PROTEINS WHICH INTERACT WITH AND REGULATE THE CHLORIDE CHANNEL, ClC-2

by

Najma Nusarat Ahmed

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Graduate Department of Institute of Medical Science

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ABSTRACT

ClC-2, a member of the ClC family of chloride channels, has been suggested to be able to functionally compensate for CFTR. However, mechanisms underlying the regulation of ClC-2 activity remain unclear. Purified, reconstituted ClC-2 exhibits constitutive channel activity, suggesting that purification may remove regulatory proteins. It is our hypothesis that ClC-2 interacts with and is regulated by cytoskeletal proteins. Interacting proteins were eluted from a purified ClC-2 column, and western blotting and MALDI-Tof analysis confirmed the presence of actin and dynein, respectively. Functional studies in *Xenopus* oocytes indicate that these interactions are functionally important. Following treatment with cytochalasin D or EHNA, a dynein inhibitor, there was an increase in ClC-2 mediated currents. Our studies indicate that ClC-2 interacts with actin via its N-terminus and that the interaction is sensitive to ionic strength. Cellular fractionation studies suggest that dynein may play a role in the movement of ClC-2 between cellular compartments.

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LIST OF ABBREVIATIONS

ATP-adenosine triphosphate

CBS-cystathione-beta-synthetase

CF- Cystic fibrosis

CFTR- Cystic fibrosis transmembrane conductance regulator

EDTA-Ethylenediaminetetraacetic acid

EGTA-Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EHNA- erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride

GABA-gamma-aminobutyric acid

GFP- green fluorescent protein

GTP-guanine triphosphate

IPTG-Isopropyl beta-D-thiogalactoside

MTS-mesitylene-2-sulfonyl

PBS-phosphate buffered saline

PFO-pentadecafluorooctanoic acid

SDS-PAGE-sodium dodecyl sulphate polyacrylamide gel electrophoresis

SOS- standard oocyte solution

sSOS- supplemented standard oocyte solution

TBS-Tris buffered saline

VSOAC- volume-sensitive organic osmolyte and anion channel

CHAPTER 1

INTRODUCTION

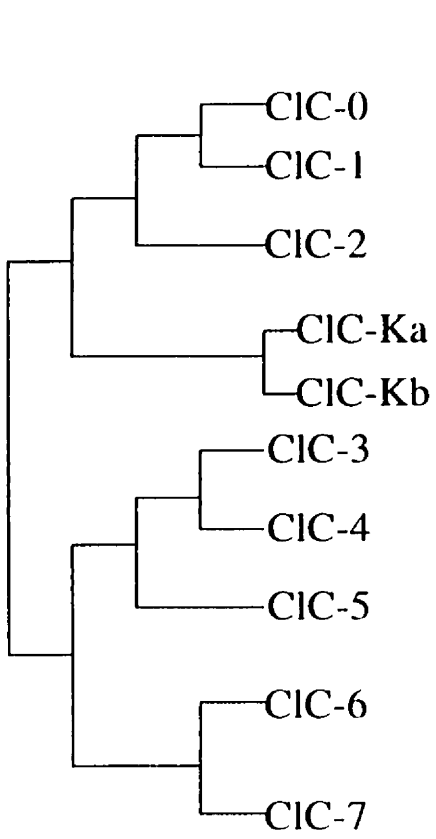
1.1 THE ClC FAMILY OF CHLORIDE CHANNELS

The ClC family of chloride channels comprises at present 9 voltage gated chloride channels. The first member of this family, ClC-0 was identified in 1990 (Jentsch *et al*, 1990). Genes encoding ClC channels have since been identified in mammals, plants, yeast and bacteria, indicating that these genes have been highly conserved in evolution, and therefore are likely functionally important in both prokaryotes and eukaryotes (Jentsch *et al*, 1997). Within the ClC family, there are three groups of channels with less than 30% homology between the groups (Figure 1.1). The first group consists of ClC-0, ClC-1, ClC-2 and the ClC-K channels. The second group includes ClC-3, ClC-4 and ClC-5 and the third branch includes ClC-6 and ClC-7. Defects in some of these proteins have previously been implicated in the pathogenesis of clinical disease.

1.2 OVERVIEW OF ClC STRUCTURE

ClC proteins have a characteristic topology with 10-12 transmembrane domains, however the exact topology is still unclear (Figure 1.2). Initial hydropathy studies in ClC-0 suggested the presence of up to 13 membrane spanning segments (D1-D13) (Jentsch *et al*, 1990). However, subsequently, it has been shown that D13 is not a transmembrane segment (Grunder *et al*, 1992). The N and C termini of these proteins are intracellular, however, the exact topology is yet to be finalized. The location of one segment termed D4 is thought to be extracellular , as cysteine residues inserted in between D3 and D4 could be modified by MTS reagents (Schmidt-Rose *et al*, 1994). Glycosylation studies have also suggested that this segment is extracellular, however, another study has suggested that this segment does cross the lipid bilayer based on cysteine scanning (Fahlke *et al*, 1997). Furthermore due to a large hydrophobic region between D9 and

Figure 1.1 The ClC family of Chloride Channels



	Tissue/species	*Proposed function*	*disease*
ClC-0	*Torpedo marmorata* Electrical organ	Stabilize membrane potential	
ClC-1	Skeletal muscle	Stabilize membrane potential	Myotonia congenita
ClC-2	ubiquitous	?cell volume regulation; maintenance of intracellular Cl- in neurons	
ClC-Ka	kidney	Transepithelial Cl- transport	Diabetes insipidus
ClC-Kb	kidney	Transepithelial Cl- transport	Bartter's syndrome
ClC-3	Many; esp. brain, Skeletal muscle	? Cell volume regulation	
ClC-4	Many; esp. brain, heart	unknown	
ClC-5	Kidney; brain, liver	?acidification of vesicles	Dent's disease
ClC-6	ubiquitous		
ClC-7	ubiquitous		

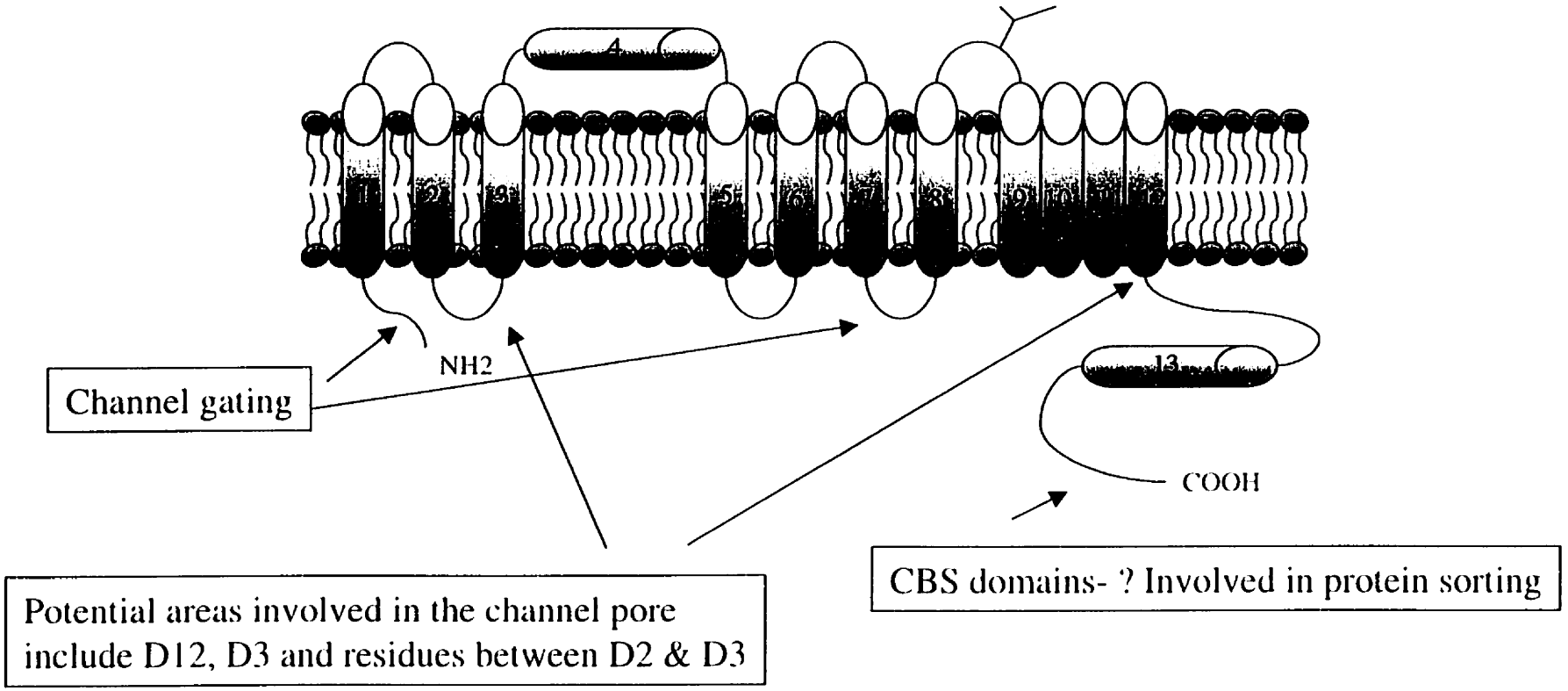
The ClC family of chloride channels can be divided into three branches. The first includes ClC-0, ClC-1 and the ClC-K channels; the second, ClC-2, ClC-3 and ClC-4 and the third branch, ClC-6 and ClC-7 (see text for details).

D12, it has been difficult to determine the exact number of transmembrane segments in this region (Schmidt-Rose *et al*, 1994). ClC channels also have two tandem C terminal CBS domains. The function of these motifs, named after their identification in cystathionine-β-synthase, is not entirely clear, however they may play a role in protein sorting (Schwappach *et al*, 1998).

Studies have also shown that ClC family members can form heterodimers. Studies in *Xenopus* oocytes injected with both ClC-1 and ClC-2 cRNA, resulted in the appearance of currents with novel properties, that were not simply summation currents from the individual channels (Lorenz *et al*, 1996). It is not clear as yet whether other ClC family members are also capable of forming heterodimers. Furthermore, ClC-0 and ClC-1 can be separated into different parts, for example, between D8 and D9, but when the parts are expressed together they still function as chloride channels (Maduke *et al*, 1998 and Schmidt-Rose *et al*, 1997). The C terminus also plays an important role in the function of these channels, since when it is truncated between the two CBS domains, the channel is non-functional. However, when the C-terminus is co-expressed, channel function resumes (Maduke *et al*, 1998 and Schmidt-Rose *et al*, 1997).

