A yeast two-hybrid mapping study previously suggested the KCC2 C-terminus is required for its self-interaction (Parvin et al., 2007), but to date the defined regions mediating KCC2 dimerization have not been identified. Given that yeast two-hybrid studies may sometimes result in false positive or negative binding results that contradict what occurs in the native state (Immink et al., 2002; Parrish et al., 2006), it was essential to examine the molecular mechanisms mediating protein dimerization using an alternative methodology. Using a combination of approaches, we for the first time report the defined regions mediating KCC2 dimerization in mammalian cells. Our molecular mapping study is also the first to identify discrete dimerization regions in any KCC member protein. Previous studies on NKCC1/2 located region(s) in their respective C-terminus that

conveys the capacity for their homo-dimerization and self-interaction (Simard et al., 2004; Parvin et al., 2007; Brunet et al., 2005). The length of the regions identified in these studies was 247 residues for NKCC1, and 146 and 189 residues for NKCC2 (Simard et al., 2004; Parvin et al., 2007; Brunet et al., 2005). As the NKCC1 dimerization region identified includes half of the NKCC1 C-terminus, it remains possible two smaller fragments exist in this region to mediate NKCC1 dimerization. In comparison to these studies, our mapping study has revealed two much narrower dimerization regions in KCC2, fragments A and B, which contain 12 and 62 residues respectively. In addition, we have generated a dimerization blocking mutant of KCC2 by mutating only two amino acids, $_{657}LL_{658}$ within fragment A, and we propose these two residues are likely involved in the formation of α-helical interaction (see below).

Our results show that fragment A and B interact between two KCC2 molecules (Fig. 3.4 and 3.5). However, neither fragment self-interacts (Fig. 3.4 and 3.6). Therefore, we propose a model for the dimer formation of KCC2, as illustrated in Fig. 3.7A, wherein fragment A of each KCC2 molecule would intermolecularly interact with fragment B of the other KCC2 molecule. Studies on the NKCC1 protein suggest distinct regions in its C-terminus may be responsible for intra and intermolecular interactions (Simard et al., 2004; Parvin et al., 2007). In the present study we did not address whether KCC2 forms intramolecular interactions and whether fragment A and B are similarly responsible for KCC2 intramolecular interactions (if any) or the formation of higher order oligomers. However, the existence of two distinct dimerization regions in KCC2 (fragments A and B) endows the advantage and likelihood for KCC2 to form higher order oligomers. This is because the reciprocal interactions between these two distinct dimerization regions allow the recruitment of as many KCC2 molecules as possible to an oligomeric complex, see Fig. 3.7B. It will be of future interest to address how intramolecular interactions occur and how KCC2 forms higher order oligomers.

What is the impact of KCC2 oligomerization on its physiological function?

Although it is now known that NCC, NKCCs, and KCCs all exist as or possess the capacity to oligomerize (de Jong et al., 2003; Starremans et al., 2003; Brunet et al., 2005; Simard et al., 2007, Blaesse et al., 2006), the functional significance of their

oligomerization remains poorly understood. NCC and NKCC1 have been shown to function as a monomer and dimer respectively, although they both exist as a homo-dimer (de Jong et al., 2003; Starremans et al., 2003). In regards to KCC2, no direct evidence of the functional impacts of KCC2 oligomerization on its transport activity has been provided. However, a potential link between the oligomeric state of KCC2 and its functional expression in LSO neurons has been implicated (Blaesse et al., 2006). The dimerization blocking mutants we described here, HA- and c-Myc-KCC2(LL657,658AA), will be useful tools for characterizing the role of KCC2 homo- and hetero-oligomerization in mediating its function and resolving the functional unit of KCC2.

What are the molecular mechanisms mediating the structure of KCC2?

As mentioned above, a role for specific regions and residues within KCC2 C-terminus in the control of KCC2 transporter activity has been reported (Strange et al., 2000; Bergeron et al., 2006; Mercado et al., 2006). However, the molecular mechanisms involved remain elusive. It is postulated that either acquired conformations or specific protein-protein interactions are involved. The secondary structures of a protein, such as α-helices and β-sheets, are the structural elements of a protein and hence are the basis for the organization of quaternary complexes. The majority of dimerization mapping studies suggests helix-helix interactions predominantly mediate dimer formation.

We utilized an online resource, J-pred program (http://www.compbio.dundee.ac.uk/~www-jpred/), to predict the secondary structural content of the KCC2 C-terminal region between a.a. 651-867, which include both fragments A and B. Firstly, we found that a major difference between CD5 (which contains fragment A) and CD6 fragment (which lacks fragment A) is the presence of an α-helical stretch in the former but not the latter (Fig. 3.8). "LxxxxxxL" is known as a commonly used dimerization motif by a number of transporter proteins, although it is usually present as heptad repeats (Sitte, 2003). Amino acid sequence alignment of CD5 and CD6 fragments reveals the sequence "$_{650}$LSAARYALL$_{658}$", which is present in the former but not the latter (Fig. 3.6A), conforms perfectly to this "LxxxxxxL" dimerization motif. Therefore, the dimerization blocking mutation, LL657,658AA, most likely exerts

its effect by disrupting this α-helical structure in CD5. Secondly, we found a complete α-helical stretch (a.a. 787-801) in fragment B. Thus, we speculate that these predicated α-helices in fragments A and B may be the secondary structural basis contributing to the acquired conformations of KCC2 C-terminus that facilitate the interaction between these two fragments and hence KCC2 dimerization. Crystallography and nuclear magnetic resonance (NMR) studies could be useful in revealing how precisely fragments A and B interact and the role of the identified amino acids $_{657}LL_{658}$ within the defined structure.

What is the function of Y_{655}?

An additional interesting finding from this mapping study was that although Y_{655} is essential for the binding of fragment A to KCC2 C-terminus in the GST pull-down assays (Fig. 3.6B), it does not have any effect on the dimerization of the full-length KCC2 protein as detected in the co-immunoprecipitation experiment (Fig. 3.6C). One possibility for this discrepancy is that when in the context of the full-length KCC2 protein Y_{655} is less important for maintaining the conformation of fragment A required for its interaction with the C-terminus. However, when in the context of the much shorter CD5 fragment (GST-CD5), this tyrosine becomes vital, presumably as the conformations of the same protein fragment can differ when placed under different structural constraints. Alternatively, the GST pull-down conditions we used may happen to resemble certain physiological conditions where besides $_{657}LL_{658}$, Y_{655} is also essential for KCC2 dimerization. These conditions could be those where the tyrosine phosphorylation state of Y_{655} may be a key factor influencing the ability of KCC2 to dimerize, although it remains unknown whether Y_{655} is a target for phosphorylation in any situation. Therefore it will be of future interest to examine whether Y_{655} is a target for phosphorylation, and if yes whether its phosphorylation state affects the ability of KCC2 to dimerize.

In conclusion, KCC2 oligomerization may represent an additional post-translational mechanism, besides membrane trafficking, by which KCC2 function and the strength of inhibitory neurotransmission may be controlled. Our studies defining the key regions and residues that mediate KCC2 dimerization are an initial step in understanding the structural determinants by which the quaternary assembly of KCC2 and related CCC member proteins may be regulated. Furthermore, given the conservation of $_{657}LL_{658}$

amongst K^+-coupled Cl^- cotransporters but absence from the functionally more divergent Na^+-coupled Cl^- cotransporters, our studies provide further implication of how these related CCC family member proteins may be differentially regulated at the post-translational level.

Figure 3.1. KCC2 dimerizes *in vivo*. Whole cell lysates from c-Myc-KCC2 (Myc), HA-KCC2 (HA), and HA-KCC2 + c-Myc-KCC2 (HA + Myc) transiently expressing HEK293 cells were immunoprecipitated (IP) with an antibody to HA (αHA, left) or c-Myc (αMyc, right), and Western blotted (WB) with antibodies to HA or c-Myc. HA-KCC2 and c-Myc-KCC2 co-immunoprecipitated only in the co-transfected cell lysates but not in the individually transfected cell lysates.

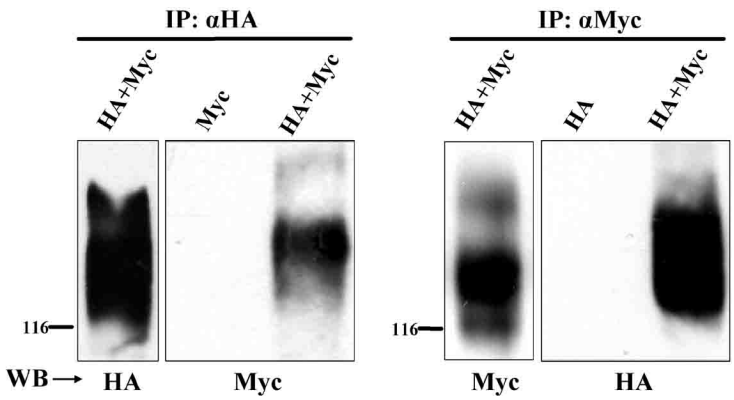

IP: αHA
IP: αMyc
HA+Myc
Myc
HA+Myc
HA+Myc
HA
HA+Myc
116
116
WB →
HA
Myc
Myc
HA

Figure 3.2. Schematic illustration of the predicted topology of KCC2, and KCC2 N- and C-terminus chimeras. (A) Schematic illustration of the predicted topology of KCC2. (B) Schematic illustration of HA-tagged Transferrin receptor (HA-TfR, left) and HA-TfR-ΔN KCC2 N-tail chimera [HA-TfRΔN-KN (Zhao et al., 2008), right]. The N-tail (amino acids 3-95) of TfR was replaced with KCC2 N-tail (amino acids 2-102) to produce HA-TfRΔN-KN. (C) Schematic illustration of the interleukin receptor 2 alpha subunit intracellular truncation mutant (Tac, left) and the Tac/KCC2 C-tail chimera [Tac-KC (Zhao et al., 2008), right]. The KCC2 C-tail (amino acids 635-1115) was fused to the C-terminus of Tac to produce Tac-KC.

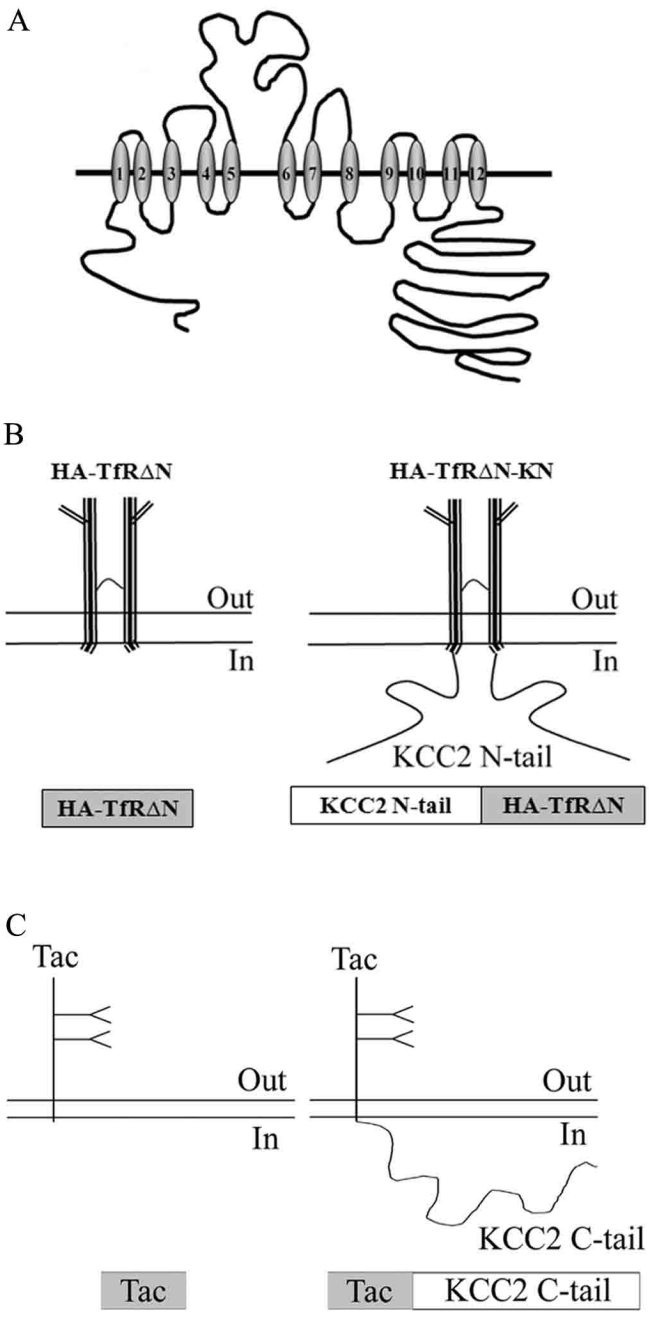

A
1
2
3
4
5
6
7
8
9
10
11
12
B
HA-TfRΔN
Out
In
HA-TfRΔN
HA-TfRΔN-KN
Out
In
KCC2 N-tail
KCC2 N-tail
HA-TfRΔN
C
Tac
Out
In
Tac
Tac
Out
In
KCC2 C-tail
Tac
Tac
KCC2 C-tail

Figure 3.3. KCC2 C-terminus mediates KCC2 dimerization. (A) KCC2 N-terminus does not contain dimerization region. Whole cell lysates from untransfected (UT), HA-TfRΔN, HA-TfRΔN-KN, KCC2 (K), HA-TfRΔN+KCC2 (HA-TfRΔN+K), and HA-TfRΔN-KN+KCC2 (HA-TfRΔN-KN+K) transiently expressing HEK293 cells were immunoprecipitated (IP) with an antibody to HA (αHA, right), and Western blotted (WB) with antibodies to HA (top right) or KCC2 (bottom right). Input represents 1% of the total expression of each transfection. No co-immunoprecipitation was observed in any of the immunoprecipitates. (B) KCC2 C-terminus encodes the dimerization capacity. Whole cell lysates from untransfected (UT), Tac, Tac-KC, c-Myc-KCC2+Tac (Myc-K+Tac), and c-Myc-KCC2+Tac-KC (Myc-K+Tac-KC) transiently expressing HEK293 cells were immunoprecipitated (IP) with an antibody to c-Myc (αMyc, right), and Western blotted (WB) with antibodies to c-Myc or Tac. Input represents 1% of the total expression of each transfection. Tac immunoreactivity was detected only in the c-Myc-KCC2 and Tac-KC co-expressing cell lysates.