The location of the pore in the ClC channels is poorly defined. Residues in D12 appear to play an important role, as the mutation K519 in this region, changes pore properties (Ludewig *et al*, 1997 and Pusch *et al*, 1995). Residues between D2 and D3, as well as within D3 also play an important role as these regions affect pore properties such as ion selectivity, single-channel conductance and rectification (Fahlke *et al*, 1997a, Schwappach *et al*, 1998, Fahlke *et al*, 1997b, Steinmeyer *et al*, 1994).

Figure 1.2 Topology of ClC-2



1.3 ClC function

1.3.1 ClC-0

ClC-0, the first member of the ClC family to be identified, was cloned from the electric organ of *Torpedo marmorata*, and encodes a protein of 90kD which is predicted to have 12 transmembrane domains (Grunder *et al*, 1992). This channel is proposed to play a role in the generation of voltage pulses by the Torpedo electric organ. It has an ion selectivity of Cl>Br>I, and structurally, this channel has been shown to exist as a dimer (Middleton *et al*, 1994, Middleton *et al*, 1996). Functionally, ClC-0 exhibits "double-barrelled" channel activity with a single channel conductance of 10pS (Ludewig *et al*, 1996). Double-barreled channels are characterized by two identical pores that can open and close independently, as well as a common gate that can close both pores together. Evidence for this structure comes from studies in which wild-type and mutant ClC-0 subunits were expressed together, and there was a change in ion selectivity and single channel conductance (Ludewig *et al*, 1996, Pusch *et al*, 1997). Furthermore, the double-barreled structure consisted of individual pores with different conductances, reflecting the wild-type and mutant subunits. ClC-0 exhibits both fast and slow gating mechanisms, reflecting the activity of the individual pore gates and the common gate, respectively. The fast gating mechanism is moderately temperature-dependent and is also dependent on cellular chloride concentrations, suggesting that the permeant ion may be important in channel gating (Fahlke *et al*, 1997b, Pusch *et al*, 1997). The slow gate is also temperature sensitive (Fong *et al*, 1998), and is activated by hyperpolarization. This activation by hyperpolarization is thought to be mediated by the carboxyl terminus of the protein, as chimeric proteins in which the carboxyl terminus was replaced by that of ClC-2 resulted in the loss of

slow gating properties (Fong *et al*, 1998). The slow gate has also been shown to be affected by extracellular zinc, which results in an inhibition of channel activity (Chen, 1998).

1.3.2 ClC-1

ClC-1 is the ClC chloride channel whose physiology has been best described. This channel was identified in skeletal muscle by homology screening and is expressed almost exclusively in this tissue (Steinmeyer *et al*, 1991). It has an ion selectivity similar to ClC-0, namely, Cl>Br>I. ClC-1 is activated by depolarization and is pH sensitive. Animal studies indicate that ClC-1 expression increases during the first few weeks after birth, in parallel with the increase in skeletal muscle chloride conductance (Fahlke *et al*, 1997c). Structurally, this channel may also exist as a dimer (Fahlke *et al*, 1997c), however this is still controversial. In single channel analysis, has also been found to exhibit double-barreled activity, with a single channel conductance of 1pS (Saviane *et al*, 1999). Other studies have however, suggested that ClC-1 has a single pore that is formed by two identical ClC-1 subunits, based on studies with cysteine mutants in the D3-D4 region. These experiments indicated that there was a decrease in current upon exposure to MTS reagents, and that the extent of the decrease was dependent upon the nature of the equivalent residue in the second subunit (Fahlke *et al*, 1998). This suggested that the residues are very close and line a common pore. However, it is likely that ClC-1 would share a common structure with ClC-0, and consequently the double-barreled model is favored.

Mutations in ClC-1 result in the muscle disease, myotonia congenita, an inherited disorder of skeletal muscle which is characterized by delayed muscle relaxation (Koch *et al*, 1992). Defects in chloride conductance result in impaired membrane repolarization leading to repetitive action potentials by a single stimulus. This disease has both autosomal recessive

(Becker) and autosomal dominant (Thomsen) forms, and mutations in ClC-1 are found in both. Over 30 different ClC-1 mutations have been identified in human myotonia, and these are found scattered over the entire length of the gene (Jentsch *et al*, 1999). In dominant myotonia congenita, with one exception, all mutations are missense mutations. Mutations in the dominant form of the disease, result in a dominant negative effect on co-expressed wild-type subunits (Steinmeyer *et al*, 1994), suggesting that the channel is an oligomer. With dominant mutations, when mutant subunits are co-expressed with wild-type subunits, there is usually a shift in voltage dependence of ClC-1 to positive potentials, such that it cannot participate in membrane repolarization (Kubisch *et al*, 1998 and Pusch *et al*, 1995b). This effect has been proposed to be mediated by disruption of the slow gate (Fahlke *et al*, 1998). In recessive disease, it is thought that either mutant subunits do not associate with their wild-type counterparts or that the resultant heteromer still retains some function (Wollnik *et al*, 1997).

1.3.3 ClC-2

1.3.3.1 ClC-2 function

The ClC-2 chloride channel is expressed ubiquitously, and is activated by hyperpolarization, cell swelling and low extracellular pH (Schmidt-Rose *et al*, 1994 and Jordt *et al*, 1997). It has approximately 50% sequence homology with ClC-0 and ClC-1. The gene for human ClC-2 has been mapped to chromosome 3 and encodes a protein of 898 amino acids with a molecular weight of 97 kilodaltons (Cid *et al*, 1995). It is expressed in pancreas, lung, liver, kidney, skeletal muscle, stomach and intestine as well as in a wide range of cell lines (Thiemann *et al*, 1992). Under resting conditions, the ClC-2 channel is usually in the closed state, but can be activated by hyperpolarization, low extracellular pH or by cell swelling (Fahlke *et al*, 1997). It is inwardly rectifying and has an ion selectivity comparable to ClC-0 and ClC-1. When *Xenopus*

oocytes expressing ClC-2 are subjected to a hypotonic stress, there is a strong increase in ClC-2 current, which returns to baseline following return to isotonicity. This property suggested a role in volume regulation, and ClC-2 has been shown to mediate regulatory volume decrease in transfected cells (Xiong *et al*, 1999).

In addition to its role in volume regulation, ClC-2 has also been implicated in the maintenance of intracellular chloride in neurons and the modulation of responses to the neurotransmitter GABA (Clayton *et al*, 1998 and Smith *et al*, 1995). In neurons which exhibit an excitatory response to GABA, ClC-2 expression is low or absent. However when such cells are transfected with ClC-2, the response to GABA becomes inhibitory (Staley *et al*, 1996). ClC-2 may consequently be important in regulating neuronal excitation by regulating the intracellular chloride concentration.

While ClC-2 has not been associated with a particular genetic disease, it may be a potential therapeutic target in the treatment of cystic fibrosis. The channel has been identified at the apical surface of epithelial cells in the lung (Murray *et al*, 1996), sharing a similar location with the cystic fibrosis transmembrane conductance regulator (CFTR). It has also been shown to be present in the intestine of CFTR knock-out mice (Joo *et al*, 1999). ClC-2 has also been suggested to play a role in lung and kidney development as well as acid secretion in the stomach (Murray *et al*, 1996 and Huber *et al*, 1998).

1.3.2.3 ClC-2 structure

Biochemical studies of ClC-2 indicate that this protein exists in the membrane as an oligomer (Ramjeesingh *et al*, 2000). Non-denaturing gel electrophoresis suggests that ClC-2 exists in the form of monomers, dimers and tetramers. Functional studies on the different

oligomeric proteins suggest that the active form of the channel is a dimer or tetramer. It appears that ClC-2 also has double-barrelled channel activity similar to that of ClC-0.

Immunolocalization studies have shown that ClC-2 localizes to the tight junction of epithelial cells, and co-localizes with the tight junction protein ZO-1 (Gyomorey *et al.* 2000, Mohammad-Panah *et al.* 2000). Indeed, these studies have also shown that ClC-2 contributes to chloride secretion in Caco-2 cells. This location may allow ClC-2 to participate in anion and fluid movement via the paracellular pathway.

1.3.3.3 ClC-2 regulation

Xenopus oocyte studies have shown that the amino terminus of ClC-2 is necessary for activation by voltage and by changes in cell volume (Fahlke *et al.* 1997a). Large deletions in the amino terminus result in constitutive channel activity, that is almost time-independent and that is unresponsive to changes in cell volume. However, the deletion of the first 15 amino acids does not affect channel activity (Fahlke *et al.* 1997a). Furthermore, transplantation of the N terminal region that is required for normal gating to the C terminus, resulted in normal gating properties, confirming the importance of this region in channel gating. These findings suggested a ball and chain model of channel regulation, with the amino terminus blocking the channel pore at rest, and being displaced by channel activation such as by hypotonicity or voltage (Fahlke *et al.* 1997a). Other mutation studies have shown that the introduction of mutations in the cytoplasmic loop between D7 and D8 results in constitutive channel activity, suggesting that this region may act as the receptor for the amino-terminal "ball" domain (Jordt *et al.* 1997). Furthermore, mutations in the last transmembrane segment, result in changes in voltage dependence and

channel rectification. This residue is likely located close to the channel pore and may be involved in determining the permeation properties of the channel.

ClC-2 also possesses multiple protein kinase C phosphorylation sites and a single protein kinase A phosphorylation site. Initial studies suggest that inhibition of PKC results in channel activation, however the details of this regulation have not yet been fully elucidated (Thiemann *et al*, 1992).

Single-channel studies of purified ClC-2 indicate that there is a loss of native gating properties. This suggests that the purification process results in the removal of associated proteins, such as cytoskeletal proteins, which may be important for the normal gating mechanism (unpublished findings, Li, C. and Bear C.E.). It is conceivable that the proposed ball-and-chain model of ClC-2 regulation involves other membrane or membrane associated proteins, however, this has not been addressed to date.

1.3.4 ClC-K channels

ClC-Ka and ClC-Kb are ClC proteins that are expressed almost exclusively in renal tissue (Adachi *et al*, 1992). In rats, these channels are referred to as ClC-K1 and K2, respectively. The precise location of these channels remains controversial, as one study showed that both ClC-K1 and K2 were at the basolateral membrane in cells in the thick ascending limb of the loop of Henle (Vanderwalle *et al*, 1997), while another study showed that ClC-K1 was at both the apical and basolateral surfaces. However, most studies have been unable to express currents from these clones. Studies by one group indicate that ClC-K1 currents are decreased by low extracellular pH and that the ion selectivity is Cl>I, similar to that of the other ClC family

members (Uchida *et al*, 1995). ClC-K2 has been also shown to be an outwardly-rectifying channel with an ion selectivity of Br>I>Cl (Vanderwalle *et al*, 1997).