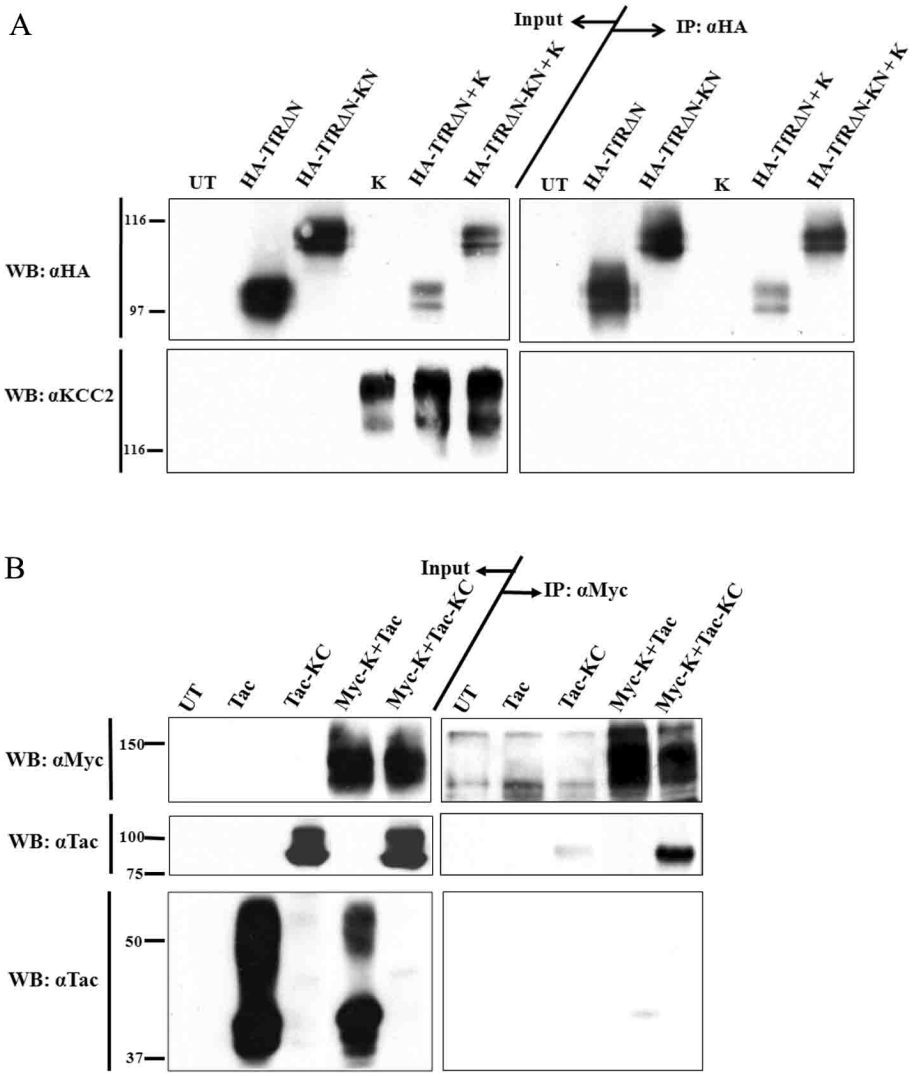
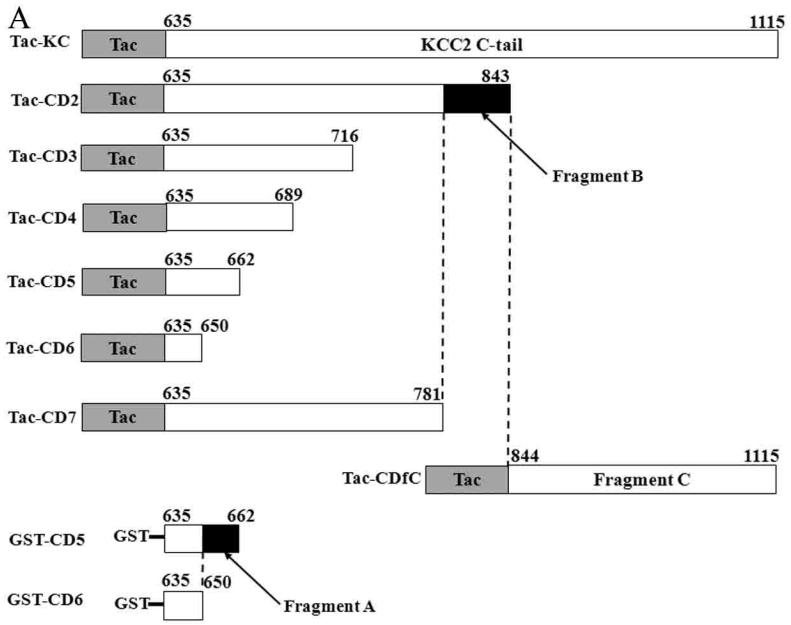
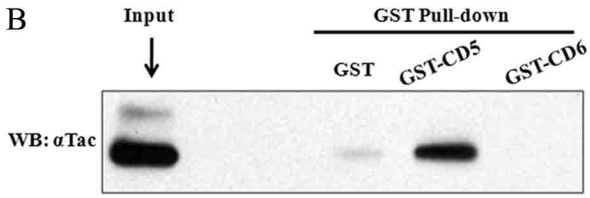
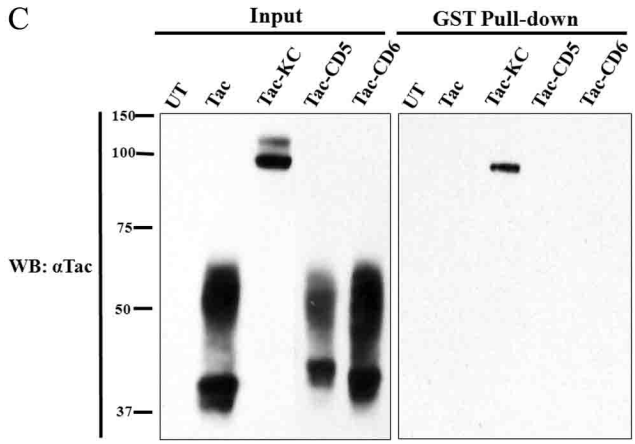

A
Input
IP: αHA
UT
HA-TfRΔN
HA-TfRΔN-KN
K
HA-TfRΔN+K
HA-TfRΔN-KN+K
WB: αHA
WB: αKCC2
116
97
B
Input
IP: αMyc
UT
Tac
Tac-KC
Myc-K+Tac
Myc-K+Tac-KC
WB: αMyc
WB: αTac
150
100
75
50
37

Figure 3.4. Identification of the proximal dimerization region in KCC2 C-terminus. (A) Schematic illustration of the Tac-KC truncation chimeras [Tac-KC, Tac-CD2, CD3, CD4 (Zhao et al., 2008), Tac-CD7, and Tac-CDfC] and KCC2 C-terminal truncation GST fusion proteins (GST-CD5 and GST-CD6). The KCC2 C-terminal amino acids contained in each construct, and the regions defining fragment A, B, and C are shown. (B) GST pull-down assays mapping the interacting region in CD5 with KCC2 C-terminus. Whole cell lysates from Tac-KC transiently expressing HEK293 cells were incubated with GST, GST-CD5, or GST-CD6 immobilized on glutathione-Sepharose beads. The bound proteins were then Western blotted (WB) with an antibody to Tac (αTac). Input represents 1% of the total Tac-KC expression from one transfection. Note Tac-KC is specifically bound to GST-CD5, but not to GST or GST-CD6. (C) GST pull-down assay testing the self-interaction of fragment A. Whole cell lysates from untransfected (UT), or Tac, Tac-KC, Tac-CD5, and Tac-CD6 transiently expressing HEK293 cells were incubated with GST-CD5 immobilized on glutathione-Sepharose beads. The bound proteins were then Western blotted (WB) with an antibody to Tac (αTac). Input represents 1% of the total expression of each transfection. Note only Tac-KC is bound to GST-CD5. (D) Schema illustrating fragment A does not self-interact. Only the C-terminus of KCC2 is shown.


A
Tac-KC
635
1115
Tac
KCC2 C-tail
Tac-CD2
843
Tac-CD3
716
Tac-CD4
689
Tac-CD5
662
Tac-CD6
650
Tac-CD7
781
Fragment B
Tac-CDfC
844
Fragment C
GST-CD5
GST
GST-CD6
Fragment A


B
Input
GST Pull-down
GST
GST-CD5
GST-CD6
WB: αTac


C
Input
GST Pull-down
UT
Tac
Tac-KC
Tac-CD5
Tac-CD6
150
100
75
50
37
WB: αTac

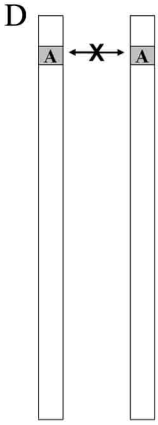

D
A
A

Figure 3.5. Identification of the central dimerization region in KCC2 C-terminus. (A) GST pull-down assays mapping the CD5 interacting region in KCC2 C-terminus. Whole cell lysates from Tac-KC, Tac-CD2, Tac-CD3, and Tac-CD4 transiently expressing HEK293 cells were incubated with GST or GST-CD5 immobilized on glutathione-Sepharose beads. The bound proteins were then Western blotted (WB) with an antibody to Tac (αTac). Input represents 1% of the total expression of each transfection. Note Tac-KC and Tac-CD2 are specifically bound to GST-CD5, but not Tac-CD3 or Tac-CD4. (B) GST pull-down assays assessing the interaction of CD5 with CD7. Whole cell lysates from Tac-CD2 and Tac-CD7 transiently expressing HEK293 cells were incubated with GST or GST-CD5 immobilized on glutathione-Sepharose beads. The bound proteins were then Western blotted (WB) with an antibody to Tac (αTac). Input represents 1% of the total expression of each transfection. Note Tac-CD2 is specifically bound to GST-CD5, but not Tac-CD7. (C) GST pull-down assays assessing the interaction of CD5 with CDfC. Whole cell lysates from Tac-CD2 and Tac-CDfC transiently expressing HEK293 cells were incubated with GST or GST-CD5 immobilized on glutathione-Sepharose beads. The bound proteins were then Western blotted (WB) with an antibody to Tac (αTac). Input represents 1% of the total expression of each transfection. Note Tac-CD2 is specifically bound to GST-CD5, but not Tac-CDfC. (D) Schema illustrating fragments A and B interact. Only the C-terminus of KCC2 is shown. Fragments A (grey) and B (black) interact reciprocally between two KCC2 molecules. Whether fragment B self-interacts is unknown at this stage.

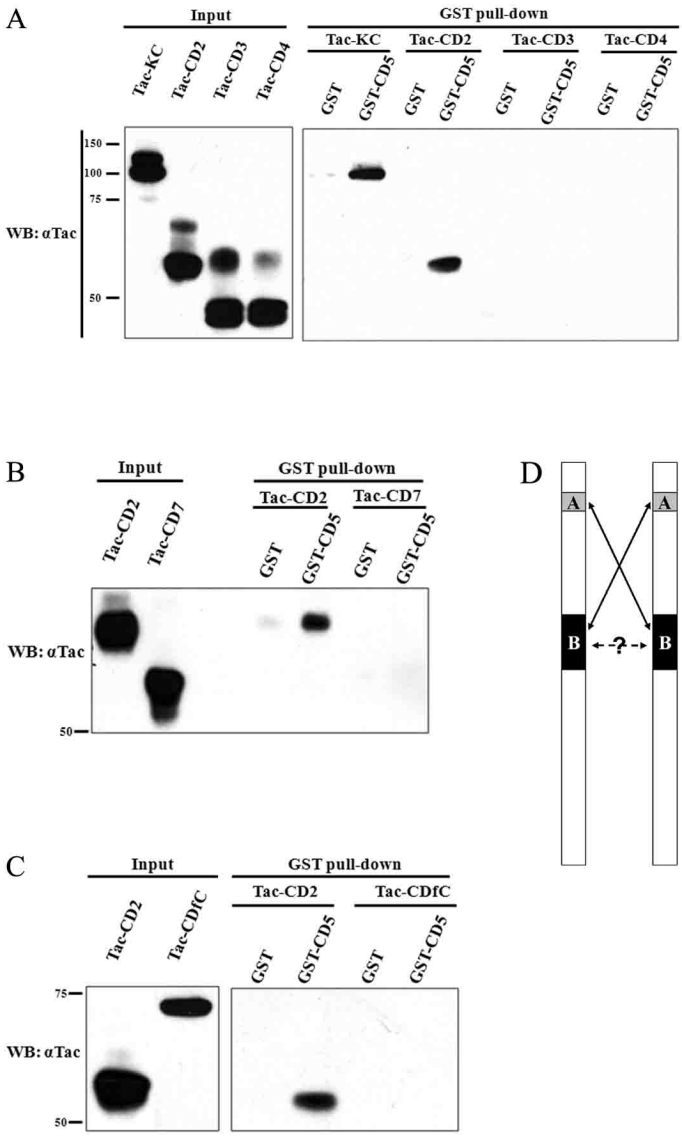

A
Input
GST pull-down
Tac-KC
Tac-CD2
Tac-CD3
Tac-CD4
GST
GST-CD5
WB: αTac
150
100
75
50
B
Input
GST pull-down
Tac-CD2
Tac-CD7
WB: αTac
50
C
Input
GST pull-down
Tac-CD2
Tac-CDfC
75
50
D
A
B
?

Figure 3.6. The LL657,658AA mutation abolishes KCC2 dimerization *in vivo*. (A) *Top*, alignment of the KCC2 amino acid sequence in the CD5 (a.a. 635-662) and CD6 (a.a. 635-650) fragments of KCC2. The amino acids examined are in bold. *Bottom*, alignment of the different GST-CD5 point mutation mutants. The alanine-substituted amino acid residues are underlined. The amino acid sequence of Fragment A is shaded. (B) GST pull-down assays assessing the interaction of the different CD5 point mutation mutants with the KCC2 C-terminus. Whole cell lysates from Tac-KC transiently expressing HEK293 cells were incubated with GST, GST-CD5 wild-type (WT), GST-CD5(Y655A) (Y/A), or GST-CD5 (LL657,658AA) (LL/AA) immobilized on glutathione-Sepharose beads. The bound proteins were then Western blotted (WB) with an antibody to Tac (αTac). Input represents 1% of the total Tac-KC expression from one transfection. Note Tac-KC is specifically bound to GST-CD5, but not to GST-CD5(Y655A) or GST-CD5(LL657,658AA). (C) The Y655A mutation had no effect on KCC2 dimerization. Whole cell lysates from c-Myc-KCC2 (Myc), c-Myc-KCC2+HA-KCC2(Myc+HA), c-Myc-KCC2(Y655A) [Myc(Y/A)], and c-Myc-KCC2(Y655A)+HA-KCC2(Y655A) [Myc(Y/A)+HA(Y/A)] transiently expressing HEK293 cells were immunoprecipitated (IP) with an antibody to HA (αHA, right), and Western blotted (WB) with antibodies to HA (top right) or c-Myc (bottom right). Input represents 1% of the total expression of each transfection. Note that similar to the wild-type c-Myc- and HA-KCC2 proteins, c-Myc-KCC2(Y655A) was specifically co-immunoprecipitated with HA-KCC2(Y655A). (D) The LL657,658AA mutation blocked KCC2 dimerization. Whole cell lysates from c-Myc-KCC2 (Myc), c-Myc-KCC2+HA-KCC2 (Myc+HA), c-Myc-KCC2 (LL657,658AA) [Myc(LL/AA)], and c-Myc-KCC2(LL657,658AA)+HA-KCC2(LL657,658AA) [Myc(LL/AA)+HA(LL/AA)] transiently expressing HEK293 cells were immunoprecipitated (IP) with an antibody to HA (αHA, right), and Western blotted (WB) with antibodies to HA (top right) or c-Myc (bottom right). Input represents 1% of the total expression of each transfection. Note that in contrast to the wild-type c-Myc- and HA-KCC2 proteins, no c-Myc-KCC2(LL657,658AA) was co-immunoprecipitated with HA-KCC2(LL657,658AA). (E) Schema illustrating that the LL657,658AA mutation abolishes KCC2 dimerization and that fragment B does not self-interact. Only the C-

terminus of KCC2 is shown. Fragment B and LL657,658AA containing fragment A (A') are indicated as black and dashed boxes respectively.

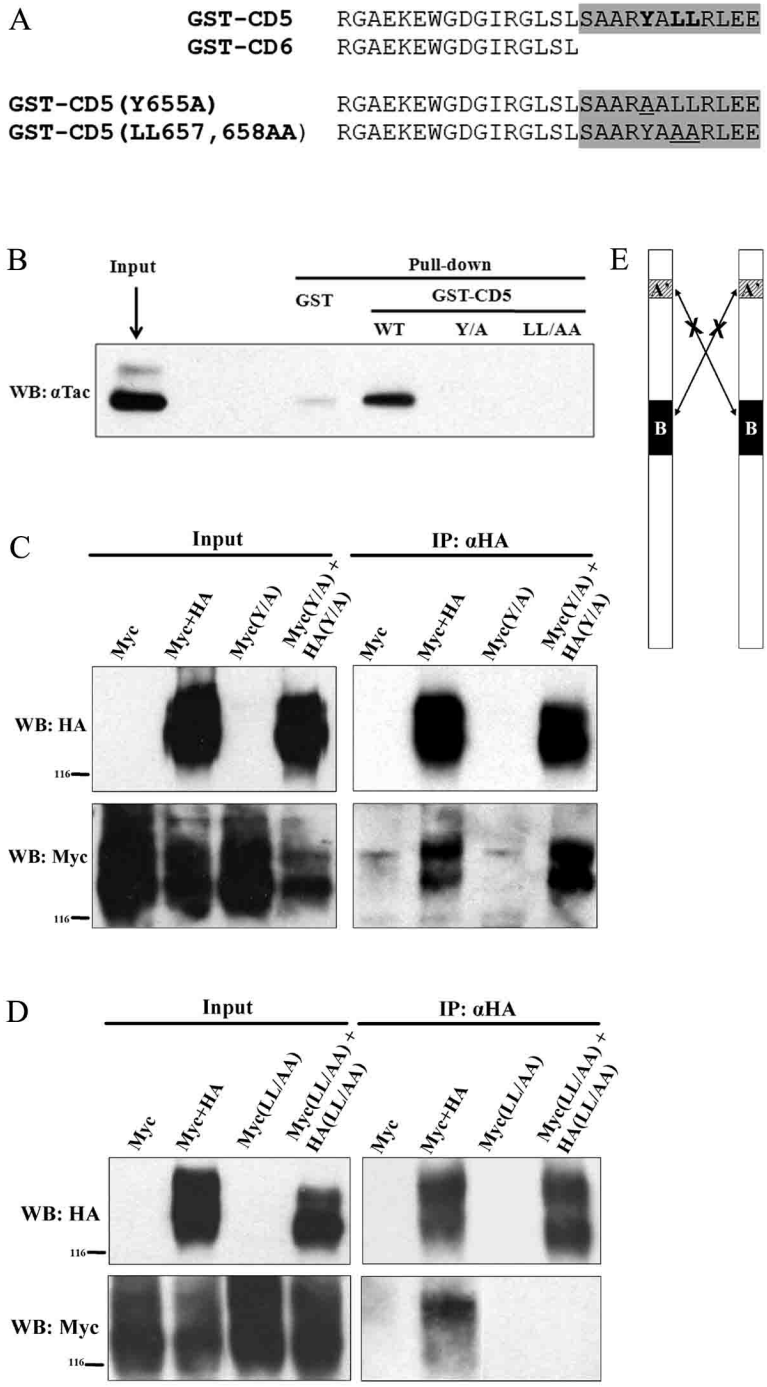
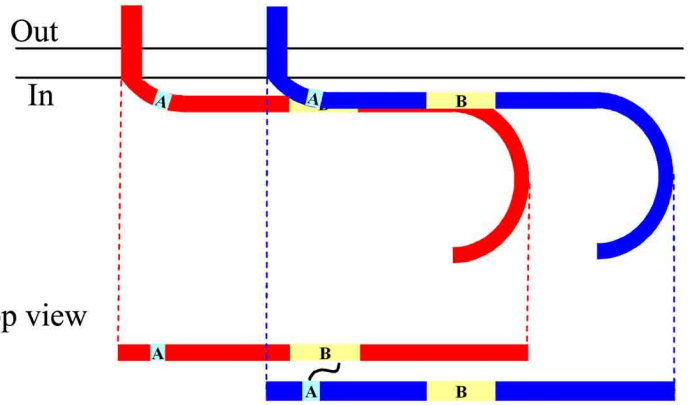

A
GST-CD5 RGAEKEWGDGIRGLSLSAARYALLRLEE
GST-CD6 RGAEKEWGDGIRGLSL
GST-CD5(Y655A) RGAEKEWGDGIRGLSLSAARAALLRLEE
GST-CD5(LL657,658AA) RGAEKEWGDGIRGLSLSAARYAARLEE
B
Input
Pull-down
GST
GST-CD5
WT
Y/A
LL/AA
WB: αTac
E
A
B
C
Input
IP: αHA
Myc
Myc+HA
Myc(Y/A)
Myc(Y/A) + HA(Y/A)
WB: HA
WB: Myc
116
D
Input
IP: αHA
Myc
Myc+HA
Myc(LL/AA)
Myc(LL/AA) + HA(LL/AA)
WB: HA
WB: Myc
116

Figure 3.7. Schematic illustration of a hypothetical configuration of KCC2 oligomerization based on the identified interacting regions within the KCC2 proximal and central C-terminus. Only the C-terminus and the last transmembrane segment of KCC2 are shown. Each colour represents a KCC2 molecule. (A) Side and top views of the potential KCC2 dimerization configuration. The fragments A and B in the proximal and central C-terminus of one KCC2 molecule reciprocally interact with the fragments B and A respectively of another KCC2 molecule. (B) Side and top views of the potential oligomerization configuration by four KCC2 molecules (shown in red, blue, green, and purple). The fragment A and B in one KCC2 molecule reciprocally interacts with the fragment B and A respectively of another KCC2 molecule.

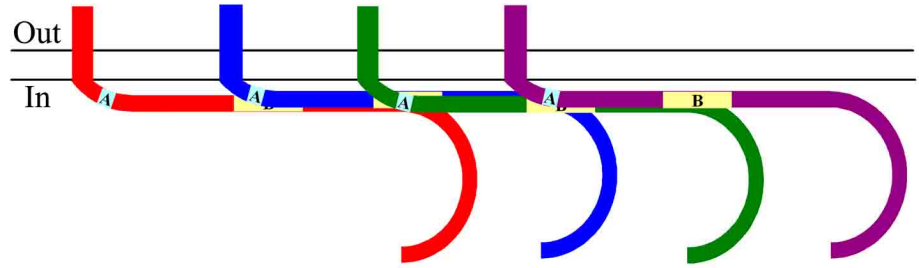
A

Side view



B

Side view



Top view

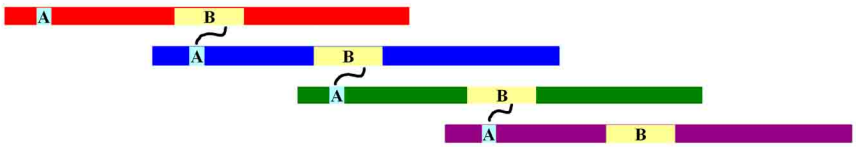


Figure 3.8. Secondary structural prediction of the KCC2 region containing amino acids 635-867 using the J-pred program (http://www.compbio.dundee.ac.uk/~www-jpred/). "H" and "E" symbolize α-helix and β-sheet, respectively. Fragments A and B are shaded.

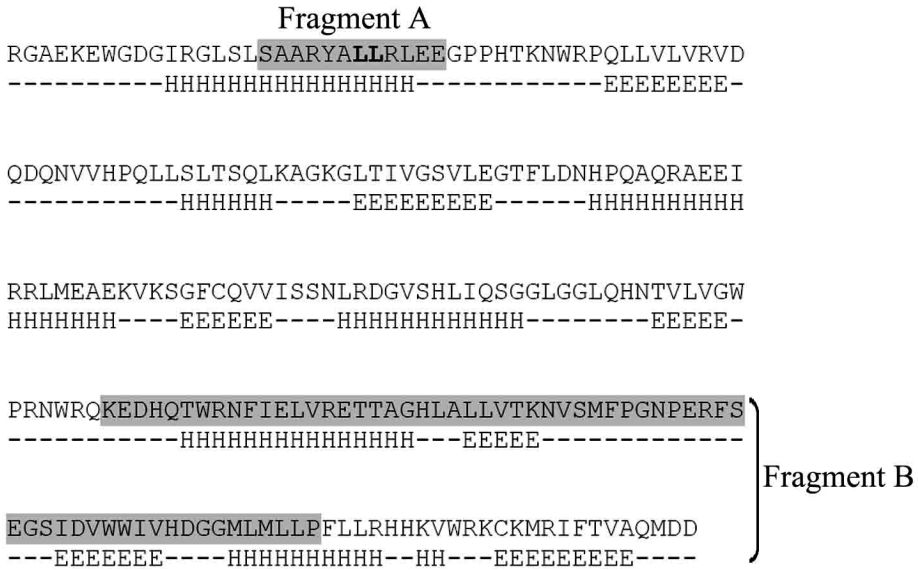

Fragment A
RGAEKEWGDGIRGLSLSAARYALLRLEEGPPHTKNWRPQLLVLVRVD
----------HHHHHHHHHHHHHHHH------------EEEEEEEE-
QDQNVVHPQLLSLTSQLKAGKGLTIVGSVLEGTFLDNHPQAQRAEEI
-----------HHHHHH-----EEEEEEEEE------HHHHHHHHHH
RRLMEAEKVKSGFCQVVISSNLRDGVSHLIQSGGLGGLQHNTVLVGW
HHHHHHH----EEEEEE----HHHHHHHHHHHH--------EEEEE-
PRNWRQKEDHQTWRNFIELVRETTAGHLALLVTKNVSMFPGNPERFS
-----------HHHHHHHHHHHHHHH---EEEEE-------------
EGSIDVWWIVHDGGMLMLLPFLLRHHKVWRKCKMRIFTVAQMDD
---EEEEEEE----HHHHHHHHHH--HH---EEEEEEEEE----
Fragment B

Table 3.1.

Oligonucleotides used to generate the different Tac-KCC2 and pGEX-KCC2 carboxyl tail constructs.

Tac-CD7 (forward)	ATATCTAGACGGGGGGCGGAG
Tac-CD7 (reverse)	AATTCTAGATTACTGCCTCCAGTTGCGAGGCC
Tac-CDfC (forward)	CGTCTAGAAAGGAGGATCATCAGACATGG
Tac-CDfC (reverse)	TATTCTAGATTAGTGGTGTCGCAGCAGGAAGG
GST-CD5 `	AGTGAATTCTCATTCCTCCAGGCGCAAGAG
GST-CD6	AAGGAATTCTTAGAGAGACAGGCCTCGGATTCC
GST-CD5 (Y655A)	ATAGAATTCTCATTCCTCCAGGCGCAAGAGAGCGC GTGC
GST-CD5 (LL657,658AA)	AATTGAATTCTCATTCCTCCAGGCGCGCGGCAGCAT AGCG

The oligonucleotides are written 5' to 3' and the *XbaI* (for Tac-CDs) or *EcoR*I (for GST-CDs) restriction site is underlined.

PREFACE TO CHAPTER 4

It is known that the induction and developmental up-regulation of KCC2 gene expression are essential for the maturation of GABA and glycine mediated synaptic inhibition in cortical and hippocampal neurons (Rivera et al., 1999; Hubner et al., 2001). However, whether additional mechanisms, such as changes in the proportion of KCC2 expressed at the neuronal surface, were involved during brain development were unknown. KCC2 gene expression can be regulated by various factors, such as the brain-derived neurotrophic factor (BDNF) dependent signalling pathways, neuronal activity, and neuronal trauma (Rivera et al., 2002; Aguado et al., 2003; Ganguly et al., 2001; Coull et al., 2003). In the course of my PhD studies, accumulating studies showed that KCC2 transport activity could be rapidly altered by BDNF, insulin, and c-Src treatments in a manner of 10~20 minutes (Woodin et al., 2003; Wardle and Poo, 2003; Kelsch et al., 2001). This suggested that changes in kinase/phosphatase activities might regulate KCC2 function via a post-translational mechanism(s), occuring on a much shorter time scale than changes in gene expression. One possible mechanism contributing to these fast effects of BDNF, insulin, and c-Src could be a change in the amount of KCC2 expression at the cell surface.

The specific aims of the present study were to examine: 1) whether the proportion of KCC2 at the cell surface is altered during neuronal maturation; and 2) whether cellular kinase/phosphatase activities elicited by BDNF, epidermal growth factor (EGF), and tyrosine phosphatase inhibitor treatment of primary cortical neurons altered KCC2 cell surface expression.

Although the data in the present study is insufficient to form a relatively complete research article for publication, it complements my findings reported in Chapters 2 and 3 and in addition suggest a starting point for future studies identifying the molecular mechanisms controlling KCC2 regulated endocytosis and oligomerization.

Modulation of K^+/Cl^- Cotransporter KCC2 Neuronal Surface Expression

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Keywords: KCC2, Chloride transporter, BDNF, EGF, Kinase, Phosphatase

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I. ABSTRACT

The neuron-specific potassium-chloride cotransporter 2 (KCC2) plays a crucial role, by controlling chloride extrusion, in the development and maintenance of inhibitory neurotransmission. It is now known that KCC2 gene expression can be regulated by various factors, such as the brain-derived neurotrophic factor dependent signalling pathways and neuronal activity. However, the cellular mechanisms underlying the post-translational regulation of KCC2, specifically its cell surface expression, remain poorly understood.

Different phosphatase (unknown) and kinase activities have been reported to regulate the chloride transport capacity of KCC2. However, how the cell surface expression of KCC2 is affected by kinase and phosphatase activities remains largely unknown. We therefore set out to examine the effect of distinct modulators of different kinase and phosphatase activities on KCC2 cell surface expression in primary cultured rat cortical neurons.

Using a surface biotinylation assay, we show the proportion of KCC2 expression at the cell surface is up-regulated during neuronal maturation. Also that sodium orthovanadate (Na_3VO_4), a general inhibitor of cellular tyrosine phosphatase activity, increases cell surface KCC2 expression. Further that brain-derived neurotrophic factor (BDNF) treatment decreases cell surface KCC2 expression, whereas epidermal growth factor (EGF) treatment increases it. Finally we show that inhibition of tyrosine phosphatase activity by Na_3VO_4 completely abolishes the negative effect of BDNF on KCC2 cell surface expression. In conclusion, our studies suggest that a fine balance of kinase and phosphatase activities regulate KCC2 cell surface expression in neurons, which may be a means for regulating the strength of synaptic inhibition.

II. INTRODUCTION

In the adult mammalian central nervous system (CNS), fast inhibitory neurotransmission is predominantly mediated by two types of ligand gated chloride (Cl^-) channels, the γ-amino-butyric acid type-A receptor ($GABA_AR$) and the glycine receptor (GlyR) (Betz, 1992; Sieghart, 2006). Cl^- influx induced by activation of these Cl^- ion channels is critically dependent on a low intracellular Cl^- concentration ($[Cl^-]_i$). The neuron-specific K^+/Cl^- cotransporter, KCC2, has now been proven to be an essential protein in establishing and maintaining low $[Cl^-]_i$ in neurons, by controlling Cl^- extrusion (Rivera et al., 1999). Consistent with the essential role of KCC2 during neuronal maturation, KCC2 knockout mice die shortly after birth due to respiratory failure caused by functional defects in respiratory motoneurons (Hubner et al., 2001).

Besides transcriptional and translational regulation, the cellular mechanisms that control the cell surface expression of many transporters can have a profound and dynamic effect on their transport capacity (Melikian et al., 1999; Ortiz, 2006). For KCC2, several different factors have been shown to regulate its cell surface expression and transport activity independently of its gene expression (Rivera et al., 2004; Khirug et al., 2005; Wake et al., 2007; Lee et al., 2007). Of particular interest, alterations in the phosphorylation state of KCC2 have been found to be accompanied by changes in its cell surface expression levels (Wake et al., 2007; Lee et al., 2007). This suggests that regulation of KCC2 cell surface expression via alterations in intracellular kinase/phosphatase activities may be an important mechanism by which the Cl^- transport capacity of KCC2 can be controlled. Similar regulatory mechanisms have also been reported for the KCC2-related cation-chloride cotransporter (CCC) family member, NKCC2 (Giménez et al., 2003). That is, application of the antidiuretic hormone vasopressin, which is known to activate NKCC2 transport capacity, caused both an increase in NKCC2 phosphorylation on its N-terminal threonine residues and plasma membrane insertion of NKCC2 (Giménez et al., 2003). Moreover, phosphorylated NKCC2 seemed to be exclusively present at the plasma membrane (Giménez et al., 2003). These observations suggest that increased phosphorylation and plasma membrane insertion may be a mechanism underlying enhanced transport activity of NKCC2 by

vasopressin. Together, altered membrane trafficking via modification of intracellular kinase/phosphatase activities may be a common mechanism by which the cell surface expression and hence the Cl^- transport capacity of CCC member proteins can be controlled.