ClC-K1 has been implicated in nephrogenic diabetes insipidus in mice. Mice in which this channel was eliminated showed a massive diuresis similar to that seen in nephrogenic diabetes insipidus, suggesting that this channel plays an important role in urinary concentration (Matsumura *et al*, 1999). ClC-Kb has been shown to be mutated in Bartter's syndrome, which results in large salt losses in the thick ascending limb of the loop of Henle (Simon *et al*, 1997). However, as of the present time, strong electrophysiological data to confirm the role of this channel is lacking.

1.3.5 ClC-3

ClC-3, ClC-4 and ClC-5 are part of a distinct branch of the ClC family, and share approximately 35% homology with the other family members. However, within this group, the proteins are almost 80% identical (Wollnik *et al*, 1997). ClC-3 is a 760 amino acid protein that is abundant in rat brain, especially in the hippocampus, olfactory bulb and the cerebellar Purkinje cells, as well as in kidney and heart (Kawasaki *et al*, 1994). Studies in *Xenopus* oocytes expressing ClC-3 and neurons stably transfected with ClC-3 indicate that the channel was outwardly rectifying and regulated by protein kinase C. In neurons, these studies also suggested that single channel currents were inhibited by rises in calcium (Kawasaki *et al*,1995). The ion selectivity of ClC-3 is slightly different to the other ClC family members, I>Br>Cl. However, some groups have reported that ClC-3 expression did not result in a detectable current (Jentsch *et al*, 1995).

Functionally, ClC-3 has also been implicated in the response to cell swelling in the heart, and may play a role in volume regulation (Duan *et al*, 1997). Activation of ClC-3 could be inhibited by PKC activation, suggesting that this pathway is likely important for channel regulation (Duan *et al*, 1999). Similarly, activation by hypotonicity has also been observed in canine colonic myocytes (Dick *et al*, 1998). It has also been suggested that ClC-3 may correspond to the volume-sensitive chloride current in rat brain endothelial cells (von Weikersthal *et al*, 1999). Taken together, these studies suggest that ClC-3 may also be important in the response to cell volume changes.

1.3.6 ClC-4

ClC-4 is expressed in many tissues including liver and lung, with high levels of expression in brain and heart, and the human gene has been mapped to Xp22.3 (Van Slegtenhorst *et al*, 1994). Initial studies of ClC-4 failed to yield currents (Duan *et al*, 1997). However, more recently studies in *Xenopus* oocytes and HEK293 cells showed the presence of an outwardly rectifying conductance at extremely positive voltages (Friedrich *et al*, 1999). The halide senstitivity in these latter studies was Cl>Br>I and the channel was also shown to be sensitive to extracellular pH. Initial studies suggest that ClC-4 is present in intracellular vesicles, however, the physiological role of ClC-4 is not yet known (Friedrich *et al*, 1999).

1.3.7 ClC-5

ClC-5 is an 83kD outwardly rectifying chloride channel expressed predominantly in the kidney, but is also present in the brain, lung and liver (Steinmetyer *et al*, 1995). It is activated at voltages greater than +20mV and exhibits the same Cl>I selectivity as the other family members.

Mutations in ClC-5 result in Dent's disease, an X-linked renal disorder characterized by proteinuria and hypercalciuria (Fisher *et al*, 1994). As a result of the hypercalciuria, patients develop kidney stones and nephrocalcinosis and may progress to renal failure. ClC-5 has also been implicated in the development of two other diseases associated with nephrolithiasis. X-linked recessive nepthrolithiasis and X-linked recessive hypophosphatemic rickets (Lloyd *et al*, 1996). The mechanism by which mutations in ClC-5 lead to nephrolithiasis is not entirely clear. However, it has been suggested that in Dent's disease, mutations in ClC-5 lead to proteinuria by effects on the endocytic pathway. ClC-5 is proposed to play a role in the acidification of vesicles in the endocytic pathway, as it colocalizes with apical early endosomes in the cells of the proximal tubule (Gunther *et al*, 1998). The proximal tubule endocytic pathway plays an important role in the resorption of low molecular weight proteins and defects in this pathway could explain the proteinuria observed in Dent's disease. Endocytic vesicles undergo progressive acidification on their way to lysosomes.(Mukherjee *et al*, 1997), through the activity of a proton pump. These vesicles also have an anionic conductance that allows for maintenance of electrical neutrality, and it has been suggested, but not proven, that ClC-5 may play an important role in this process (Gunther *et al*, 1998). In the cortical collecting duct, ClC-5 localizes to the apical membrane, however it's function in these cells is not yet clear. It also remains unclear as to how mutations in ClC-5 result in hypercalciuria, although a recent study indicates that ClC-5 expression is under the regulation of parathyroid hormone (Silva *et al*, 2000).

1.3.8 ClC-6 and ClC-7

ClC-6 and ClC-7 represent the third branch of the ClC family and are both ubiquitously expressed . The two channels are 40% identical, but share only 25-30% homology with the other

ClC family members (Brandt *et al*, 1995). Initial studies of these channels in *Xenopus* oocytes failed to produce detectable currents. However, co-injection of ClC-6 RNA and the RNA encoding pICln, induced an outwardly rectifying chloride conductance, however the functional significance of this is not clear (Buyse *et al*, 1997). It has been suggested that ClC-6 may be located within organelles, thereby leading to difficulty in measuring channel function.

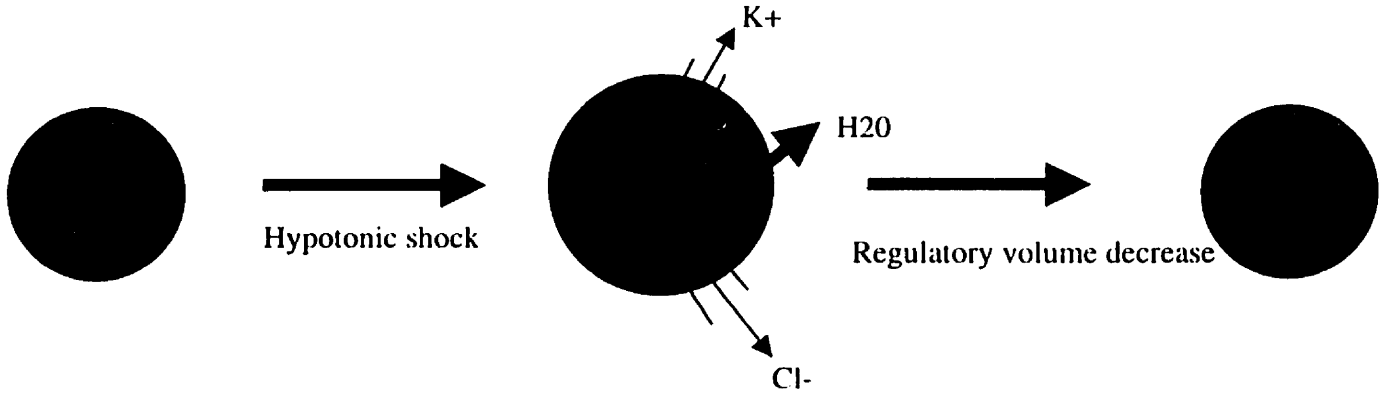
The genes for human ClC-6 and ClC-7 have been mapped to chromosomes 1p6 and 16p13 respectively (Brandt *et al*, 1996). However, neither of these channels have been associated with a particular disease.

1.4 THE ROLE OF CIC CHLORIDE CHANNELS IN VOLUME REGULATION

Cells are able to maintain a fixed cell volume in the face of an osmotic stress. Changes in osmolarity result in the influx or efflux of water from the cell and this is reflected by cell swelling or shrinkage. However, homeostatic mechanisms are able to restore the cell to its resting volume, via the activation of membrane transporters. Activation of such proteins leads to the loss or gain of osmotic solutes, allowing for the passive movement of water. The movement of ions across the cell membrane allows for the rapid, efficient adjustment of cell volume. The loss of solutes from the cell, in an attempt to maintain cell volume is termed regulatory volume decrease (RVD), and the accumulation of solutes is termed regulatory volume increase (RVI). (Strange *et al*, 1996). In animal cells, cell swelling results in the activation of pathways that lead to the efflux of K+, Cl- and organic osmolytes out of the cell (Figure 1.3). Organic osmolytes include amino acid derivatives, sorbitol, and methylamines, and are extremely important in volume regulation in the kidney where high osmotic gradients exist. In contrast, in regulatory volume increase, different transporters have been implicated and these include the Na/K/2Cl-cotransporter, the sodium-hydrogen exchanger and the chloride bicarbonate exchanger, and the organic osmolyte transporters (Lang *et al*, 1998).

In many mammalian cells, an outwardly rectifying chloride conductance is activated by cell swelling (Strange *et al*, 1995). However, the protein or proteins which mediate this conductance have not been clearly identified. This channel has been termed, VSOAC, the volume-sensitive organic osmolyte and anion channel, and several candidates have been proposed to mediate this conductance which is anion selective and has an ion selectivity of NO3->Br>Cl-. It is inhibited by a variety of pharmacologic agents, including NPPB, stilbenes, fatty acids, and tamoxifen and by millimolar concentrations of extracellular nucleotides (Jackson *et al*,

Figure 1.3 Model of Regulatory volume decrease



In response to hypotonic shock, cells swell and then return to their resting volume through the efflux of ions which then leads to the passive movement of water out of the cell (see text for details).

1995). It is also voltage sensitive, and is inactivated by depolarization. Osmotic stress results in channel activation and chloride efflux. This conductance has also been implicated in the movement of organic osmolytes out of the cell in response to cell swelling (Strange *et al*, 1996).

Icln is a ubiquitously expressed protein that was first cloned in 1992, (Paulmichl *et al*, 1992). whose function is yet unclear. Initially studies suggested that this protein was a functional chloride channel and was responsible for the swelling activated chloride conductance. When overexpressed in *Xenopus* oocytes, ICln produces an outwardly rectifying anion conductance that is constitutively active. Its ion selectivity is I>Br>Cl and it is inactivated by strong depolarization (Paulmichl *et al*, 1992). It has been implicated in the activation of a volume-activated chloride current in ciliary epithelial cells (Paulmichl *et al*, 1992). However, other studies indicated that pICln and ClC-6 induced similar chloride conductances in *Xenopus* oocytes, and suggested that this protein was not a functional chloride channel, but was perhaps indirectly related to such a channel (Buyse *et al*, 1997). Furthermore, Icln has been found to reside mainly in the cytoplasm (Krapivinsky *et al*, 1994), and while one study has demonstrated the ability of this protein to translocate to the membrane (Musch *et al*, 1998), others have not (Emma *et al*, 1998). Given the conflicting results, the role of this channel in volume regulation is unclear, and most of the evidence suggests that it is not a chloride channel and it may not be related at all to such a conductance (Clapham, 1998).

P-glycoprotein has been suggested to function as volume-sensitive chloride channel in addition to an ATP dependent pump. This was based on studies that showed that overexpression of this protein gave rise to a volume-activated anion conductance, and that inhibitors of P-glycoprotein inhibited swelling-activated anion conductances (Valverde *et al*, 1992). Furthermore, substrates for P-glycoprotein inhibited this conductance and activation of the

chloride conductance by cell swelling inhibited drug efflux (Gill *et al*, 1992). However, other studies have failed to confirm these results (Ehring *et al*, 1994, Luckie *et al*, 1994). Other evidence against the role of this protein in volume regulation is the presence of an outwardly rectifying chloride conductance in *Xenopus* oocytes which lack P-glycoprotein (Ackerman *et al*, 1994, Castillo *et al*, 1990). While most of the evidence suggests that P-glycoprotein is not itself a swelling activated anion channel, it may play a role in regulating such channels (Hardy *et al*, 1995).

ClC-2 and ClC-3 are members of the ClC family of chloride channels as discussed previously. ClC-2 is activated by cell swelling in *Xenopus* oocytes and activation occurs with volume increases of 10-15% in these cells (Strange *et al*, 1996). Furthermore, ClC-2 has been shown to mediate regulatory volume decrease in Sf9 cells (Xiong *et al*, 1999). However, although it is activated by swelling, it is not clear that this is its physiologic stimulus, and ClC-2, although it plays a role in volume regulation is not considered a candidate for VSOAC, as it does not exhibit the properties of VSOAC. The N-terminus of ClC-2 plays an important role in the response to cell swelling, as certain mutations within this region result in constitutively active channels which are either unresponsive to cell swelling or respond to a lesser extent (Fahlke *et al*, 1998). ClC-2 is ubiquitously expressed, and therefore is well suited to function in volume regulation in many cell types.

ClC-3 has also been implicated in volume regulation in the heart. In NIH/3T3 cells, expression of this protein results in a basally active outwardly rectifying chloride conductance that is sensitive to cell volume (Duan *et al*, 1997). Furthermore, ClC-3 has been suggested to play a role in volume regulation in canine colonic myoctes and rat brain endothelial cells (von

Wiekersthal *et al*, 1999, Dick *et al*, 1998). However, other studies have failed to elicit currents with this protein (Jentsch *et al*, 1995), so the final role of ClC-3 in volume regulation is unclear.

Final conclusions regarding the nature of the volume-sensitive chloride conductance have yet to be reached. There may be more than one anion channel that plays a role in the response to changes in cell volume, and this may vary between cell types.

1.5 THE ROLE OF THE CYTOSKELETON IN VOLUME REGULATION

1.5.1 THE ROLE OF THE ACTIN CYTOSKELETON IN VOLUME REGULATION

The actin cytoskeleton has been known to play an important role in cell motility and changes in cell shape (Small *et al.* 1999). It has also been implicated in the regulation of cell volume. Initial evidence that suggested that actin played a role in volume regulation came from early studies in multiple cell types in which disruption of the actin cytoskeleton by cytochalasins impaired the cell's ability to undergo regulatory volume increase or decrease (Foskett *et al.* 1985, Linshaw *et al.* 1991, Cornet *et al.* 1988). It was hypothesized that the actin cytoskeleton may play a role in limiting cell swelling, or in the activation of channels involved in regulatory volume decrease or in cell contraction to restore normal volume (Henson, 1999). The effect of cytoskeletal disruption on regulatory volume change has since been confirmed in studies in Ehrlich ascites tumor cells and HT-3 cells (Pedersen *et al.* 1999, Shen *et al.* 1999).

The effect of cytoskeletal disrupting drugs on volume regulation was confirmed through fluorescence experiments. Rearrangement of the actin cytoskeleton was observed to occur with changes in cell volume. In most cells, actin bundles are disrupted with cell swelling, and depolymerize into more distinct foci, and then reorganize following regulatory volume decrease (Emma *et al.* 1998). This response is, however, not uniform. For example, in glial cells there is an increase in actin filament bundles with cell swelling (Mountain *et al.* 1998). In some cell types there is an increase in actin synthesis in response to an osmotic stress (Clapham, 1998), while in others there is no detectable increase in actin (Henson *et al.* 1997).

The actin cytoskeleton has also been hypothesized to play a role in sensing volume changes within the cell. It is suggested that the cross-linked actin cytoskeleton acts as a sensor which is sensitive to the local osmotic environment, and is involved in crosstalk with membrane

transporters, such that changes in the cell volume, result in changes in the submembranous cytoskeleton and lead to the activation of these proteins (Cantiello *et al*, 1997). However, other studies do not show a consistent relationship between actin structure and ion transport, or changes in membrane tension (Okada, 1997). Actin may also relay changes in osmolarity to membrane transporters indirectly via actin binding proteins. For example, cell swelling stimulates binding of the actin binding protein, ankryin, to the membrane protein Band III, an anion-exchanger (Musch *et al*, 1996).

A number of studies have demonstrated linkages between the actin cytoskeleton and membrane transporters. Studies of epithelial sodium channels have demonstrated that short actin filaments are associated with channel activation (Cantiello *et al*, 1991, Berdiev *et al*, 1996, Rehn *et al*, 1998). In neurons, functional linkage between calcium channels and actin filaments have also been demonstrated (Rosenmund *et al*, 1993), and in MDCK cells, the Na/K ATPase has also been functionally linked to the cytoskeleton (Cantiello *et al*, 1995). Furthermore, the actin crosslinking protein, filamin has been shown to inhibit CFTR and the actin-regulated Na channel (Luckie *et al*, 1994 and Prat *et al*, 1995). While these transporters are not thought to play a major role in volume regulation, they provide evidence that the actin cytoskeleton is involved in the regulation of membrane transporters. Other channels which are thought to play a role in volume regulation have also been shown to be either structurally or functionally related to the actin cytoskeleton. These include the swelling activated chloride channel in regulatory volume decrease (Schwiebert *et al*, 1994) and the Na/K/2Cl cotransporter in regulatory volume increase (Jessen *et al*, 1992). In renal cells, the swelling activated chloride conductance was activated by disruption of the actin cytoskeleton by cytochalasin, and inhibited by stabilization of the actin cytoskeleton with phalloidin (Jessen *et al*, 1992). A similar response has been observed in other

cell types, including astrocytes and intestinal epithelial cells (Tilly *et al*, 1996, Lascola *et al*, 1998). In regulatory volume increase, the Na/K/2Cl- cotransporter has also been linked to the actin cytoskeleton, in a number of cell types, including renal cells and Ehrlich ascites tumor cells (Tilly *et al*, 1996, Wu *et al*, 1994). Disruption of the actin cytoskeleton with cytochalasin activates the Na/K/Cl- cotransporter in intestinal cells, while stabilization of actin filaments inhibits the channel (Matthews *et al*, 1997). Similarly, there is evidence for cytoskeletal regulation of the Na/H exchanger, which is also involved in regulatory volume increase (Goss *et al*, 1994).

The mechanism for the coupling of changes in the actin cytoskeleton in response to cell swelling to the activation of transporters leading to regulatory volume change is likely complex. The Rho family of regulatory proteins has been implicated in the organization of the actin cytoskeleton (Hall, 1998). Rho can be activated by extracellular ligands, and this results in the formation of actin-myosin stress fibers and focal adhesion complexes (Ridley *et al*, 1992). The involvement of this family of small GTPases in cytoskeletal reorganization suggested that they may play a role in mediating the response to cell swelling (Ridley *et al*, 1992). Studies in which epithelial cells were treated with an inhibitor of Rho resulted in a reduction of swelling-induced anion efflux (Henson, 1999). Furthermore, hypotonicity induces tyrosine phosphorylation of the rho-dependent kinase, p125FAK (focal adhesion kinase), and the activation of its substrate PI-3 kinase (Tilly *et al*, 1996). Other studies have implicated the src family of tyrosine kinases in the response to cell shrinkage. Cell shrinkage resulted in the activation of p59fyn and inhibition of src, and phosphorylation of the actin-binding protein cortactin (Kapus *et al*, 1999). Recently, it has been shown that in lymphocytes, the tyrosine kinase p56lck, mediates the activation of swelling-induced chloride channels (Uhlemann *et al*, 2000, Lepple-Wienhues et al, 1998).

Other potential mechanisms for the coupling of volume changes to changes in transporter function, are through other actin binding proteins. The ezrin/radixin/moesin (ERM) family of actin-binding proteins, are also regulated by the rho family of GTPases and may provide a link between volume changes and membrane proteins (Mackay *et al*, 1998).

While there is significant evidence that the actin cytoskeleton is involved in the response to regulatory volume decrease and may directly or indirectly modulate transporters involved in the restoration of cell volume, the mechanisms by which this occurs are not clear. This modulation of transporter function may involve actin-binding proteins and signalling pathways including the rho family of GTPases or other kinases such as the src family of proteins.

1.5.2 THE MICROTUBULE CYTOSKELETON AND VOLUME REGULATION

1.5.2.1 THE MICROTUBULE CYTOSKELETON AND MOLECULAR MOTORS

Microtubule based transport plays an important role in membrane-trafficking in many different cell types. Initially, study of vesicle transport by molecular motors focussed on neuronal cells, however, it is now clear that this system plays a role in multiple cell types, especially within epithelial cells. The microtubule based transport system has been implicated in axonal transport, acinar secretion in lacrimal glands, lactation in mammary gland, release of atrial natriuretic peptide in cardiac myocytes and the release of cytotoxic granules from T cells (Hirokawa, 1998). Microtubules are polarized filaments that serve as the tracks for the transport of vesicles and organelles in polarized cells. Microtubules consist of α/β tubulin heterodimers arranged into thirteen parallel protofilaments. The microtubule has a fast growing end (+), and a minus end. In most epithelial cells, the + end is oriented towards the basolateral surface. In non-polarized cells, microtubules are usually oriented in a radial fashion from the center of the cell with the + ends oriented towards the periphery (reviewed by Hamm-Alvarez and Sheetz, 1998). Proteins and organelles are transported along microtubules by specialized motor proteins. Three superfamilies of motor proteins have been identified: the myosin, dynein and kinesin families. The myosin family of motor proteins move along actin filaments, and are involved in morphological changes in the cell, as well as muscle contraction and vesicle movement (Hoyt *et al*, 1997). Members of the dynein and kinesin families move along microtubules and are involved with the movement of vesicles and organelles, the movement of flagella and cilia and cell division (Hoyt *et al*, 1997). In general, movement along microtubules is for long-range transport, while movement along actin filaments is used for shorter distances (Goode *et al*,

2000). Recently, it has been shown through the yeast two-hybrid system, that there is a direct interaction between microtubule and actin based motors (Huang *et al*, 1999).