How KCC2 plasma membrane expression is controlled, specifically how kinase/phosphatase activities regulate the cell surface expression of KCC2, is yet largely unknown. Therefore, in the present study we examined the effect of intracellular kinase/phosphatase signalling mediated by neurotrophic/growth factors and tyrosine phosphatase inhibitors on the cell surface expression of endogenous KCC2 in neurons.

III. MATERIALS AND METHODS

Antibodies

The following antibodies were used, rabbit polyclonal anti-KCC2 (Upstate, Lake Placid, NY, USA), mouse monoclonal anti-human anti-transferrin receptor (Zymed Laboratories, South San Francisco, CA, USA), and donkey anti-mouse and anti-rabbit antibodies conjugated to peroxidase (Jackson Labs, Bar Harbor, Maine, USA).

Neuronal culture

High-density cortical neuronal cultures were prepared from embryonic rats at embryonic day 19 (E19). All procedures with animals were performed in accordance with the "Canadian Council on Animal Care" guidelines for the use of animals in research. Briefly, E19 embryos were removed and cortices were microdissected in dissection media consisting of 10 mM HEPES and 10% Hank's Balanced Salt Solution (HBSS, Invitrogen) under sterile condition. To dissociate neurons, isolated cortices were digested with 0.25% trypsin for 15 min at 37 °C, washed three times of 5 min in dissection media, and triturated with two fire-polished pasteur pipettes with gradually reducing sizes.

Dissociated neurons were plated onto poly-D-lysine coated P100 Nunc petri dishes, incubated in attachment media composed of Minimum Essential Media (Invitrogen) supplemented with 1 mM sodium pyruvate, 0.6% glucose, and 10% horse serum (Invitrogen), and oxygenated in 5% CO_2 and 95% O_2 at 37 °C. 24 h after plating, attachment media was replaced with maintenance media composed of Neurobasal A media (Invitrogen) supplemented with 2% B27, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Neurons were then fed once a week by replacing half of the maintenance media. Cortical cultures were maintained for 10 days *in vitro* before experimentation.

Isolation of acute hippocampal slices

Postnatal day 35 rats were anaesthetized with halothane (Sigma-Aldrich) and decapitated. Hippocampi were isolated, and transverse slices (300 µm) were collected using a vibrating blade vibratome (Leica VT1000S, Leica, Wetzlar, Germany) in ice-cold

slicing solution containing (in mM): 250 sucrose, 4.0 $MgCl_2$, 2.5 KCl, 1.25 NaH_2PO_4, 0.1 $CaCl_2$, 25 $NaHCO_3$, 0.5 Na-ascorbate, 2.0 Na-pyruvate, and 10 glucose equilibrated with 95% O_2 and 5% CO_2). Slices were then incubated for 1 h at room temperature in the artificial cerebrospinal fluid (ACSF) containing (in mM): 125mM NaCl, 1.0 $MgCl_2$, 2.5 KCl, 1.25 NaH_2PO_4, 2.0 $CaCl_2$, 25 $NaHCO_3$, 0.5 Na-ascorbate,2.0 Na-pyruvate, and 10 glucose equilibrated with 95% O2 and 5% CO2.

Surface biotinylatioin assay

10 DIV primary cultured cortical neurons were placed on ice, washed twice with phosphate buffer saline^{++} (PBS^{++} contains 1 mM $CaCl_2$ and 1 mM $MgCl_2$), incubated 20 min with 1 mg/ml Sulfo-NHS-SS-biotin-PBS^{++}, washed three times with PBS^{++} containing 0.1% BSA, then twice with PBS^{++} alone and incubated in the appropriate treatment media at 37 °C for different times as indicated. In the case where acute hippocampal slices were analysed, acutely isolated hippocampal slices were recovered in ACSF for 1 h, and then submerged in ice-cold 1 mg/ml Sulfo-NHS-SS-biotin-ACSF for 1 h with continuous oxygenation. Primary cortical neurons or acute hippocampal slices were then washed two times in PBS^{++} and lysed in lysis buffer (20 mM Tris-HCl pH7.6, 50 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.1% SDS, 1 mM Na_3VO_4, 50 mM NaF, and 10 mM $Na_4P_2O_7$, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for 1 hour at 4°C. Nuclei and insoluble material were removed by centrifugation at 14,000 g, 15 min, 4 °C. Biotinylated proteins were purified on NeutrAvidin-coated beads (Pierce) at 4 °C for 2 h, washed three times with lysis buffer and eluted in Laemmli buffer at 95 °C 5 min. Proteins were separated on 8% SDS-PAGE gels, followed by Western blotting with specific antibodies as indicated. Intensities of immunoreactive bands were quantified by densitometry analysis of exposed films using a Biorad GS-800 densitometer. Background intensity readings were subjected from all readings and results were expressed as a percentage of the total surface labelling for each clone.

IV. RESULTS

KCC2 expression at the cell surface is up-regulated during development

It is well established that the induction and up-regulation of KCC2 gene expression are essential for the maturation of inhibitory neurotransmission mediated by $GABA_A$ and glycine receptors during the development of the central nervous system (CNS). Several studies have, however, shown that the levels of KCC2 expression are not proportional to its transport activity in certain types of neurons during development (Kelsch et al., 2001; Balakrishnan et al., 2003; Zhang et al., 2006), suggesting that post-translational mechanisms most likely contribute to the functional activation and up-regulation of KCC2 during neuronal maturation in these neurons. Given that KCC2 is a transmembrane ion cotransporter, we reasoned that increased levels of KCC2 expression at the plasma membrane may be a mechanism contributing to its functional up-regulation during development. Therefore, we examined the surface expression of KCC2 in cortical or hippocampal neurons from different developmental stages. For this, we used primary cultured cortical neurons isolated from embryonic 19 (E19) rat brains as the model for immature neurons and acute hippocampal slices from postnatal day 35 (P35) rat brains as the model for mature neurons. To quantitatively determine the proportion of KCC2 protein at the cell surface, we employed a surface biotinylation approach. Primary cultured cortical neurons of 5 and 10 day *in vitro* (DIV), and acute hippocampal slices from P35 mouse brain were incubated with an impermeable biotin reagent to label all the cell surface proteins. Biotinylated KCC2 was then detected by streptavidin purification followed by Western blot detection with a KCC2 antibody. The cell surface level of KCC2 was normalized to its total expression at each stage. As shown in Fig. 4.1, there is a gradual up-regulation of the proportion of KCC2 at the cell surface during development, with only 1% of KCC2 expressed at the cell surface in 5DIV neurons but 5.4% in the adult. These results strongly suggest that the proportion of KCC2 expression at the neuronal surface is up-regulated during neuronal maturation.

The activity of an endogenous tyrosine phosphatase(s) suppresses KCC2 expression at the cell surface

Protein tyrosine kinases (PTKs) are implicated in a wide variety of cellular events, such as cell proliferation, growth, differentiation and synaptogenesis (Schlessinger and UIIrich, 1992; Levitzki and Gazit, 1995; Catarsi and Drapeau, 1993). With respect to KCC2, protein tyrosine kinase activity induced by the co-application of the insulin-like growth factor 1 (IGF-1) and c-Src rapidly activates KCC2 transport activity in immature cultured hippocampal neurons, which express an inactive form of KCC2 (Kelsch et al., 2001). Consistently, tyrosine kinase inhibitors reduced KCC2 activity in mature hippocampal neurons (Kelsch et al., 2001). These observations suggest KCC2 transport activity is positively regulated by relatively high levels of tyrosine kinase activity. We speculated that one possible mechanism mediating this effect could be through an increase in the cell surface expression levels of KCC2. To test this hypothesis, we examined the cell surface expression of KCC2 upon treatment with a pan tyrosine phosphatase inhibitor, sodium orthovanadate (Na_3VO_4). 10DIV primary cortical neurons were either untreated or treated with Na_3VO_4 (1 mM) for 20 minutes, and the cell surface expression of KCC2 was evaluated by surface biotinylation. As shown in Fig. 4.2, the total expression level of KCC2 was comparable between untreated and treated neurons. However, Na_3VO_4 treatment resulted in a ~ 2 fold increase in KCC2 expression at the cell surface, demonstrating that suppression of endogenous tyrosine phosphatase activity may enhance the cell surface expression of KCC2. In other words, an endogenous protein tyrosine phosphatase(s) may intrinsically suppress KCC2 cell surface expression in neurons.

Suppression of general tyrosine phosphatase activity prevents the down-regulation of KCC2 cell surface expression by BDNF

Brain-derived neurotrophic factor (BDNF) has been shown to be an important growth factor regulating the gene expression of KCC2 during development as well as in the adult brain (Aguado et al., 2003; Carmona et al., 2006; Rivera et al., 2002; Rivera et al., 2004). BDNF has also been shown to down-regulate KCC2 transport activity and plasma membrane expression in hippocampal neurons (Rivera et al., 2002 and 2004).

How BDNF affects the cell surface expression of KCC2 in cortical neurons is however largely unknown. Therefore, we examined the effect of BDNF on the levels of KCC2 cell surface expression in 10DIV primary cortical neurons. Neurons were incubated with BDNF (100 ng/ml, PeproTech, Rocky Hill, NJ, USA) for 0, 2, 5, 15, 30, or 60 minutes, and KCC2 cell surface expression was assessed by surface biotinylation. As shown in Fig. 4.3A, 2 minutes of BDNF treatment resulted in a loss of 27.5 ± 16.1% of cell surface KCC2, and by 30 minutes 61.2 ± 11.5% was lost. This result demonstrates that BDNF acutely down-regulates the cell surface expression of KCC2 in cultured cortical neurons, and this effect occurs in the manner of minutes.

BDNF is known to trigger downstream signalling pathways predominantly through the activation of its high affinity receptor, the tyrosine receptor kinase B (TrkB), a member of the receptor tyrosine kinase (RTK) family (Benito-Gutiérrez et al., 2006**).** Therefore, we reasoned that inhibition of tyrosine phosphatase activity, which would enhance the activity of TrkB, may facilitate the down-regulating effect of BDNF on KCC2 surface expression. To test this hypothesis, 10DIV cortical neurons were pre-incubated with Na_3VO_4 (1mM) for 20 minutes, followed by BDNF treatment (100 ng/ml) for 0, 5, 30, and 60 minutes in the continuous presence of Na_3VO_4. Mock treatment was included as control. As shown in Fig. 4.3B**,** 20 minutes of Na_3VO_4 pre-incubation significantly increased KCC2 expression at the cell surface. Surprisingly, however, the presence of Na_3VO_4 not only prevented the loss of KCC2 surface expression by BDNF but also increased it more than 2 fold after 30 minutes, which sustained until the end of the longest time point we examined (1 hour). These results suggest that suppression of endogenous tyrosine phosphatase activity not only enhances the surface expression of KCC2 under basal conditions, but also abolishes the down-regulating effect of BDNF on the cell surface expression of KCC2.

EGF up-regulates KCC2 cell surface expression

The epidermal growth factor (EGF) has been shown to modulate a number of cellular processes, such as cell proliferation, differentiation, and survival, and has been indicated to be involved in ontogenesis (Herbst, 2004). EGF activates the ErbB family of RTKs and triggers several downstream signalling cascades. EGF-dependent c-Src kinase

activation has been observed in fibroblasts and epithelial carcinoma cell lines (Osherov and Levtizki, 1994; Oude Weernink**,** 1994; Wilde et al., 1999). The positive effect of c-Src on KCC2 transport activity mentioned above could be accomplished by enhancing KCC2 cell surface expression. If so, we speculated that EGF treatment, which presumably activates c-Src in cortical neurons, may increase KCC2 cell surface expression. To test this, we examined the effect of EGF on KCC2 surface expression in 10DIV cortical neurons. Cortical neurons were treated with EGF (100 ng/ml, Upstate Biotechnology; Lake Placid, NY, USA) for 0, 2, 5, 15, and 30 minutes, and the cell surface level of KCC2 expression was evaluated by surface biotinylation. Interestingly, we observed a transient increase in KCC2 surface expression, peaking at 5 minutes and returning to the basal level at 30 minutes (Fig. 4.4). The level of cell surface KCC2 was increased by 1.63±0.22 fold after 5 minutes of EGF treatment (Fig. 4.4). As a control, we also examined the cell surface expression of the transferrin receptor (TfR) by surface biotinylation, and we did not observe an apparent change throughout the treatment period. Together, these results suggest that EGF transiently increases KCC2 expression at the neuronal surface.

V. DISCUSSION

KCC2 has been established as an essential protein in the vast majority of CNS neurons for the development and maintenance of inhibitory neurotransmission mediated by $GABA_A$ and glycine receptros (Rivera et al., 1999; Hubner et al., 2001; Coull et al., 2003; Nabekura et al., 2002; Woo et al., 2002). In addition, loss of KCC2 expression and function is associated with several neuropathological conditions including chronic pain (Coull et al., 2003), nerve injury (Nabekura et al., 2002), and epilepsy (Woo et al., 2002). Therefore it is particularly significant to understand the cellular mechanisms regulating the amount of KCC2 and its transport activity. Several reports have shown KCC2 activity can be regulated by cellular kinase and phosphatase activities (Fiumelli et al., 2005; Khiurg et al., 2005; Kelsch et al., 2001; Rivera et al., 2004; Lee et al., 2007; Wake et al., 2007; Inoue et al., 2005). However, the mechanisms behind these effects remain poorly understood. In the present study, we examined the plasma membrane expression of KCC2 in neurons at different developmental stages as well as primary cortical neurons treated with reagents that modify the activity of certain cellular kinases and phosphatases. We show that (i) the proportion of KCC2 cell surface expression is up-regulated during development, (ii) endogenous tyrosine phosphatase activity suppresses KCC2 surface expression, (iii) BDNF down-regulates KCC2 cell surface expression, and that this effect can be reversed by inhibition of endogenous tyrosine phosphatase activity, and (iv) EGF transiently increases KCC2 surface expression. Together our results demonstrate that the cell surface expression of KCC2 in neurons is developmentally up-regulated and can be dynamically regulated by tyrosine phosphatase activity and neurotrophic/growth factor dependent signalling cascades.

KCC2 cell surface expression level is up-regulated during development.

A number of studies have shown KCC2 gene expression is central for its Cl^- extrusion capacity and the maturation of GABAergic and glycinergic neurotransmission (Rivera et al., 1999; Hubner et al., 2001; Coull et al., 2003; Woo et al., 2002). However, in certain types of neurons the transport activity of KCC2 does not match its developmental gene expression pattern (Balakrishnan et al., 2003; Zhang et al., 2006).

Specifically, in the lateral superior olive (LSO) of rats and mice, KCC2 mRNA and protein levels are unchanged during both depolarizing and hyperpolarizing stages, and the glycine mediated depolarizing responses in immature neurons are not attributed to the gene expression of the Cl^- uptaker NKCC1 (Balakrishnan et al., 2003). Immunohistochemical studies have revealed plasma membrane insertion of KCC2 from the cytosol in LSO neurons during neuronal maturation (Balakrishnan et al., 2003). Similarly, delayed functional activation of KCC2 has also been observed in retinal neurons (Zhang et al., 2006). These reports strongly suggest gene expression is not sufficient for the functional activation of KCC2 in some types of neurons, and that additional post-translational mechanisms such as plasma membrane insertion, as discussed above are required. In the present study, we show although a great amount of KCC2 protein was already expressed in primary cortical neurons as early as 5DIV, there was only 1% present at the cell surface (Fig. 4.1). With development, there was a 5.4 fold increase in the proportion of KCC2 at the cell surface (Fig. 4.1). This suggests that increased KCC2 cell surface expression may be an important contributing factor in the functional up-regulation of KCC2 during neuronal maturation.