THE KINESIN SUPERFAMILY

Kinesin, a plus end directed motor protein, was the first microtubule based motor to be identified (Vale *et al*, 1985). It is an ATPase and consists of 2 heavy chains and 2 light chains. The heavy chains form three separate domains: globular heads which bind to microtubules and confer the ATPase activity, a stalk domain and a C-terminal tail domain that fans out from the stalk to associate with the light chains. The light chains of kinesin are thought to play an important role in binding to its cargo (Skoufias *et al*, 1994). It is thought that the diversity that exists in the tail region may be responsible for interaction with distinct cargo. The kinesins form a superfamily of at least ninety proteins which can be classified according to the position of the motor domain, either at the N-terminus, the C-terminus or in the middle of the molecule. While most kinesins are homodimers, they can also form heterodimers, tetramers and monomers (Cole *et al*, 1995). This diversity allows for the movement of cargo and varying speeds towards the positive end of microtubules, however some kinesins also move to minus ends (Saito *et al*, 1997).

Initially, kinesin was mainly associated with the transport of membranous organelles in nerve axons (Hirokawa *et al*, 1991). In other cell types, kinesin has been shown to associate with the endoplasmic reticulum, Golgi apparatus, mitochondria, endosomes and lysosomes (Hirokawa, 1995). One member of the kinesin family, KIF1A has been implicated in the transport of synaptic vesicles along axons (Okada *et al*, 1995). Mice lacking this motor show a marked reduction in the density of synaptic vesicles in nerve terminals, and exhibit sensory and

motor disturbances (Hirokawa *et al*, 1991). Other kinesins have also been associated with specific functions, such as KIF 1B, which transports mitochondria (Nangaku *et al*, 1994).

CYTOPLASMIC DYNEIN

Cytoplasmic dynein belongs to the dynein superfamily of proteins. Dynein was first identified for its role in the movement of flagella and cilia (Gibbones *et al*, 1965). Subsequently, it was found that a distinct form of dynein played a role in the movement of organelles along microtubules, as a minus-end directed ATPase (Paschal *et al*, 1987a, Paschal *et al*, 1997b). To date, only a few isoforms of cytoplasmic dynein have been identified, in contrast to the large kinesin family (Vaisberg *et al*, 1996, Vaughan *et al*, 1995). Dynein consists of 2 heavy chains (~500kD each), 3 intermediate chains (70-74kD) and 4 light chains (8-22kD). The heavy chains are predicted to form globular domains which interact with microtubules and confer the ATPase and motor activities. Similarly to kinesin, the heavy chains also form a stalk domain and the light chains are presumed to interact with cargo. Dynein is also associated with a protein complex, dynactin, that is required for most of dynein's activities. Dynactin consists of 10 subunits, p150Glued, p135Glued, p62, dynamitin (actin-related protein 1 (Arp1)), actin, acting capping protein alpha and beta subunits, p27 and p24 (Hirokawa , 1998). This complex is bound to the dynein intermediate chain via p150Glued, and may also play a role in linking dynein to its cargo via an interaction mediated by p150Glued-Arp1 (Vaughan *et al*, 1995).

Cytoplasmic dynein has been implicated in the retrograde transport of organelles in neuronal cells. It also plays a role in the distribution of late endosomes and lysosomes (Lin *et al*, 1992), trafficking between early and late endosomes (Aniento *et al*, 1993), movement of phagosomes (Blocker *et al*, 1997) and the localization of the Golgi complex (Corthesy-Thelauz *et al*, 1992) as well as the transport of Golgi derived membranes to the apical surface of intestinal

epithelial cells (Fath *et al*, 1994). Studies in dynein heavy chain knockout mice, showed that these mice died as early embryos, and that blastocyst cells exhibited a diffuse distribution of Golgi, endosomes and lysosomes instead of their characteristic perinuclear localization (Harada *et al*, 1998).

In neurons, dynein plays a role in fast axonal transport, as well as neuronal growth and development (Waterman-Storer *et al*, 1997, Ahmad *et al*, 1998). It has been suggested that mutations in the dynein-dynactin complex may in fact play a role in the development of certain neurological disorders, such as Huntington's disease. It has been determined that the p150Glued subunit of dynactin interacts with Huntingtin-associated protein which in turn associates with the product of the Huntingtin gene, and may therefore play a role in the development of this disorder (Engelender *et al*, 1997).

Dynein has been implicated in the sorting of endocytotic vesicles in hepatocytes. Following receptor mediated endocytosis, vesicles containing ligand bind to cytoplasmic dynein while those containing receptors do not, therefore suggesting that this motor may play a role in the movement of vesicles between cellular compartments (Oda *et al*, 1995). More recently, dynein has been implicated in the transport of rhodopsin containing vesicles in photoreceptor cells of the eye (Tai *et al*, 1999). However, the extent of dynein's role in vesicle transport is still not clear, and it is possible that this may be important in the movement of many different proteins between cellular compartments.

1.5.2.2 THE MICROTUBULE CYTOSKELETON AND VOLUME REGULATION

The effect of cell volume changes on microtubule structure has not been well studied. There is some evidence that the microtubule network also undergoes changes in response to cellular volume changes. In the liver, cell swelling results in stabilization of the microtubule

network and increased synthesis of tubulin (Haussinger *et al*, 1994). Furthermore, in certain cell types including Jurkat cells, HL-60 cells and peripheral blood neutrophils (Downey *et al*, 1995), microtubule disruption by drugs such as colchicine results in an impaired ability to undergo regulatory volume decrease. However, in other cell types, this response is not affected by disruption of the microtubule network (Krapivinsky *et al*, 1994). Studies in PC-12 cells undergoing hypo-osmotic shock, did not show any change in the microtubule network by immunostaining, and also did not show any impairment of regulatory volume decrease by microtubule disruption with colchicine (Krapivinsky *et al*, 1994). In contrast, studies in the cervical cancer cell line, HT-3, showed that stabilization of microtubules by treatment with taxol, inhibited the activation of volume regulated chloride channels, and impaired the ability of these cells to undergo regulatory volume decrease (Shen *et al*, 1999). This study, therefore, suggests that microtubule polymerization interferes with this process, and conflicts with other studies that suggest that microtubule depolymerization impairs the process of regulatory volume decrease.

The activity of molecular motors in response to volume change has not been well characterized. Studies in Tetrahymena, where one of two dynein genes were knocked-out, showed a loss of regulation of cell size and shape, however the significance of this on regulatory volume changes was not evaluated (Lee *et al*, 1999). It would be plausible that the molecular motors may be involved in the redistribution of membrane transporters involved in either regulatory volume decrease or increase, however further study is needed to evaluate this.

THESIS INTRODUCTION

The ClC-2 chloride channel has been localized to the epithelia of the lung (Murray *et al*, 1995) and gastrointestinal tract (Joo *et al*, 1999) and it has been suggested that this channel may

be able to functionally compensate for CFTR. The regulation of this channel however, remains unclear. It is well established that the cytoskeleton plays an important role in volume regulation (reviewed in Henson *et al*, 1999), and given that ClC-2 has been shown to play a role in volume regulation (Xiong *et al*, 1999), it is likely that the cytoskeleton plays a role in its regulation. Furthermore, studies of the purified protein have indicated that this channel exhibits constitutive channel activity, suggesting that other proteins are important in ClC-2 regulation (Li, C. and Bear CE, unpublished findings). It is the hypothesis of the work presented in this thesis that ClC-2 interacts with and is regulated by cytoskeletal proteins.

CHAPTER 2

MATERIALS AND METHODS

A. 2.1 BIOCHEMICAL METHODS

B. *2.1.1 Expression of ClC-2 in Sf9 cells*

ClC-2 protein was expressed in Sf9 cells by Yanchun Wang using clone B12-2, encoding the full length cDNA of rat ClC-2, from T. Jentsch (Hamburg, Germany). The open reading frame (ORF) was amplified using the oligonucleotides 5'- CTAGGATCCGAGATGGCGGCCGCAAC – 3' and 5' – GGAATTCACTGGCACTTGTCATCA – 3', which overlap the start and stop codon respectively, of rat ClC-2 and contain BamHI and EcoRI sites respectively, for cloning purposes. The amplified fragment was then ligated into pBlueBacHisB, a baculoviral vector which incorporates an N-terminal, polyhistidine tag (Invitrogen, California, USA). The first 50 amino acids of the expected protein are:

[MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPSSRSE**MAAATAAAATVA**] (ClC-2 protein is indicated in bold).

The entire tagged ClC-2 ORF was then introduced either into the pBlueBac4 vector for high-level expression in the baculoviral system, or into a high efficiency oocyte expression vector, as described by Lorenz et al. The bulk of the ORF was then replaced with unamplified DNA from the original B12-2 clone, using the restriction sites NotI and BspEI. Regions that were not replaced by this procedure were sequenced to ensure that no errors were introduced during amplification. The resulting clones encode an unambiguous ORF representing rat ClC-2 with an N'-terminus hexa-histidine tag driven from either the baculovirus polyhedrin promoter for Sf9 cell expression.

2.1.2 Purification of ClC-2 protein

Sf9 insect cells expressing recombinant ClC-2 were harvested from 1 litre of medium by centrifugation at 2000g at 4C and the cell pellet was washed once with PBS. The cell pellet was resuspended in 70mLs of PBS containing protease inhibitors (aprotinin 10ug/ml, leupeptin 10ug/ml, benzamidine 1mM, E64 10uM and dithiothreitol 2mM). Cells were lysed using a French Press (Spectronic Inst. NY, USA) at a pressure setting of 1000 psi. Lysed cells were spun at 4000g for 10 minutes to pellet nuclei and cell debris. The supernatant was spun at 100000g for 90 minutes to obtain a crude membrane pellet containing associated proteins. The pellet was then resuspended in 25 volumes of ice-cold 10mM NaOH and 0.5mM EDTA to extract peripheral proteins. The suspension was then spun at 100000g for 2 hours. The pellet containing integral membrane proteins were solubilized using 100mLs of a detergent solution, containing 8% pentadecafluorooctanoic acid (PFO) and 25mM PO4, pH 8.. and stirred overnight at room temperature. Insoluble material was pelleted by centrifugation at 60 000g for 1 hour at foom temperature, and the supernatant was then filtered using a 22uM filter prior to application to the nickel agarose column (Qiagen, CA, USA).

Nickel affinity chromatography

A freshly generated 25mL nickel column was attached to an FPLC column and the ClC-2 containing sample was applied at 1 mL per minute at room temperature. Then, the column was washed with 100mLs of a buffer containing 25mM phosphate, 100mM NaCl and 4% PFO at pH 8.0 (buffer 1). Buffers containing PFO were prepared by adding the free acid, pentadecafluorooctanoic acid to the buffer and titrating with NaOH to the required pH. A pH gradient was applied to the column titrating buffer #1 with buffer #2 (20mM phosphate, 4% PFO at pH 6), going from 0% buffer #2 to 100% buffer #2 in 100mLs. Three mL fractions were

collected and analyzed by dot-blot. Immunopositive fractions were then analyzed by Western blotting (10uL of each fraction) and by silver staining (50uL) of each fraction. Fractions containing ClC-2 protein eluting about pH 6.8 were pooled and concentrated in an Amicon Centriprep 50 concentrator (#4310) to a final volume of 1 mL. This sample was diluted 10 times with a buffer containing 10mM HEPES, 0.5 mM EGTA and 0.025% sodium azide at pH 7.2, and re-concentrated to a volume of 600uL.

Preparation of Sf9 cell membranes

Sf9 insect cells expressing recombinant ClC-2 were harvested from 1 litre of medium by centrifugation at 2000g at 4C and the cell pellet was washed once with PBS. The cell pellet was resuspended in 70mLs of PBS containing protease inhibitors (aprotinin 10ug/ml, leupeptin 10ug/ml, benzamidine 1mM, E64 10uM and DTT 2mM). Cells were lysed using a French Press (Spectronic Inst. NY, USA) at a pressure setting of 900 psi. The lysate was centrifuged at 3000rpm for 10 minutes and the supernatant was then centrifuged at 100 000g for 1 hour at 4 degrees. The pellet was then resuspended in 10mLs of PBS with inhibitors, and the protein concentration determined by a modified Lowry protein assay.

2.1.3 Protein Quantitation

Protein quantitation was performed by a modified Lowry assay. A duplicate series of standards were prepared using BSA, and 10% deoxycholate was added to each sample and the final volume adjusted to 1 mL. Samples for analysis were prepared by taking a fixed volume of each sample in duplicate and adding deoxycholate as above and then adjusting the volume to 1 mL. The samples and standards were vortexed and kept at room temperature for 10 minutes. The proteins

were then precipitated with 70% trichloroacetic acid, vortexed and centrifuged at 13000rpm for 5 minutes. The pellets were resuspended in 400uL of CTC mix containing equal amounts of NaOH, 10% SDS, CTC reagent and water. A further 400uL of deionized water was added and the samples incubated for 10 minutes at room temperature. Folin reagent (Sigma) was then added to each sample, followed by vortexing, and the samples were then incubated for 30 minutes at room temperature. The OD750 was then measured using a Beckman Spectrophotometer. Standard curves were generated using Prism software and linear regression analysis was performed to obtain protein concentrations for the samples.

2.1.4 Preparation of GST fusion peptides

Purification of GST peptides for the N and C terminus of ClC-2

Nucleotide sequences corresponding to amino acids 31-74 for the N-peptide, and amino acids 869-907 for the C peptide, were amplified from clone B 12-2 (from T.J. Jentsch), ligated into pGEX-2T (Pharmacia) and sequenced. GST fusion protein expression was induced as per the following protocol. Overnight cultures were setup from a single colony grown from bacterial stocks. The overnight culture was then diluted into 500 mLs of LB media and grown until the OD600 was between 0.5 and 1. Induction was then done with a final concentration of 0.1mM IPTG (Sigma). The culture was then grown for a further 3 hours and the cells were then spun at 4000rpm for 10 minutes at 4C. The bacterial pellet was resuspended in 25 mLs of PBS and sonicated 3 times. Triton-X-100 was then added to the sonicate at a final concentration of 1% and the samples nutated for 30 minutes at 4C. The sonicate was spun at 10 000rpm for 20 minutes at 4C. Glutathione agarose (Sigma) was prepared as a 50% slurry and 500uL added to the supernatant and nutated for 30 minutes at 4C. The beads were then spun down in a clinical centrifuge and were washed 3 times with PBS. To elute the protein, 1mL of elution buffer

(10mM reduced glutathione, 50mM Tris-HCl, pH 8) was added to the beads and nutated for 10 minutes at room temperature. The beads were spun down and the supernatant contains the eluted protein. The purified peptide is then analyzed by SDS-PAGE on 12% precast gels, and stained with Coomassie blue.

2.1.5 Preparation of purified protein columns

Amylase column preparation

A 1 mL Hi-Trap (Amersham Pharmacia Inc.) column was washed three times with 2 mLs of 1 mM ice cold HCl. The column was then injected with ligand, either 10mg amylase (Sigma) in 1mL coupling buffer (0.5M NaCl, 0.2M NaHCO3) or purified ClC-2 in coupling buffer. Coupling was carried out at room temperature for 30 minutes. The column was then washed with two buffers: buffer A (0.5M ethanolamine, 0.5M NaCl, pH8.3), buffer B (0.1M acetate, 0.5M NaCl, pH 4). First the column was washed three times with 2 mLs of buffer A, followed by 3 washes with buffer B and a final 3 washes with buffer A. The column was then left at room temperature for 30 minutes and then washed 3 times with buffer B, then buffer A and a final wash with buffer B. The column was then injected with a buffer containing 25mM Tris and 0.05% azide.

Control column preparation

A 1 mL Hi-Trap column was washed three times with 2 mLs of 1mM ice cold HCl. The column was then washed with two buffers: buffer A (0.5M ethanolamine, 0.5M NaCl, pH8.3), buffer B (0.1M acetate, 0.5M NaCl, pH 4). First the column was washed three times with 2 mLs of buffer A, followed by 3 washes with buffer B and a final 3 washes with buffer A. The column was then

left at room temperature for 30 minutes and then washed 3 times with buffer B, then buffer A and a final wash with buffer B. The column was then injected with a buffer containing 25mM Tris and 0.05% azide.

Application of samples to ClC-2 affinity column

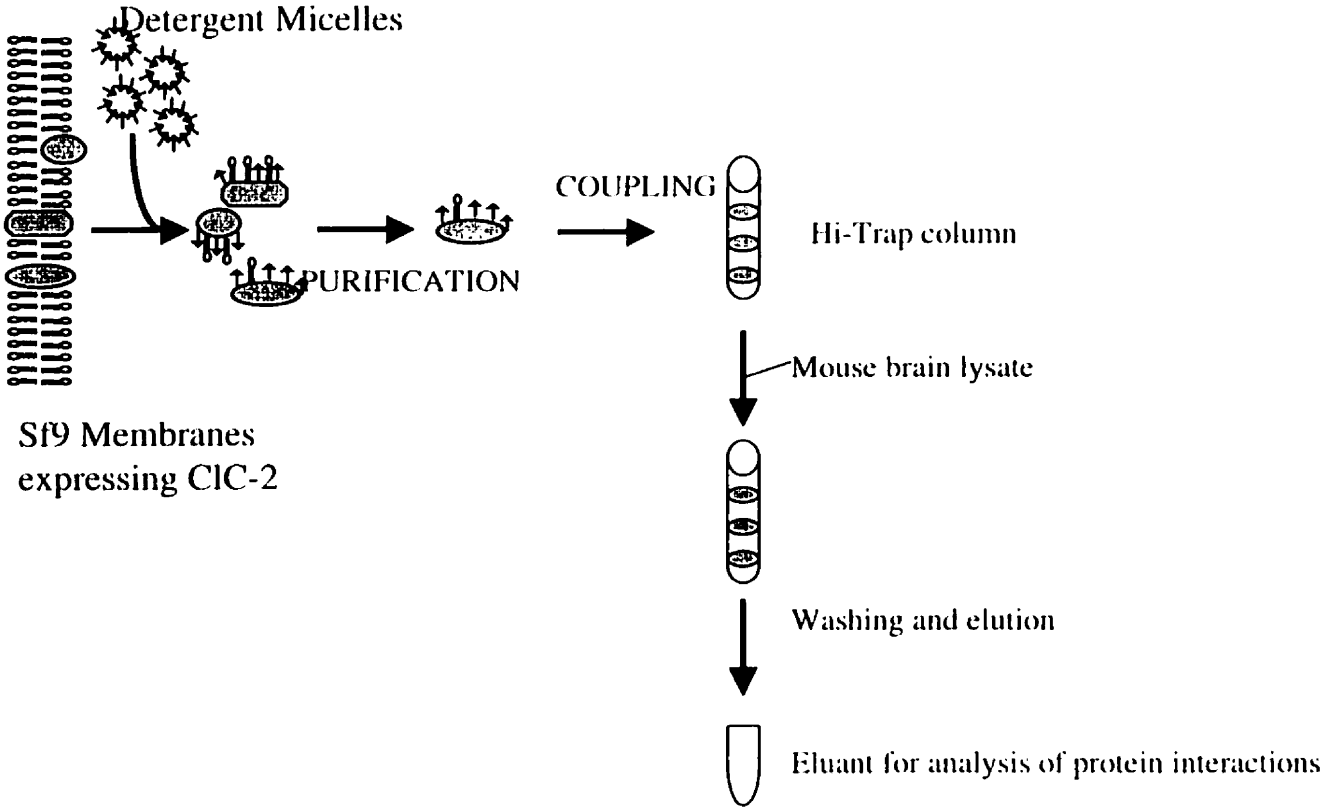
Mouse brain lysate was prepared using 3 brains (UNC or Balb/C) obtained from mice sacrificed for other purposes. The tissue was homogenized in a PBS containing 1% Triton and protease inhibitors (aprotinin 10ug/ml, leupeptin 10ug/ml, benzamidine 1mM, E64 10uM and DTT 2mM), using a Dounce homogenizer. The homogenate was spun at 100000g for 2 hours and the supernatant was passed through the affinity column (Figure 2.1). The column was first washed with 25 mLs of PBS/1% triton pH 8. The filtered sample was then applied to the column twice. The column was then washed with 25 mLs of PBS/1% Triton and the bound proteins were eluted using a buffer containing 4% NaPFO, 100mM phosphate, pH4. One mL fractions were collected, and the OD 280 was measured. Fractions containing protein were pooled and concentrated in an Amicon Centriprep 50 concentrator, and protein concentration was determined using a modified Lowry protocol.