The effect of tyrosine kinase/phosphatase activities on KCC2 phosphorylation state, cell surface expression, and transport activity.

Alteration of cellular kinase/phosphatase activities has been shown to be a common mechanism by which the transport capacity of CCCs can be modulated (Mount et al., 1998; Gamba et al., 2005). As for KCC2, several different kinase activities and neurotrophic/growth factor triggered cellular signalling pathways have been shown to regulate its activity (Khiurg et al., 2005; Kelsch et al., 2001; Wardle and Poo, 2003; Rivera et al., 2004; Wake et al., 2007). The effects of IGF-1, c-Src, and tyrosine kinase inhibitors on KCC2 transport activity (Kelsch et al., 2001) mentioned above suggested a relatively high level of tyrosine kinase activity in neurons may be required for the functional expression of KCC2. We speculated this could be accomplished by facilitating KCC2 cell surface expression. Indeed, in the present study we show inhibition of general tyrosine phosphatase activity using a pan tyrosine phosphatase inhibitor, Na_3VO_4, increased the cell surface expression of KCC2 in a manner of 20 minutes (Fig. 4.2 and

4.3B). Our findings suggest that an endogenous tyrosine phosphatase(s) may intrinsically suppress KCC2 cell surface expression. In other words, a relatively high level of a cellular tyrosine kinase(s) versus tyrosine phosphatase(s) activity may be necessary for the optimal level of KCC2 cell surface expression. The rapid effect of Na_3VO_4 on KCC2 cell surface expression is unlikely to involve protein synthesis (which requires a much longer time scale), and is likely due to either accelerated membrane insertiono or recycling, or decelerated endocytosis, or a combination of two or more of these pathways.

Direct evidence for a correlation between KCC2 tyrosine phosphorylation state and its cell surface expression emerged from a study performed by Wake et al. (2007). In this study, they showed that the tyrosine phosphorylation state of KCC2 can be modified by oxidative stress and seizure activity in hippocampal neurons, and that the levels of tyrosine phosphorylated KCC2 parallel its cell surface expression (Wake et al., 2007). This suggests the level of KCC2 tyrosine phosphorylation is a positive regulator for KCC2 surface expression. It will be of future interest to identify the specific KCC2 tyrosine residue(s) subject to phosphorylation modifications upon alteration of cellular tyrosine kinase/phosphatase activities.

Tyrosine phosphatase(s) activity downstream of BDNF signalling mediates KCC2 cell surface expression

BDNF can regulate KCC2 on many different levels, including its gene expression, phosphorylation, membrane trafficking, and transport activity (Aguado et al., 2003; Carmona et al., 2006; Rivera et al. 2002 and 2004; Wake et al., 2007). Specifically, epileptic activity induced by Mg^{2+} withdrawal in acute hippocampal slices shortened the half life of KCC2 at the neuronal surface from about 20 to 10 minutes, and this effect seemed to be mediated by the BDNF receptor, tyrosine receptor kinase B (TrkB), dependent signalling pathways (Rivera et al., 2004). However, it remains unclear what is the kinase(s)/phosphatase(s) downstream of BDNF signalling that mediates this process. In agreement with this report, we show BDNF similarly caused a rapid loss of surface KCC2 expression in primary cultured cortical neurons (Fig. 4.3A), suggesting that the mechanisms contributing to the down-regulation of surface KCC2 expression by BDNF

are shared in both neuronal systems. Given that TrkB is a tyrosine receptor kinase, we therefore postulated that application of the tyrosine phosphatase inhibitor Na_3VO_4 may enhance the activity of TrkB (by acting on tyrosine phosphatases that dephosphorylate TrkB), which in turn would further accelerate the loss of KCC2 from the cell surface by BDNF. To our surprise, however, we found that the addition of Na_3VO_4 not only blocked the effect of BDNF but also increased KCC2 surface expression, which resembles the effect of Na_3VO_4 alone (Fig. 4.3B). The effect of Na_3VO_4 on BDNF we show is reminiscent of the observations from another report which shows that Na_3VO_4 completely prevented the down-regulation of cell surface KCC2 by the oxidative stressor, H_2O_2 (Wake et al., 2007). Given that BDNF down-regulates KCC2 cell surface expression via a TrkB-dependent pathway (Rivera et al., 2004), our results suggest that Na_3VO_4 may act on a tyrosine phosphatase(s) downstream of TrkB to regulate the cell surface expression of KCC2. It will be of future interest to identify the specific tyrosine phosphatase(s) that is sensitive to Na_3VO_4 and controls the cell surface expression of KCC2.

What is the role of EGF in regulating KCC2 and synaptic inhibition?

The role of EGF in the regulation of KCC2 has never been addressed. Interestingly, our data shows that EGF transiently increases KCC2 surface expression in primary cortical neurons (Fig. 4.4). Many questions remain to be addressed. For example, what is the specific downstream signalling pathway involved? Is the phosphorylation state of KCC2 altered by EGF-dependent signalling? What is the functional significance of EGF on KCC2 transport activity and GABAergic and glycinergic responses? Understanding these questions will potentially reveal a novel function for EGF in controlling KCC2 function and the strength of inhibitory neurotransmission in neurons. Finally, an interesting observation from our present study is that BDNF and EGF treatments lead to completely opposite outcomes for the cell surface expression of KCC2 (compare Fig. 4.3A and 4.4), although they are known to activate fairly similar downstream signalling cascades in cells. Therefore it will be of future interest to address what is the signalling component that distinguishes the effect of BDNF and EGF in the process of regulating KCC2 cell surface expression. Understanding this will also help to

provide insight into how neurotrophins and growth factors coordinately regulate the strength of neurotransmission in neurons.

Figure 4.1. The proportion of KCC2 expression at the cell surface is up-regulated during development. (A) Comparison of the cell surface expression levels of KCC2 at different ages. Cell surface biotinylation was performed using the non-permeable biotinylation reagent Sulfo-NHS-SS-Biotin (1 mg/ml) on 5 and 10 DIV primary cortical neurons and adult acute hippocampal slices as described in materials and methods. Immunoblots were detected using an anti-KCC2 antibody. "Input" represents 1/20 of the total KCC2 expression from $8X10^6$ of primary cortical neurons or 1 mg of hippocampal slice lysate, and "pull-down" represents the total cell surface expression under each condition examined. (B) Quantification of cell surface KCC2 at each developmental stage examined. Western blots were quantified by densitometry. The cell surface expression levels of KCC2 were normalized to its total expression at each stage, producing the proportion of KCC2 expression at the cell surface.

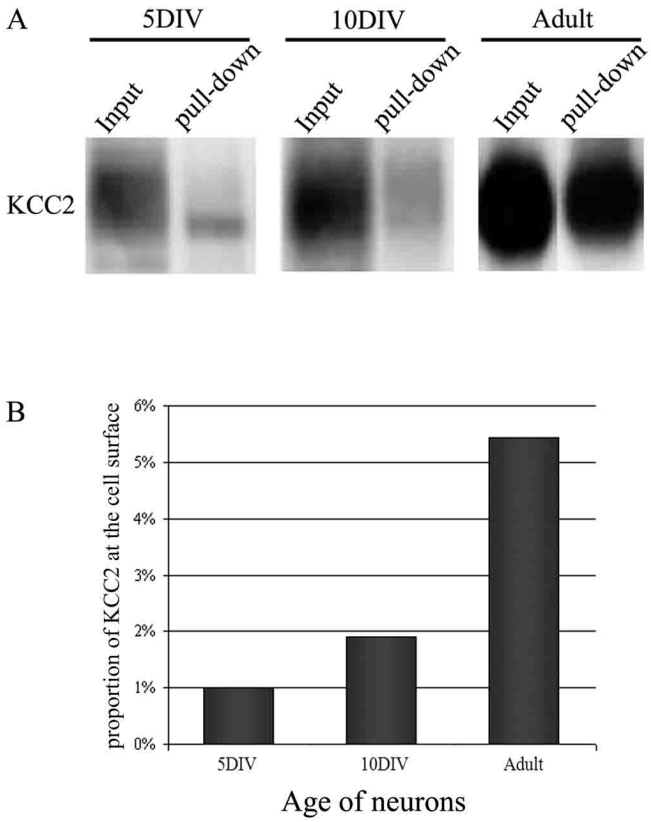

A
5DIV
10DIV
Adult
Input
pull-down
Input
pull-down
Input
pull-down
KCC2
B
proportion of KCC2 at the cell surface
6%
5%
4%
3%
2%
1%
0%
5DIV
10DIV
Adult
Age of neurons

Figure 4.2. The cell surface expression levels of KCC2 are increased upon treatment with a pan tyrosine phosphatase inhibitor, Na_3VO_4. 10DIV primary cortical neurons were untreated (0') or treated with Na_3VO_4 (1 mM) for 20 min (20'), followed by surface biotinylation as described in materials and methods. Immunoblots were detected using an anti-KCC2 antibody. "Input" represents 1/50 of the total KCC2 expression from $1.6X10^7$ of neurons, and "pull-down" represents the total cell surface expression under each condition examined. Note that cell surface KCC2 is increased by Na_3VO_4 treatment.

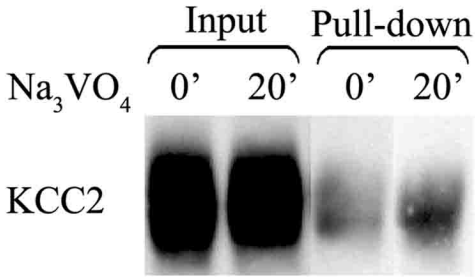

Input
Pull-down
Na_3VO_4
0'
20'
0'
20'
KCC2

Figure 4.3. Na_3VO_4 prevents the decrease of KCC2 cell surface expression by BDNF. (A) Cell surface KCC2 is decreased by BDNF treatment. 10DIV primary cortical neurons were untreated (0') or treated with BDNF (100 ng/ml) for different times (2', 5', 15', 30', and 1 h), followed by surface biotinylation as described in materials and methods. Immunoblots were detected using an anti-KCC2 antibody. "Input" represents 1/20 of the total KCC2 expression from $1.6X10^7$ of neurons, and "pull-down" represents the total cell surface expression under each condition examined. Note the surface expression levels of KCC2 were rapidly reduced by BDNF treatment, which was detectable as early as 2'. (B) Na_3VO_4 and BDNF co-treatment increased cell surface KCC2. 10DIV primary cortical neurons were preincubated with Na_3VO_4 (1 mM) for 20 min, followed by BDNF (100 ng/ml) treatment for different times (2', 5', 15', 30', and 1 h) in the continuous presence of Na_3VO_4. The surface expression levels of KCC2 under each condition were evaluated by surface biotinylation as described in materials and methods. Immunoblots were detected using an anti-KCC2 antibody. "Input" represents 1/50 of the total KCC2 expression from $1.6X10^7$ of neurons, and "pull-down" represents the total cell surface expression under each condition examined. Note Na_3VO_4 prevented the decrease of KCC2 cell surface expression by BDNF compared to (A).

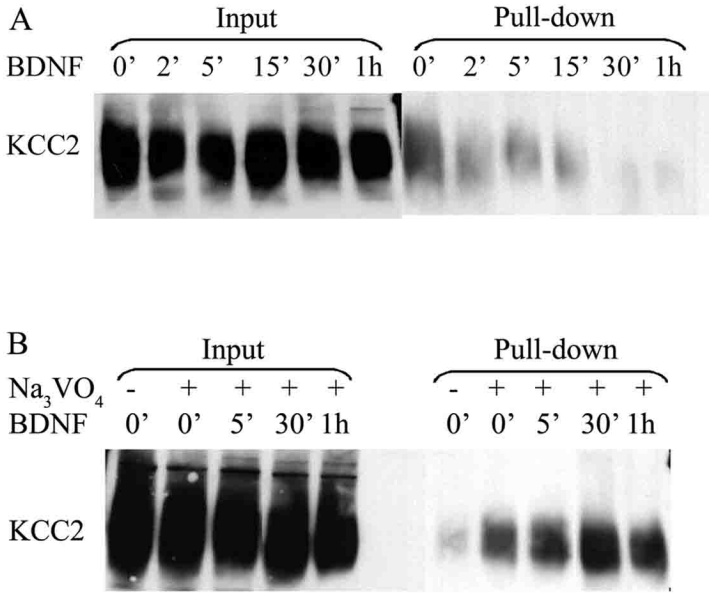
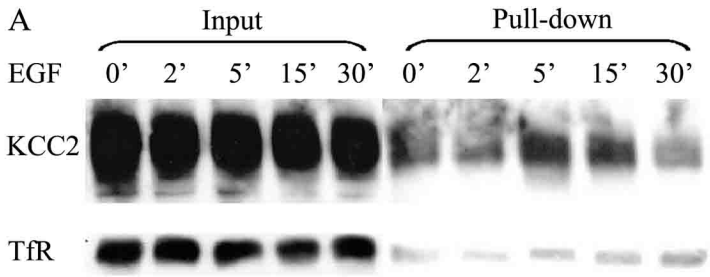
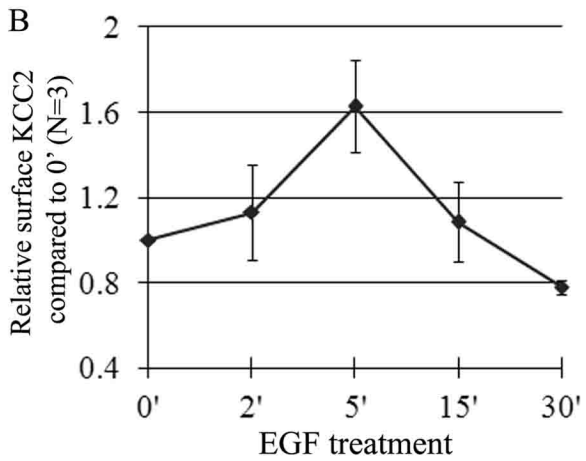

A
Input
Pull-down
BDNF
0' 2' 5' 15' 30' 1h
0' 2' 5' 15' 30' 1h
KCC2
B
Input
Pull-down
Na3VO4
- + + + +
- + + + +
BDNF
0' 0' 5' 30' 1h
0' 0' 5' 30' 1h
KCC2

Figure 4.4. EGF transiently increases KCC2 cell surface expression. Cell surface KCC2 is transiently increased by EGF treatment. 10DIV primary cortical neurons were untreated (0') or treated with EGF (100 ng/ml) for different times (2', 5', 15', and 30'), followed by surface biotinylation as described in materials and methods. Immunoblots were detected using antibodies to KCC2 or transferrin receptor (TfR). "Input" represents 1/50 of the total KCC2 expression from $1.6X10^7$ of neurons, and "pull-down" represents the total cell surface expression under each condition examined. Typical immunoblot is shown. Note the surface expression levels of KCC2 were transiently increased by EGF treatment, peaking at 5' and dropping back to the basal level at 30'. (B) Quantification of cell surface KCC2 upon EGF treatment. Western blots were quantified by densitometry. Data are expressed as the fold of increase in KCC2 cell surface expression compared to 0' condition and presented as mean ± s.e. (n=3).


A
Input
Pull-down
EGF
0’
2’
5’
15’
30’
0’
2’
5’
15’
30’
KCC2
TfR


B
Relative surface KCC2 compared to 0’ (N=3)
2
1.6
1.2
0.8
0.4
0'
2'
5'
15'
30'
EGF treatment

GENERAL DISCUSSION

While it is now well established that KCC2 gene expression is regulated by a wide variety of factors, including specific transcription factors, neurotrophic factors, and neuronal activity, far less is still known about the post-translational mechanisms regulating KCC2. At the start of my PhD studies, nothing had yet been reported on what post-translational mechanisms regulate KCC2 protein expression or function.