2.1.6 Purification of antibodies

Affinity purification

GST-fusion proteins were coupled to Hi-trap columns as specified by the manufacturer. The column was then washed with 10mLs of binding buffer (Bio-Rad) containing 0.2M NaHCO3, 0.5M NaCl pH 8.3. Serum obtained from immunized animals was then diluted 5 fold in binding buffer and applied to the column three times. The column was then washed with 25mLs of

Figure 2.1 Protocol for ClC-2 purification from Sf9 cells



ClC-2 was purified from Sf9 cells over-expressing ClC-2. The purified protein was coupled to a column, and murine brain tissue was passed through the affinity column. Bound proteins were eluted using a pH gradient and studied using silver staining and western blot analysis to study ClC-2 protein interactions.

binding buffer and the last 6 mLs of washing collected. The bound antibodies were then eluted using 24 mLs of elution buffer (Bio-Rad) containing 100mM glycine, 50mM NaCl, pH 3.0. Three mL fractions were collected and the OD280 was measured for each. Positive fractions (OD>0.1) were concentrated and stored in 50% glycerol.

2.1.6 Silver stain analysis and Western blotting

Silver Stain analysis

Samples were combined with SDS loading dye and 50uL of sample were run in each lane on a precast 8% gel (Helixx Technologies) at 130mV. The gel was removed from the casing and silver stained using a standard protocol from Sigma. The gel was fixed using a solution containing 10% glacial acetic acid and 30% ethanol for 2- 30 minute intervals. The fixed gel was washed three times with deionized water (ddH2O) for 10 minutes. The gel was placed in silver equilibration solution (1.5mL silver concentrate (Sigma) in 300mLs ddH2O) for 30 minutes, followed by 3 brief washes with ddH2O. The gel was then developed using a developer solution (Sigma Developer 1-20mL, Sigma Developer 2-113uL in 180cc ddH2O). The developing was stopped using a 1% glacial acetic acid solution.

Western blotting

Samples for SDS-PAGE were run on precast tris-glycine gels (Helixx Technologies) at 130mV. Gels were then removed from the casing and transferred to nitrocellulose at 100mV for 1 hour in transfer buffer containing 20% methanol, Tris-base, and glycine. Nitrocellulose membranes were then blocked for 1 hour using a solution of 5% skim milk powder in PBS-0.1% Tween. The blots were then incubated with the appropriate primary antibody diluted in blocking solution for 2-4 hours at room temperature or overnight at 4 degrees C. The membranes were then

washed 5 times for 5 minutes with PBS-0.1% Tween, and then incubated with the horseradish-peroxidase conjugated secondary antibody in blocking solution for 1 hour. After a series of 5-5 minute washes, the membranes were developed using enhanced chemiluminescence (Amersham Pharmacia, Inc.).

2.2 Electrophysiology

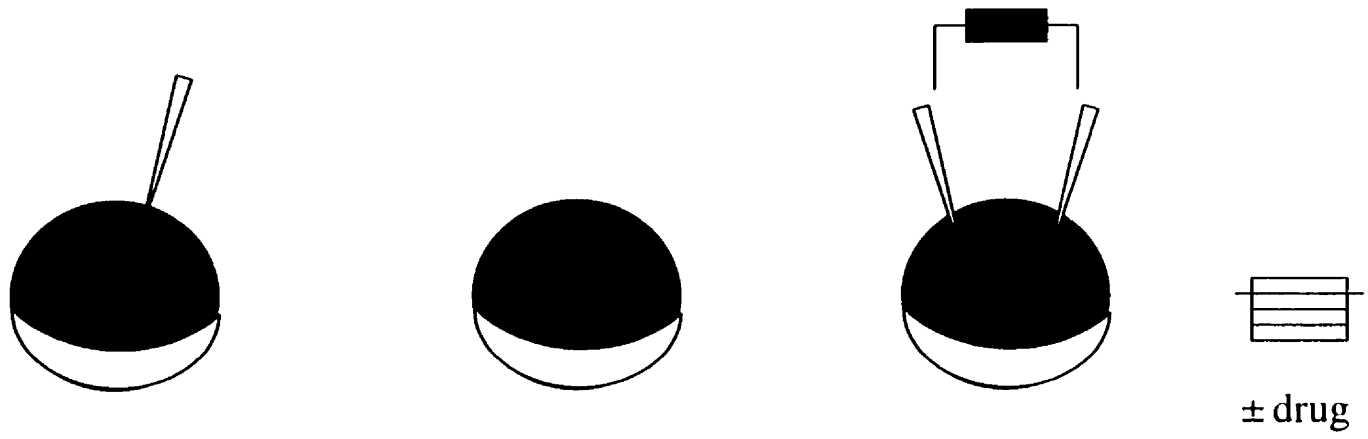
2.2.1 Harvesting of Xenopus oocytes

Stage VI oocytes were surgically removed from the abdomen of *Xenopus Laevis* (*Xenopus* One, Michigan, USA) frogs under anesthesia. Frogs were anesthetized using 0.17% Tricaine (3-amino acid ethyl ester methanesulfonate salt, Sigma) solution. The oocytes were manually separated and then subjected to collagenase (2mg/mL, Sigma) treatment for 30 minutes to further degrade intercellular matrix. The oocytes were then defolliculated using a solution containing 0.1M potassium phosphate, pH 6.5 for 10 minutes. Healthy oocytes were selected and stored in standard oocyte solution (SOS), (100mM NaCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 2mM KCl, 5mM HEPES pH 7.6) at 18C. Oocytes were microinjected with 50nL of cRNA coding for ClC-2 at a concentration of 1-3ng/nL, and stored in pyruvate (2.5mM) and gentamicin (50mg/L) supplemented SOS (sSOS). Approximately 18 hours after injection, oocytes were then used for two-electrode voltage clamp studies (Figure 2.2).

2.2.2 Voltage clamp experiments

Whole-cell current measurements were performed using a Geneclamp 500 amplifier (Axon Instruments, Inc.) and the Digidata 1300a interface (Axon Instruments, Inc.). Electrodes were tested to ensure resistances between 1 and 5 MΩ, and were filled with a 3M KCl solution. *Xenopus* oocytes were subjected to a series of 5 voltage steps between -140mV and +20mV from a holding potential of -30mV, and current is recorded over time (Pclamp 7 software, Axon Instruments Inc.). In certain experiments oocytes were treated with cytochalasin D (10uM),) latrunculin B (1uM), EHNA (0.125mM) to evaluate the effect of changes in the cytoskeleton on channel function.

Figure 2.2 Expression of Ion Channel in Xenopus Oocytes



Stage VI Xenopus oocytes were harvested from Xenopus Laevis frogs as described (see text). Oocytes were injected with ClC-2 cRNA and incubated for 18-24 hours prior to voltage clamp studies.

2.3 Immunofluorescence

Caco-2 cells were grown on coverslips to the desired confluence. Cells were washed 3 times with ice-cold PBS and then fixed with 4% paraformaldehyde for 10 minutes. The fixed cells were permeabilized with TBS containing 0.5% Triton for 30 minutes. The coverslips were washed 3 times with TBS containing 0.05% Triton and then blocked with a 0.05% solution of normal goat serum (Vector Laboratories, California USA) in TBS/0.05% Triton for 30 minutes. ClC-2 antibody was prepared in blocking solution at a dilution of 1/400, and applied to the coverslips for 2-4 hours. The coverslips were then washed 6 times with TBS/0.05% Triton and the cells are incubated with the secondary antibody, FITC-anti-rabbit (Molecular Probes) at a dilution of 1/100 in blocking solution for 1 hour. The coverslips were then washed 6 times with TBS/0.05% Triton and mounted on microscope slides using commercial mounting media (DAKO, California USA or Vectashield, Vector Laboratories, California USA). For phalloidin staining, phalloidin Texas Red (Molecular Probes Inc.) was applied to the coverslips for one hour along with the secondary antibody.

2.4 Statistical Methods

Statistical analysis was performed using Prism Software. For electrophysiological data, the student's t-test was used for evaluation of the effect of the drugs used.

CHAPTER 3

BIOCHEMICAL AND FUNCTIONAL INTERACTION OF THE CHLORIDE CHANNEL ClC-2 WITH THE ACTIN CYTOSKELETON

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CHAPTER 3. BIOCHEMICAL AND FUNCTIONAL INTERACTION OF THE CHLORIDE CHANNEL ClC-2 WITH THE ACTIN CYTOSKELETON

3.1 INTRODUCTION

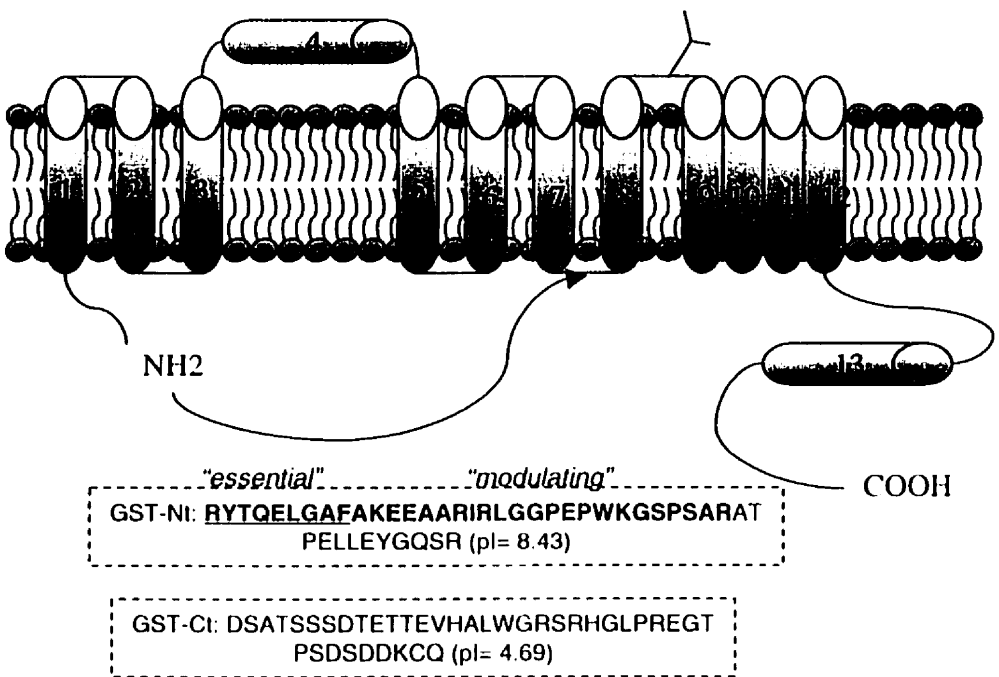
Volume regulation allows cells to maintain cell volume when faced with a hypotonic or hypertonic environment. This process involves the efflux or influx of ions, followed by the passive movement of water. In response to cell swelling, chloride and potassium efflux allows for regulatory volume decrease (Lang *et al*, 1996). While the final nature of the swelling activated chloride conductance involved in this response remains controversial, a number of candidate chloride channels have been proposed to mediate chloride efflux in response to hypo-osmotic stress (Strange *et al*, 1996).