The observations that neuronal spiking as well as BDNF can alter KCC2 transport activity in a manner of 10-20 minutes (Woodin et al., 2003; Wardle and Poo, 2003) were suggestive that KCC2 was regulated by post-translational mechanisms rather than by regulated gene expression, which generally requires a much longer time scale. Therefore, post-translational mechanisms likely also play an important role in controlling overall KCC2 activity and synaptic inhibition. As such, in the course of my PhD studies I have been focused on addressing the questions of what are the post-translational mechanisms regulating KCC2 and how these processes are controlled at the cellular and molecular levels.

In brief the major findings of my thesis are as follows. Firstly, using a heterologous expression system, I provide evidence demonstrating that KCC2 is constitutively endocytosed via the clathrin-mediated endocytosis pathway. My mapping studies on identifying the KCC2 endocytic motif(s) have revealed a novel acidic di-leucine endocytic motif, $_{657}$LLXXEE$_{662}$, which is essential for the constitutive endocytosis of KCC2 and for its interaction with the clathrin-binding adaptor protein AP-2 complex (**Chapter 2**). Secondly I have identified two fragments in the proximal and central C-terminus of KCC2, namely fragments A and B, that mediate KCC2 dimerization. I show also that the LL657,658AA mutation within fragment A is sufficient to abolish KCC2 dimerization, most likely by disrupting an α-helical based interaction between fragments A and B (**Chapter 3**). Finally, I show using primary cultured cortical neurons and acute hippocampal slices that KCC2 expression at the neuronal surface is developmentally up-regulated and can be dynamically regulated by neurotrophic/growth factor dependent signalling pathways and changes in the levels of cellular tyrosine phosphatase activity (**Chapter 4**).

In the following sections, I will discuss the major implications of my findings, the potential relevance and contributions of my work to the field, as well as prospective future directions from my studies.

I. Membrane trafficking as a means for controlling the level of KCC2 cell surface expression

A large number of studies have demonstrated the functional significance and the molecular mechanisms governing the membrane trafficking of neurotransmitter receptor and transporter proteins (Melikian and Buckley, 1999; Ortiz, 2006; Esteban, 2003; Michels and Moss, 2007; Al-Hasani et al., 2002; Holton et al., 2005; Schmidt et al., 2006). Prior to commencing my PhD studies, little was known of the cellular and molecular mechanisms controlling KCC2 endocytic trafficking and cell surface expression, although these mechanisms could potentially have a profound effect on the function of KCC2. A previous report had indicated endogenous neuronal KCC2 does undergo constitutive endocytosis (Rivera et al., 2004). However, my study was the first to report the cellular and molecular mechanisms governing the constitutive endocytosis of KCC2 (Zhao et al., 2008; **Chapter 2**). I have reported that KCC2 constitutive endocytosis is mediated by the CME machinery, and endocytosed KCC2 is sorted to the recycling pathway. It is interesting to speculate that KCC2 endocytosis and recycling could provide rapid and effective means by which the number of KCC2 molecules at the neuronal membranes can be controlled. In addition, that the coordination of these two pathways could be central for precisely controlling the stable amount of KCC2 cell surface expression, and hence its transport activity and finally the strength of KCC2 dependent synaptic inhibition. It will therefore be of future interest to determine the relative rates of KCC2 constitutive endocytosis and recycling, as well as the functional significance of maintaining the balance between these two pathways.

A previous report has shown BDNF treatment and Mg^{2+} withdrawal can lead to an acceleration of KCC2 endocytosis and a reduction in the amount of KCC2 protein respectively (Rivera et al., 2004), suggesting that endocytosed KCC2 may be targeted to the proteolytic degradation pathway by these treatments. I show constitutively endocytosed KCC2 is targeted to the recycling pathway (Zhao et al., 2008; Fig. 2.2,

Chapter 2). Therefore these findings suggest that distinct sorting mechanisms may be involved following constitutive versus regulated endocytosis of KCC2. That is, KCC2 is sorted to the recycling pathway following constitutive endocytosis, whereas it is targeted for degradation following regulated endocytosis triggered by certain conditions such as BDNF treatment and Mg^{2+} withdrawal (Fig. 5.1).

My findings outlined in **Chapter 4** show inhibition of cellular tyrosine phosphatase activity by Na_3VO_4 not only raises the basal level of KCC2 surface expression in primary cultured cortical neurons, but also prevents the down-regulation of surface KCC2 by BDNF treatment. In addition, I show that the EGF dependent signalling pathway can transiently enhance KCC2 cell surface expression, although it remains elusive what are the physiological roles for this effect of EGF. It is unclear however whether these increases in KCC2 cell surface expression upon Na_3VO_4 or EGF treatment are due to an alteration in sorting decision (recycling versus degradation, for instance) following KCC2 endocytosis. This will be an interesting question to address in future studies. In addition, it will also be interesting to examine the relative kinetics of the different membrane trafficking pathways of KCC2 under these conditions that I have shown to alter KCC2 cell surface expression (Na_3VO_4, BDNF and EGF). These proposed studies will potentially add to our knowledge on the cellular mechanisms controlling KCC2 cell surface expression via membrane trafficking.

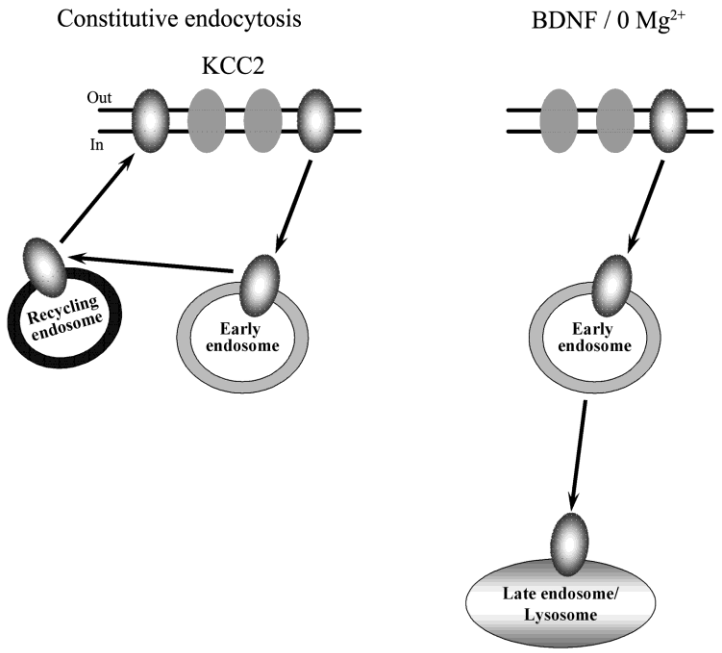


Figure 5.1 KCC2 is targeted for recycling and degradation, following constitutive endocytosis and BDNF/0 Mg^{2+} triggered regulated endocytosis respectively.

An additional observation from my studies is that a very small proportion of KCC2 protein is expressed at the cell surface. Even in adult hippocampal neurons, where KCC2 transport capacity is maximal, there is only approximately 5% of the total KCC2 protein expressed at the cell surface under resting conditions (Fig. 4.1, **Chapter 4**). This suggests the number of KCC2 molecules at the neuronal surface is tightly controlled. This may not be surprising considering that KCC2 acts as the major Cl^- extrusion pathway controlling Cl^- homeostasis in CNS neurons, and therefore very subtle changes in KCC2 surface expression may lead to significant functional outcomes. This data also suggests the majority of KCC2 is retained within intracellular pools. Deglycosylation experiments suggest this is not due to an effect on the maturation of the KCC2 protein (data not shown). Therefore interesting questions arising from these above findings include, in which intracellular compartment(s) is KCC2 found, and what is the physiological role for this intracellular pool of KCC2? A likely scenario is intracellular KCC2 may serve as a "ready-to-use" internal supply, which can to be translocated to the cell surface upon "urgent" situations, such as neuronal swelling occurring during neuronal spiking.

Colocalization studies using markers for different cellular compartments will be a start to address these questions. An additional question that will be interesting to address is whether the proportion of the cell surface expression of the other CCC family proteins is also developmentally regulated.

II. Identification of the molecular determinants for KCC2 constitutive endocytosis

My endocytic motif mapping study presented in **Chapter 2** is not only the first to report a defined endocytic motif that mediates the constitutive endocytosis of KCC2, but also the first to report an endocytic motif in any CCC family member proteins. I identified a key 12 amino acid region $_{651}$SAARYALLRLEE$_{662}$ within the KCC2 proximal C-terminus, containing the autonomous endocytic signal. Moreover, I showed the di-leucine motif in this region, $_{657}$LL$_{658}$, is essential for the constitutive endocytosis of KCC2. However, this di-leucine sequence is not present within a classical acidic di-leucine endocytic motif, (E/D)XXXL(L/I) (Bonifacino and Traub, 2003), in which an acidic residue is usually present at position −4 from the first leucine residue. Rather, I show two glutamic acid residues $_{661}$EE$_{662}$, which are downstream of the di-leucine sequence are required for the full strength of this endocytic motif. My results suggest the proximal availability of acidic residues may be more important than their position (upstream or downstream of the di-leucine residues), in influencing the strength of a di-leucine endocytic motif.

My studies have also shown endogenous KCC2 interacts with the clathrin-binding partner AP-2 complex and that $_{657}$LL$_{658}$ is essential for this process in heterologous HEK293 cells, which explains how the blockade of KCC2 constitutive endocytosis occurs upon mutation of these two leucine residues (Zhao et al., 2008; **Chapter 2**). A number of plasma membrane proteins have been shown to utilize di-leucine based endocytic motifs to internalize, and the [DE]XXXL[LI] consensus motif has been shown to preferentially bind to the μ and/or β subunits of AP complexes (Bonifacino and Traub, 2003). Therefore it will be of future interest to determine which subunit of the AP-2 complex KCC2 is specifically bound to.

III. What is the molecular motif(s) mediating the regulated endocytosis of KCC2?

Several studies have shown KCC2 cell surface expression can be regulated by a variety of factors, such as epileptic activity induced by 0 Mg^{2+}, the neuronal stressor H_2O_2, the protein tyrosine phosphatase inhibitor Na_3VO_4, and PKC (Rivera et al., 2004; Wake et al., 2007; Lee et al., 2007). In addition, it has been shown 0 Mg^{2+} and PKC can either reduce or increase KCC2 cell surface expression, via accelerating or decelerating the rate of KCC2 endocytosis respectively (Rivera et al., 2004; Lee et al., 2007). The molecular motif(s) mediating the accelerated KCC2 endocytosis by 0 Mg^{2+} is presently unknown. A serine residue in KCC2, Ser^{940}, has been identified as a target for PKC phosphorylation and PKC-dependent deceleration of KCC2 endocytosis (Lee et al., 2007). This suggests phosphorylation of certain residue(s) is a mechanism to regulate the rate of KCC2 endocytosis. These phosphorylated residues may exert their effect by modulating the interaction of KCC2 to the endocytosis machinery or establishing a specific conformation that facilitates the recruitment of KCC2 to the endocytosis machinery.

In addition to the role of PKC dependent Ser^{940} phosphorylation of KCC2 in controlling its endocytosis, tyrosine phosphorylation of KCC2 may also regulate KCC2 endocytosis. It has been shown that changes in KCC2 cell surface expression and transport activity occur upon exposure to the oxidative stressors and tyrosine phosphatase inhibitors (Wake et al., 2007). This suggests certain tyrosine residue(s) in KCC2 is the direct target(s) for phospho-modification under specific conditions. In addition, it suggests that the phosphorylation state of this tyrosine residue(s) in KCC2 is correlated with the level of KCC2 cell surface expression. Thus, one possible mechanism underlying this is through an alteration in the rate of KCC2 endocytosis.

To date no tyrosine residues in KCC2, to my knowledge, have been identified as the direct target for phospho-modification or a molecular motif mediating KCC2 endocytosis. However, I observed from my mapping study that the autonomous endocytic motif containing region in KCC2, $_{651}$SAARYALLRLEE$_{662}$, also contains a potential tyrosine-based endocytic motif, $_{655}$YALL$_{658}$, which conforms perfectly to the consensus tyrosine-based endocytic motif, YxxΦ (where x represents any amino acid and Φ represents a bulky hydrophobic amino acid residue). Although to my surprise I did not

detect any effect of this tyrosine residue on the constitutive endocytosis of KCC2, I speculated it might have alternative targeting function for KCC2 (Zhao et al., 2008; Fig. 2.6 and 2.7, **Chapter 2**). Therefore, this tyrosine residue is a good candidate for tyrosine phosphorylation analysis and future studies on identifying the molecular motif(s) mediating the regulated endocytosis of KCC2, under conditions such as exposure to H_2O_2, Na_3VO_4, BDNF, 0 Mg^{2+}, or EGF treatment. It will also be of interest to examine the role of the constitutive endocytosis motif I identified, $_{657}LL_{658}$, in controlling the rates of KCC2 endocytosis under these conditions. Addressing these questions will potentially reveal distinct molecular mechanisms governing KCC2 constitutive versus regulated endocytosis.

IV. Cellular kinase and phosphatase activities regulate KCC2 cell surface expression in neurons

Several studies have shown KCC2 activity can be regulated by intracellular kinase and phosphatase activities, including tyrosine kinases/phosphatases, PKC**,** creatine kinase, and WNK kinases (Fiumelli et al., 2005; Khiurg et al., 2005; Kelsch et al., 2001; Rivera et al., 2004; Lee et al., 2007; Wake et al., 2007; Inoue et al., 2005). However, the molecular mechanisms involved remain largely unknown.

As mentioned above, my data in **Chapter 4** shows in primary cortical neurons, BDNF treatment decreases KCC2 cell surface expression whereas EGF treatment transiently increases it. Moreover, the tyrosine phosphatase inhibitor Na_3VO_4 not only increases the basal level of KCC2 cell surface expression but also reverses the negative regulatory effect of BDNF on it. These findings are analogous to the observation that Na_3VO_4 prevented the down-regulation of KCC2 cell surface expression by the oxidative stressor H_2O_2 treatment in primary hippocampal neurons (Wake et al., 2007). Together, these observations strongly suggest that an endogenous tyrosine phosphatase(s) activity may intrinsically suppress KCC2 cell surface expression in both hippocampal and cortical neurons.

Given that EGF and BDNF trigger fairly similar signalling cascades, it is slightly surprising I found they have distinct effects on KCC2 surface expression. At present it is unknown what is the component(s) downstream of EGF and BDNF signalling that

distinguishes their effects on KCC2 cell surface expression in neurons. Therefore it will be of future interest to identify the tyrosine kinase/phosphatase(s) involved in the control of KCC2 cell surface expression and understand how they impact KCC2 endocytic trafficking (endocytosis, recycling, and degradation). In addition, whether cell surface targeting via the secretory pathway is a mechanism by which KCC2 cell surface expression can be controlled will also need to be addressed. Addressing these questions will potentially provide further insights into the regulation of KCC2 function and the strength of synaptic inhibition by distinct kinase and phosphatase activities via different cellular mechanisms, such as the endocytic and secretory pathways.

V. Identification of the molecular and structural basis mediating KCC2 dimerization

It is now known that all the CCC member proteins, except CIP and CCC9 which are yet to be examined, exist as or encode the capacity to form quaternary structures (de Jong et al., 2003; Starremans et al., 2003; Brunet et al., 2005; Simard et al., 2007; Blaesse et al., 2006). However, the molecular determinants mediating their quaternary assembly remain largely unknown. The majority of dimerization mapping studies so far has been focused on the NKCC1/2 proteins, and the regions mediating their self-interaction/dimerization have been mapped to their respective C-termini (Simard et al., 2004; Parvin et al., 2007; Brunet et al., 2005). With regards to the KCC member proteins, the importance of the C-termini in mediating their self-interaction has also been indicated, from yeast two-hybrid mapping studies (Simard et al., 2007). However, to date defined regions mediating their dimerization have not been reported.

In my work presented in **Chapter 3**, I employed a combination of experimental approaches such as the construction of chimeric proteins, site-directed mutagenesis, *in vitro* GST binding assay, and *in vivo* co-immunoprecipitation assays to map the molecular regions mediating KCC2 dimerization. I have identified two small regions in the proximal and central C-terminus of KCC2, fragment A (12 a.a.) and fragment B (62 a.a.) respectively, that mediate KCC2 dimerization. These dimerization regions in KCC2 are much narrower than previously identified for NKCC1 and NKCC2 (Simard et al., 2004; Parvin et al., 2007; Brunet et al., 2005). My results also suggest a hypothetical model for

the dimerization of KCC2, in which fragments A and B in one KCC2 molecule reciprocally interact with fragments B and A in another KCC2 molecule (Fig. 3.7, **Chapter 3**). These reciprocal interactions between fragments A and B suggest a great likelihood for KCC2 to form higher order oligomers (Fig. 3.7, **Chapter 3**). In addition, the amino acid sequence and functional homology amongst KCC member proteins suggest that similar oligomeric architectures may apply to the other KCCs and that hetero-oligomerization many occur between different KCC isoforms. To understand how physically fragments A and B may mediate the interaction between two KCC2 molecules, I conducted secondary structural prediction analysis. Interestingly, my results reveal fragments A and B each contains a predicted α-helical stretch, suggesting that a helix-helix based interaction between these fragments is most likely the structural basis mediating KCC2 dimerization (Fig. 3.8, **Chapter 3**).

My mutagenesis mapping studies revealed a dimerization blocking mutation of KCC2, LL657,658AA. Secondary structural analysis predicts this mutation may exert its effect through disrupting of a potential "LxxxxxxL"-type dimerization motif ($_{650}$LSAARYALL$_{658}$), and hence the α-helical interaction between fragments A and B. Other molecular motifs, such as GxxxG, QxxS, and aromatic-xx-aromatic, have also been suggested to be involved in α-helical interactions (Sitte et al., 2003; Sal-Man et al., 2005 and 2007). These motifs are apparently not found in fragment A. However, I noticed fragment B contains a potential α-helical interaction sequence, $_{788}$WRNF$_{791}$, which conforms to the aromatic-xx-aromatic motif. This sequence will therefore be a good candidate target for future mapping analysis to precisely delineate the key residues in fragment B that mediate KCC2 dimerization.

Together, my data presented in **Chapter 3** is the first to report defined molecular regions mediating the oligomerization of KCC2 or any other related KCC member protein and present a working model for the structural basis for the required intermolecular interactions. It also opens up the field in understanding the structural basis underlying the quaternary assembly of CCC member proteins.

VI. Conservation of $_{657}LL_{658}$ amongst KCCs-indication of evolutionarily conserved post-translational regulations?

An important finding common in both of my endocytosis and dimerization studies is that the di-leucine sequence, $_{657}LL_{658}$, is highly conserved amongst KCC2 related KCC family members but is absent from the analogous region of the more distally related NKCC1, NKCC2 or NCC proteins (**Chapter 2 and 3**). This suggests the mechanisms controlling the constitutive endocytosis and oligomerization of KCC family members may be evolutionarily conserved. Given the opposing physiological function of KCC versus NKCC and NCC proteins, in mediating either Cl^- efflux or influx respectively, the presence or absence of these two leucine residues provides further indication of how these related protein families may be similarly or differentially regulated at the post-translational level. My mapping studies are also an initial step in uncovering the molecular basis that differentiates the physiological roles exerted by distinct branches of the CCC member proteins, provided that my mapping studies will be extended to the other CCC member proteins.

VII. KCC2 displays different features of quaternary assembly from NCC and NKCCs

Studies on NCC and NKCC1 have so far suggested they preferentially form homo-dimers at the cell surface (de Jong et al., 2003; Moore-Hoon et al., 2000; Starremans et al., 2003). In contrast to these Na^+-coupled Cl^- cotransporters, KCC2 has been shown to predominantly exist as a mixture of dimers, trimers, and tetramers in mature brain stem LSO neurons (Blaesse et al., 2006), see Fig. 5.2. In addition, the developmental increase in the proportion of oligomeric KCC2 parallels the functional up-regulation of KCC2 in rat brain stem LSO neurons between E18-P30 (Blaesse et al., 2006). However, the levels of total KCC2 expression or protein maturation are comparable throughout this period, and both active and inactive forms of KCC2 are present at the plasma membrane (Blaesse et al., 2006). This suggests that unlike NCC and NKCC1, higher order quaternary assembly may be necessary for the optimal transport activity of KCC2 in LSO neurons. It is known that in hippocampal neurons the increase in KCC2 protein expression underlies the functional up-regulation of KCC2 and the

maturation of inhibitory neurotransmission (Rivera et al., 1999). However, it remains unclear whether post-translational mechanisms, such as quaternary assembly of KCC2, are also involved in the increase in KCC2 transport activity during the neuronal maturation of forebrain neurons, as observed in brain stem LSO neurons. Therefore it will be of future interest to examine the oligomeric state of KCC2 in the developing forebrain regions, such as the cortex and hippocampus.

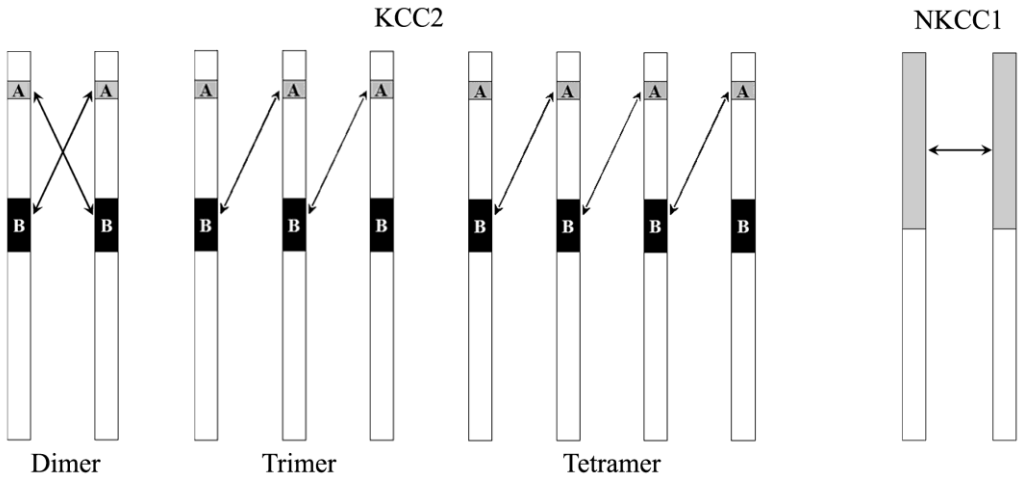


Figure 5.2 Comparison of the major differences between the features of quaternary assembly of KCC2 and NKCC1. Only the C-termini of KCC2 and NKCC1 are shown.

My molecular mapping study on KCC2 dimerization regions and the previous related studies on NKCC1, suggest their quaternary complexes may be assembled in different ways, see Fig. 5.2. As discussed above, I show KCC2 dimers (or higher order oligomers) are formed through reciprocal interactions between fragments A and B in the KCC2 C-terminus. In contrast, the mapping studies on NKCC1 dimerization regions have so far suggested NKCC1 dimers are formed through interactions between the same region in the NKCC1 C-terminus (Parvin et al., 2007).

Biochemically, KCC2 also exhibits different features from NCC and NKCC1. The dimerization of NCC and NKCC1 is achieved via noncovalent interactions that are sensitive to non-ionic detergent such as 0.4% Triton X-100 or the anionic detergent 0.01% SDS (Moore-Hoon and Turner, 2000; de Jong et al., 2003). In contrast, KCC2 oligomers are resistant to 4% Triton X-100 or 1% SDS but sensitive to sulfhydryl-

reducing agents such as β-mercaptoethanol (Blaesse et al., 2006), suggesting that covalent interactions are involved in the control of KCC2 oligomerization. My data suggests the LL657,658AA mutation may abolish KCC2 dimerization by disrupting of potential α-helical non-covalent based interactions. Therefore it is possible that both covalent and noncovalent interactions may be involved in maintaining the stability of KCC2 oligomeric complexes. This is an issue that needs to be addressed in the future.

Together, the above observations suggest that distinct mechanisms may be involved in regulating the quaternary assembly of the K^+-coupled versus Na^+-coupled Cl^- cotransporters. Given the opposing physiological function of KCC versus NKCC and NCC proteins, in mediating either Cl^- efflux or influx respectively, quaternary assembly may represent a post-translational mechanism by which these two branches of CCC member proteins may be differentially regulated. As regards to KCC2, it has yet to be addressed what is the functional significance for KCC2 to form higher order oligomers? Also, do transitions between different orders of KCC2 oligomers occur in mature neurons and if so, do these transitions occur spontaneously or are they only triggered by certain conditions? Finally if transitions between different orders of KCC2 oligomers occur at the cell surface, this could be an efficient means by which KCC2 transport activity can be modulated. Addressing these questions will potentially provide a better understanding of the role for KCC2 quaternary assembly in the control of KCC2 function and KCC2 dependent synaptic inhibition.

VIII. Does KCC2 form hetero-oligomers with related CCC member proteins?

It has been shown that different isoforms of CCC member proteins can form hetero-dimers when expressed in *X. laevis* oocytes (Simard et al., 2007). In particular, KCC2 can form hetero-dimers with its functionally homologous CCC proteins, KCC1, KCC3, and KCC4 when they were co-expressed in *X. laevis* oocytes (Simard et al., 2007). However, it is unknown whether these hetero-dimers are also formed in CNS neurons (where KCC2 is exclusively expressed), and if so what is the physiological significance for KCC2 to dimerize with the other KCCs in these tissues.

Hetero-dimerization between K^+- and Na^+-coupled Cl^- cotransporters has also been observed between KCC1 and NKCC1 as well as KCC4 and NKCC1, when they

were co-expressed in *X. laevis* oocytes (Simard et al., 2007). More importantly, a functional consequence of KCC4-NKCC1 hetero-dimerization has been detected (Simard et al., 2007). Under conditions that stimulate NKCC1 transport activity, NKCC1 function was increased 2 fold by KCC4 co-expression as compared to without KCC4 (Simard et al., 2007). Although it is yet to be determined whether similar effects occur in naïve tissues, this study suggests hetero-oligomerization can indeed occur between certain CCCs and that the availability of one monomer within a hetero-oligomeric complex may affect the function of another.

KCC2 and NKCC1 are the two major CCCs controlling Cl^- homeostasis in CNS neurons. It is known that in the vast majority of brain neurons, the late induction of KCC2 gene expression and the concomitant down-regulation of NKCC1 during development result in the decrease in $[Cl^-]_i$, and consequently the switch of GABA-mediated synaptic responses from excitatory to inhibitory (Payne et al., 2003). The complementary temporal gene expression profiles of KCC2 and NKCC1 in brain neurons indicates it is unlikely for these two proteins to hetero-oligomerize during very early development or in the adult. However, it remains possible during the transition period when both proteins are expressed. Unlike brain neurons, in the spinal cord both KCC2 and NKCC1 are expressed and functional during early development between E11.5-13.5 (mouse), when GABAergic responses are excitatory (Delpy et al., 2008). This could be due to NKCC1 Cl^- uptake activity overriding KCC2 Cl^- extrusion activity, while they both function independently. Alternatively, they may hetero-oligomerize and the Cl^- transport activity of one isoform may affect the other, which results in the overall functional outcome as raising $[Cl^-]_i$ and hence excitatory GABAergic responses.

Therefore, it will be of future interest to examine whether KCC2 and NKCC1 hetero-oligomerize in neurons of different CNS regions, and if so whether their hetero-oligomeric state is altered during neuronal maturation. Also, if KCC2 and NKCC1 do form hetero-oligomeric complexes, what are the contributions of each monomer to the overall Cl^- transport capacity of the entire complex. Understanding these questions will potentially reveal a novel mechanism controlling cellular Cl^- homeostasis via modulating the hetero-oligomeric state of different CCC member proteins.

IX. What is the functional unit of KCC2 at the cell surface?

A large number of plasma membrane proteins are known to exit as dimers or higher order oligomers, such as the GPCR family proteins (Milligan, 2008), TRKs (Hubbard and Miller, 2007), neurotransmitter transporters (Sitte and Freissmuth**,** 2003), and ion channels and transporters (Kittler et al., 2002; Greger et al., 2007; Taniguchi et al., 2001; Sachs et al., 1995; Fafournoux et al., 1994; Canessa et al., 1994; Agre et al., 1998; Hastrup et al., 2003). Quaternary assembly is crucial for the function of ion channels at the cell surface, such as glutamate receptors and $GABA_A$ receptors (Kittler et al., 2002; Greger et al., 2007). Many transporter proteins have been shown to exist as complex quaternary structures (Veenhoff et al., 2002). However, evidence for both monomeric and multimeric proteins acting as their functional unit has been provided (Fafournoux et al., 1994; Gerchman et al., 2001; Torres et al., 2003; Bonde et al., 2006; Starremans et al., 2003). For instance, the Na^+/H^+ exchanger (NHE) family member proteins exist as oligomers, but their functional unit is a monomer (Fafournoux et al., 1994; Gerchman et al., 2001). In contrast, the dopamine transporter (DAT) both exits and functions as at least a dimer (Torres et al., 2003). Similarly, the metabotropic glutamate-like GPCRs also exist and function as dimers (Bonde et al., 2006).

The functional unit of the CCC member proteins remains largely unknown. Relevant functional studies have so far only addressed NCC and NKCC2. These studies have suggested when heterologously expressed in *X. laevis* oocytes, the functional units of NCC and NKCC2 are a monomer and dimer respectively, although they can both exist as a dimer (de Jong et al., 2003; Starremans et al., 2003). In the NCC study, $^{22}Na^+$ uptake was measured on *X. laevis* oocytes expressing concatameric proteins consisting of two wild-type NCC monomers or one wild-type and one partially retarded G980R (a mutation identified in Gitelman's syndrome) monomer. Comparable transport activity was observed in both types of concatamers, suggesting the functional unit of NCC is a monomer although it can exist as a homo-dimer (de Jong et al., 2003). Hence dimerization does not play an important role in mediating the function of NCC and its relevance at present is unknown. Similar functional assays were performed in the NKCC2 study, where concatameric constructs consisting of two wild-type monomers or a wild-type and a loss-of-function mutant monomer of NKCC2 were expressed in *X. laevis* oocytes. The

transport activity of the wild-type-mutant concatemer was reduced by half relative to the wild-type-wild-type concatamer, suggesting that NKCC2 functioned at least as a dimer in this system (Starremans et al., 2003). As discussed above, KCC2 can exist as dimers and higher order oligomers in both LSO neurons and heterologous HEK293 cell, and there is a seemingly good correlation between the levels of KCC2 quaternary assembly and neuronal maturation in these neurons (Blaesse et al., 2006). However, no direct evidence for the functional unit of KCC2 at the cell surface has so far been provided. Given the above studies on characterizing the functional unit of NCC and NKCC2, possibilities of both KCC2 functioning as a monomer and an oligomer exist. As I have shown the LL657,658AA mutation is sufficient to block KCC2 dimerization (Fig. 3.6, **Chapter 3**), this KCC2 mutant will provide a useful tool for future studies on characterizing the functional unit of KCC2.

X. How does KCC2 dimerization impact its endocytosis?

The most striking result obtained from my PhD studies presented in **Chapters 2** and **3** is that the LL657,658AA mutation is sufficient to block both KCC2 constitutive endocytosis and dimerization. This seeming coincidence has brought up a very interesting question, which is how these two leucine residues are sufficient to mediate both processes of KCC2? One possibility is that the mutation LL657,658AA may disrupt the structural basis (α-helices, for instance) for KCC2 quaternary assembly, and in the meanwhile also block KCC2 binding to the AP-2 complex and hence its constitutive endocytosis. In this scenario, KCC2 constitutive endocytosis and dimerization are two independent processes mediated by $_{657}LL_{658}$, through distinct mechanisms. Alternatively, the blockade of KCC2 constitutive endocytosis by LL657,658AA may be a secondary effect of the blockade of KCC2 dimerization.

It is known that dimerization has a significant impact on the membrane trafficking of a number of plasma membrane proteins, such as G-protein coupled receptors (GPCRs, Jordan et al., 2001; Terrillon and Bouvier, 2004; Milligan, 2008) and the tyrosine receptor kinase (TRK) family proteins (Wang et al., 2005; Heldin, 1995). For example, it is known that nearly all TRKs, when in their inactive state, exist as monomers at the cell surface. Binding of ligand results in conformational changes in the receptors, which leads to their

tyrosine trans-autophosphoryation and the formation of active dimeric receptors (Hubbard and Miller, 2007). A major consequence of this is to permit recruitment of the dimeric receptors to clathrin-coated pits and subsequent receptor endocytosis and signalling transductions (Vieira et al., 1996). Therefore, the conformational changes of the receptor seem to be the fundamental signal directing the transitions between two cellular processes of the receptor. Although KCC2 is not a ligand binding protein as are the TRKs, similar mechanisms utilizing conformational changes of the protein to signal its "dimerization to endocytosis" process may also be applicable to KCC2. That is, the $_{657}$LL$_{658}$ residues may, through the formation of potential α-helices (**Chapter 3**), act to establish a conformation of the KCC2 C-terminus which facilitates the interaction between fragments A and B (most likely through α-helical interactions) and hence KCC2 dimerization. This conformation of the KCC2 C-terminus acquired by KCC2 dimerization may in turn allow the endocytic motif $_{657}$LL$_{658}$ to be exposed and recognized by the AP-2 complex, whereby targeting KCC2 to the CME pathway.

Due to the simultaneous requirement for $_{657}$LL$_{658}$ in both KCC2 dimerization and constitutive endocytosis, it demands an alternative dimerization deficient mutant of KCC2, rather than KCC2(LL657,658AA), to address whether dimerization is a prerequisite for KCC2 constitutive endocytosis. Given my identification of fragment B as a second KCC2 dimerization region, we can potentially generate a dimerization deficient mutant of KCC2 by mutating fragment B while maintaining fragment A and hence the endocytic motif $_{657}$LL$_{658}$ intact. We can then analyze the constitutive endocytosis of this dimerization mutant. Alternatively, we may disrupt the interaction between fragments A and B by mutating a distinct residue(s), rather than $_{657}$LL$_{658}$, within fragment A. As discussed in **Chapter 3**, the sequence "$_{650}$LSAARYALL$_{658}$", which conforms perfectly to the "LxxxxxxL" dimerization motif, may be the molecular basis contributing to the binding of fragment A to B. If this is the case, we can theoretically disrupt KCC2 dimerization by mutating only L$_{650}$ while sparing the endocytic motif $_{657}$LL$_{658}$. We can then utilize this dimerization deficient mutant to examine the role of KCC2 dimerization in mediating its endocytosis.

My studies uncovering this seemingly coincidence, that is the simultaneous function of $_{657}$LL$_{658}$ in both KCC2 dimerization and constitutive endocytosis, have

provided a starting point to understand how oligomerization may impact KCC2 endocytosis, and perhaps the cell surface expression and physiological function of KCC2 and related CCC member proteins. In addition, our results may also hint on how the quaternary assembly, endocytosis, surface expression, and function of a protein may be linked at the molecular level. An interesting question to be addressed in the future is whether KCC2 exists as a quaternary complex throughout its life along the endocytic pathway.

XI. Does KCC2 endocytose as a monomer or oligomer?

Many transporter and cotransporter proteins have been shown to exist as a dimer or higher order oligomer (de Jong et al., 2003; Moore-Hoon and Turner, 2000; Starremans et al., 2003; Simard et al., 2007; Taniguchi et al., 2001; Sitte and Freissmuth, 2003; Hastrup et al., 2003), but the oligomeric state of these proteins following their endocytosis is poorly understood. A study performed by Chen and Reith (2008) showed that dopamine transporter (DAT) substrates (dopamine and amphetamine) led to not only a reduction in DAT cell surface expression but also a dissociation of oligomeric DAT in heterologous HEK293 cells. In addition, inhibition of DAT endocytosis by phenylarsine oxide or high sucrose (450 mM) prevented the effect of dopamine and amphetamine in reducing oligomeric DAT (Chen and Reith, 2008). Although no direct evidence for the oligomeric state of DAT following substrate-induced endocytosis was provided in this study, it has provided a hint that DAT potentially endocytoses as a monomer.

The oligomeric state of plasma membrane proteins following their endocytosis is better understood for some GPCR proteins (Cao et al., 2004; Overton and Blumer, 2000). Some experiments performed in these studies provide a great strategy for future studies to examine whether KCC2 is endocytosed as a monomer or oligomer. For instance, by co-expressing the wild-type α-factor receptor, the product of the *STE2* gene in the yeast *Saccharomyces cerevisiae*, with its endocytosis deficient mutant receptor, Overton and Blumer (2000) have shown that α-factor receptor endocytoses as a dimer. Given our observation that co-expression of wild-type Myc-KCC2 and HA-KCC2(LL657,658AA) resulted in normal dimerization of the KCC2 protein (data not shown), we can co-express

these two KCC2 constructs in HEK293 cells and examine whether the endocytosis of HA-KCC2(LL657,658AA) can be (partially) rescued.

Fluoresence resonance energy transfer (FRET) is regarded as a fine approach to detect protein-protein interactions (including dimerization), protein-DNA interactions, and protein conformational changes especially in live cells. For example, in order to monitor the dimerization between two protein molecules, one of them is labelled with a donor fluorophore (CFP, for instance) and the other with an acceptor fluorophore (YFP, for instance). These two fluorophore labelled molecules are co-expressed in cells. If they do not interact, the donor emission is detected upon the donor excitation. However, if they are close enough (1-10 nm) to interact (dimerize), the acceptor emission will be observed upon the donor excitation because of the intermolecular FRET from the donor to the acceptor. This approach has been successfully employed to detect the dimerization of the α-factor receptor in yeast (Overton and Blumer, 2000). The major advantage of using this technique to study protein dimerization is that one can visualize the process in live cells. In addition, it is also possible to visualize the cellular localization where dimerization occurs such as by examining the co-localization of FRET signal with intracellular compartment markers. Therefore FRET is a good candidate methodology that could be employed in future studies to better understand the fate of KCC2 oligomeric complexes in live cells.

As mentioned above, the di-leucine sequence in KCC2, $_{657}$LL$_{658}$, is not only an AP-2 binding site (Zhao et al., 2008; Fig. 2.8, **Chapter 2**), but also most likely participates in the formation of a potential "LxxxxxxL" type α-helical dimerization motif (Fig. 3.8, **Chapter 3**). As discussed above, KCC2 dimerization may establish certain C-terminal conformations that facilitate the recruitment of KCC2 to the AP-2 complex. This could then lead to two potential scenarios. In the first (Fig. 5.3A), AP-2 binding could be competitive, resulting a disruption of the interaction between fragments A (which contains the endocytic motif) and B. This would lead to the dissociation of dimeric KCC2 into monomers at the initial stage of or following endocytosis (Fig. 5.3A). In the second scenario (Fig. 5.3B), AP-2 binding would be noncompetitive with the fragments A and B interaction. In this case, if one fragment A is unused for KCC2 oligomerization, AP-2 would bind to this site, hence having no effect on the integrity of the KCC2 oligomer.

Consequently, KCC2 would remain as an oligomeric complex after being endocytosed from the plasma membrane (Fig. 5.3B). Utilizing a combination of different approaches, such as the biotinylation assays and FRET discussed above, these hypothetical scenarios could be tested in the future.

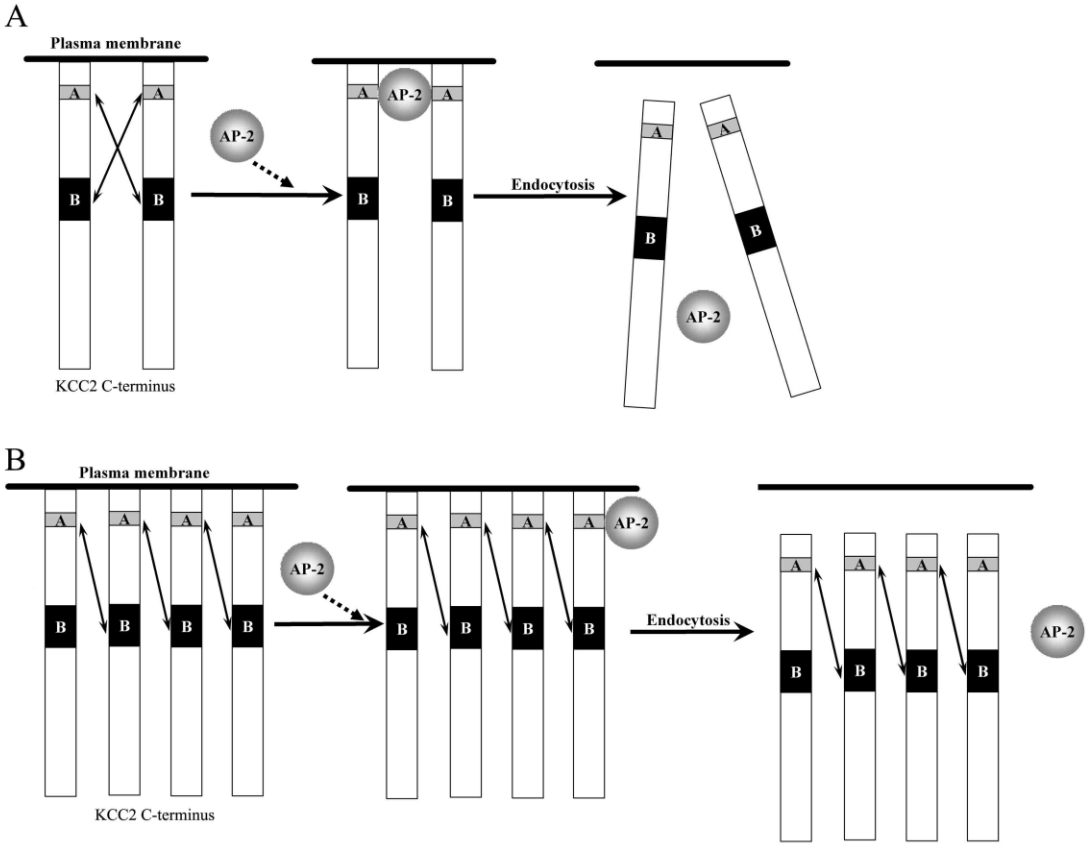


Figure 5.3 Hypothetical illustration for two possible KCC2 quaternary assemblies following endocytosis. Only the C-terminus of KCC2 is shown.

XII. What is the pathological relevance of KCC2 post-translational regulations?

As discussed in **Chapter 1**, KCC2 plays a central role in mediating a number of neuronal processes in the CNS, including promoting neuronal maturation of synaptic inhibition during development, controlling neuronal excitability in the adult brain, potentially mediating Cl^- homeostasis and cell volume at excitatory synapses, and bi-directionally cotransporting K^+/Cl^- or NH_4^+/Cl^-. Deficient gene expression of KCC2 has been linked to the neuropathology of epileptogenesis, neuronal trauma, and neuropathic

pain (Woo et al., 2002; Huberfeld et al., 2007; Palma et al., 2006). However, all of the above findings are associated with a functional outcome upon changes in KCC2 gene expression. It remains unclear whether disruption of KCC2 post-translational regulations, such as membrane trafficking or oligomerization, also has any physiological relevance and whether they are associated with pathological conditions.

Aberrant endocytic trafficking or deficient oligomerization of other membrane transporter/cotransporters and ion channels is associated with certain human diseases (Neufeld et al., 2004; Marr et al., 2002; AbdAlla et al., 2005). For instance, a deficiency in the late endocytic trafficking of the ATP binding cassette transporter A1 (ABCA1) has been associated with Tangier disease, which is an inherited disorder characterized by a severe reduction in the amount of the high density lipoprotein (Neufeld et al., 2004). Hetero-oligomerization of the wild-type Aquaporin-2 (AQP2) with a mutant AQP2-727δG results in mistargeting of the oligomeric complex to the late endosomes/lysosomes instead of to the plasma membrane (Marr et al., 2002). This is indicated in the pathogenesis of dominant nephrogenic diabetes insipidus (NDI) (Marr et al., 2002), a disease in which the kidney is unable to concentrate urine in response to vasopressin. Also, inhibition of hetero-dimerization of the angiotensin II receptors AT1/B2 results in increased angiotensin II-stimulated endothelin-1 secretion of mesangial cells, and is associated with the pathogenesis of hypertension (AbdAlla et al., 2005). As such, it is reasonable to speculate that the interference of KCC2 oligomerization or endocytosis, which may affect its functional expression, could also lead to certain neuropathological conditions such as where the neuronal homeostasis of Cl^- or NH_4^+ is disrupted.

My identification of the molecular determinants for KCC2 constitutive endocytosis and dimerization are an initial step in understanding the molecular basis controlling KCC2 function via post-regulation of its gene expression. The KCC2 constitutive endocytosis and dimerization deficient mutants I have generated will therefore provide useful tools for future studies on the physiological significance and pathological relevance of KCC2 post-translational regulations.

CONCLUSIONS

KCC2 is an essential protein controlling neuronal Cl^- homeostasis and hence synaptic inhibition in the CNS. While gene expression is crucial for KCC2 function, I show endocytosis and oligomerization are two important post-translational mechanisms regulating KCC2. The work presented in this thesis is the first to describe the cellular mechanisms and molecular determinants regulating KCC2 constitutive endocytosis and dimerization. My molecular mapping analysis identifying the constitutive endocytosis motif and dimerization regions in KCC2 provides important information on how KCC2 function may be controlled at the molecular level. My studies also indicate how KCC2 and its functionally homologous or divergent CCCs may be similarly or differentially regulated at the post-translational level. Finally, my findings in addition shed some light on the cellular mechanisms underlying the control of KCC2 cell surface expression by neurotrophic/growth factors and cellular kinase/phosphatase activities.

Hence, my studies provide a strong starting point for future studies on characterizing the molecular mechanisms regulating other aspects of KCC2 post-translational regulation, such as regulated endocytosis and regulated oligomerization. The endocytosis and dimerization deficient mutants of KCC2 as well as related constructs I have generated will be essential tools for these future studies. In a broader sense, my studies are also applicable to the analysis of the molecular mechanisms by which other KCC2 related CCC member proteins might be controlled.

Finally an interesting issue for future research will be to understand how transcriptional/translational and post-translational mechanisms are coordinated in order to tightly control the function and efficiency of KCC2 mediated Cl^- transport. Investigation of this issue will deepen our knowledge about how KCC2 function and hence the efficacy of inhibitory neurotransmission and synaptic plasticity may be controlled at different regulatory levels.

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APPENDIX

Other publications arising from this thesis:

Ryan J. Petrie, **Beibei Zhao**, Fiona Bedford and Nathalie Lamarche-Vane. 2009 Compartmentalized DCC signaling is distinct from DCC localized to lipid rafts. *Biol Cell.* **101**(2):77-90.