ClC-2 and ClC-3 are members of the ClC chloride family which have been implicated in volume regulation (Strange *et al*, 1996 and Duan *et al*, 1997). The role of ClC-3, however, remains controversial as not all studies have been able to elicit currents with this protein (Jentsch *et al*, 1995). In contrast, studies of ClC-2 indicate that the channel is ubiquitously expressed, and is in the closed state under resting conditions (Thiemann *et al*, 1992). It has been shown that ClC-2 can be activated by cell swelling, hyperpolarization and low extracellular pH (Jordt *et al*, 1997). In *Xenopus* oocytes subjected to hypotonic shock, ClC-2 is activated and then returns to its basal state following a return to isotonicity. Functionally, ClC-2 has been shown to mediate regulatory volume decrease (Xiong *et al*, 1999), and it has also been implicated in the maintenance of intracellular chloride concentrations in neuronal cells (Smith *et al*, 1995 and Staley *et al*, 1996). Evidence for the role of ClC-2 in volume regulation, comes from studies in Sf9 cells transfected with ClC-2. Transfected cells exhibit a chloride

conductance that is activated by hypotonic shock, and is different from endogenous swelling-activated currents. Furthermore, Sf9 cells expressing ClC-2 showed a more rapid volume decrease than mock-transfected cells (Xiong *et al.* 1999). These studies support a role for ClC-2 in mediating regulatory volume decrease. However, the mechanisms by which ClC-2 is activated by hypotonicity are yet to be elucidated. Furthermore, studies of ClC-2 like currents in certain cell types, such as epithelial T84 cells, pyramidal neurons, parotid gland cells and cardiac myocytes reveal similar, but not identical channel regulation (Duan *et al.* 1997, Staley *et al.* 1996, Park *et al.* 1998, Cid *et al*, 1995). For example, ClC-2 like currents in neurons are not stimulated by hypotonicity, and the voltage required for activation of the channel is less hyperpolarized than in the *Xenopus* oocyte system (Staley *et al.* 1996). This variation in the activation properties of ClC-2 currents in different cell types have been suggested to be due to differences in the membrane environment, intracellular chloride concentration or the extent of association with elements of the cytoskeleton (Pusch *et al.* 1999).

ClC-2 has been evaluated to identify domains which are important in the response to hypotonic shock. An "essential" region has been defined within the N terminus of the protein (Grunder *et al.* 1992). Deletion of this region results in constitutive channel activity and loss of regulation by cell volume. Deletion of the "modifier" region results in an intermediate open phenotype (Figure 3.1). Furthermore, when the N-terminus is transplanted to the C-terminus of the protein, the channel retains normal gating properties. These studies suggested that the N-terminus of ClC-2 may function as a "ball-region" in a ball-and-chain model of channel regulation (Grunder *et al.* 1992). This model of channel regulation was proposed initially by Armstrong and Bezanilla, and has

Figure 3.1 Proposed model for ClC-2 regulation



The proposed model for ClC-2 regulation is a "ball and chain" model in which the N-terminus interacts with D7-D8 intracellular loop. The sequences used for the N and C terminus GST fusion peptides are also shown.

been implicated in the regulation of the Shaker potassium channel (Hoshi *et al*, 1990, Zagotta *et al*, 1990). In the proposed model of ClC-2 regulation, a putative docking site for the N-terminal ball region has been suggested to exist in the D7-D8 intracellular loop of the protein (Jordt and Jentsch, 1997). Deletions within this region also result in constitutive channel activity, suggesting that this region is also critical for regulation by cell swelling.

Other clues regarding the regulation of ClC-2 by cell swelling, are present in studies of the purified protein. Single channel analysis of the ClC-2 protein indicate a loss of normal gating mechanisms when the protein is studied in a lipid bilayer. The channel, instead of being mainly in the closed state, exhibits a predominantly open phenotype (Li C, and Bear CE, unpublished findings). These studies suggest that other proteins may be involved in ClC-2 regulation, but are removed during the purification process, thus resulting in a change in gating properties. Furthermore, studies in excised patches of ClC-2 occasionally result in an open phenotype, suggesting the presence of other regulatory mechanisms.

The mechanism of ClC-2 regulation by a ball-and-chain model have not been evaluated. The loss of normal gating properties in studies of the purified protein suggest the loss of associated proteins which may participate in channel regulation. Other channels implicated in either regulatory volume increase or regulatory volume decrease have been shown to be interact with cytoskeletal proteins, including the Na/K/2Cl cotransporter and the Na/H exchanger (Henson, 1999 and Matthews *et al*, 1997). The cytoskeleton has been proposed to play a role in restricting various transporters to specific membrane regions and for the regulation of these proteins (Cantiello, 1995). The

swelling activated chloride conductance has also been previously shown to be activated by disruption of the actin cytoskeleton and inhibited by stabilization of actin in renal cells (Schwiebert *et al*, 1994). The role of the cytoskeleton in the regulation of this conductance has been confirmed in astrocytes and in intestinal epithelial cells (Mountain *et al*, 1998, Tilly *et al*, 1996, Lascola *et al*, 1998). Consequently, it is biologically plausible that ClC-2, a volume-sensitive chloride channel, may also be regulated by cytoskeletal proteins.

Immunolocalization studies have shown that ClC-2 is located at the apical surface of the plasma membrane in epithelial cells and co-localizes with the tight junction protein ZO-1. (Gyomorey *et al*, 2000 Mohammad-Panah *et al*, 2000). This unique location, at the tight junction, places the channel in a region where a large number of cytoskeletal proteins are present, which may be important for channel regulation.

The results presented indicate that disruption of the actin cytoskeleton mimics the the effect of hypotonicity and induces an "open phenotype" in the ClC-2 chloride conductance. Furthermore, biochemical studies suggest that this may be due to the disruption of an electrostatic interaction between the actin cytoskeleton and the amino terminus of ClC-2.

METHODS

3.2.1 Electrophysiology

Stage VI *Xenopus* oocytes were injected with ClC-2 cRNA and studied at least 18 hours after injection. Two-electrode voltage clamp studies were performed as described previously (page 42). Voltage step protocols were applied from a holding potential of –30mVat increments of 40mV between -140mV and +20mV. Baseline hyperpolarization activated currents were recorded, and the oocyte was then treated with 10μM cytochalasin D by direct addition of the drug to the bath solution. Similar experiments were performed with uninjected oocytes. Chloride selectivity was assessed on the basis of the measured reversal potential and the current-voltage relationship of the activated whole cell current.

3.2.2 Identification of ClC-2 interacting proteins

Purified protein columns for ClC-2, amylase and a control column without bound protein were generated as discussed previously. Mouse brain lysate was prepared as described in chapter 2 (page 39), using 3 brains (UNC or Balb/C), obtained from mice sacrificed for other purposes. The lysate was passed through the column and eluted proteins were analyzed by silver stain analysis and western blotting following the determination of protein concentration using a modified Lowry protocol.

3.2.3. Pull-down experiments

The GST fusion proteins for the N and C termini of ClC-2 (GST-Nt or GST-Ct), as well as that for GST alone, were coupled to glutathione agarose beads (Pharmacia). Equal

volumes of the beads were then incubated with increasing concentrations of F-actin overnight at 4 degrees. Purified actin (Cytoskeleton Inc.) was polymerized according to the manufacturer's instructions to produce a stock of F-actin. Briefly, 250μg of lyophilized actin was resuspended in 600mLs of A buffer (1 mL G buffer with 2μl of 100mM ATP), on ice for 30 minutes to dissemble oligomers. The actin was the polymerized using pre-warmed actin polymerization buffer (Cytoskeleton Inc.) for 1 hour at room temperature. The beads were then spun down and washed three times with PBS/0.1% Tween and then once with PBS. The beads were resuspended in SDS loading buffer (63mM Tris HCl, 10% glycerol, 2% SDS, 25 mM dithiothreitol, pH6.8), and analyzed by SDS-PAGE on 12% gels. The gels were stained with Coomassie Blue and the intensity of the bands was evaluated using NIH Image software.

3.2.4 Actin Cosedimentation experiments

Polymerized actin (Cytoskeleton Inc.), 10uM, was incubated with the ClC-2 N-terminus fusion protein at an equimolar concentration for 30 minutes at room temperature. Cosedimentation experiments were performed at varying sodium chloride concentrations from 0.1M to 0.4M to evaluate the effect on the interaction. The samples were then subjected to centrifugation at 100 000g for 20 minutes. Supernatants were carefully extracted and the pellets were resuspended in SDS loading buffer. Equal volumes of supernatant and pellet were then analyzed by SDS-PAGE and stained with Coomassie Blue (Sigma). The relative intensity of the bands was measured using NIH Image Software.

3.2.5 Immunofluorescence

Immunofluorescence studies were performed on Caco-2 cell monolayers as described in chapter 2. Staining was performed for ClC-2 using an affinity purified antibody and for actin using Texas Red conjugated Phalloidin (Molecular Probes).

3.3 RESULTS

3.3.1 ClC-2 is activated by disruption of the actin cytoskeleton in *Xenopus* oocytes

Typically, following expression in *Xenopus* oocytes, ClC-2 mediates chloride currents that are activated in a time-dependent fashion by hyperpolarizing voltage steps of −140mV. To assess the effect of disruption of the actin cytoskeleton on ClC-2 function, *Xenopus* oocytes expressing ClC-2 were treated with 10uM cytochalasin D or 1uM latrunculin B following the acquisition of a baseline recording of activation by hyperpolarization. Cytochalasin D causes gradual depolymerization of actin filaments by binding to their fast growing ends, and unlike cytochalasin B, is unlikely to influence the metabolic status of the oocyte (Spector *et al*, 1989). Latrunculin, a compound that is unrelated to cytochalasin, forms complexes with actin monomers and thereby reduces actin polymerization (Spector *et al*, 1989). Following treatment with cytochalasin D, a gradual increase in ClC-2 current activation by hyperpolarization was observed at both hyperpolarizing and depolarizing voltage steps (Figure 3.2a). The response was detectable at five minutes after treatment and was maximal at 20-25 minutes after exposure to the drug. Typically, ClC-2 currents exhibit a very slow rate of activation with hyperpolarization, however with cytochalasin D treatment, the currents were observed to activate immediately after the application of a similar hyperpolarization step (Figure 3.2b). Treatment of oocytes expressing ClC-2 with 1μM latrunculin B also caused a significant increase in currents at both hyperpolarizing (-140mV), and depolarizing voltages (+20mV), of 173±20%(SE), and 175±30% (SE), respectively (Figure 3.2c). Thus, disruption of the actin cytoskeleton by both drugs resulted in the

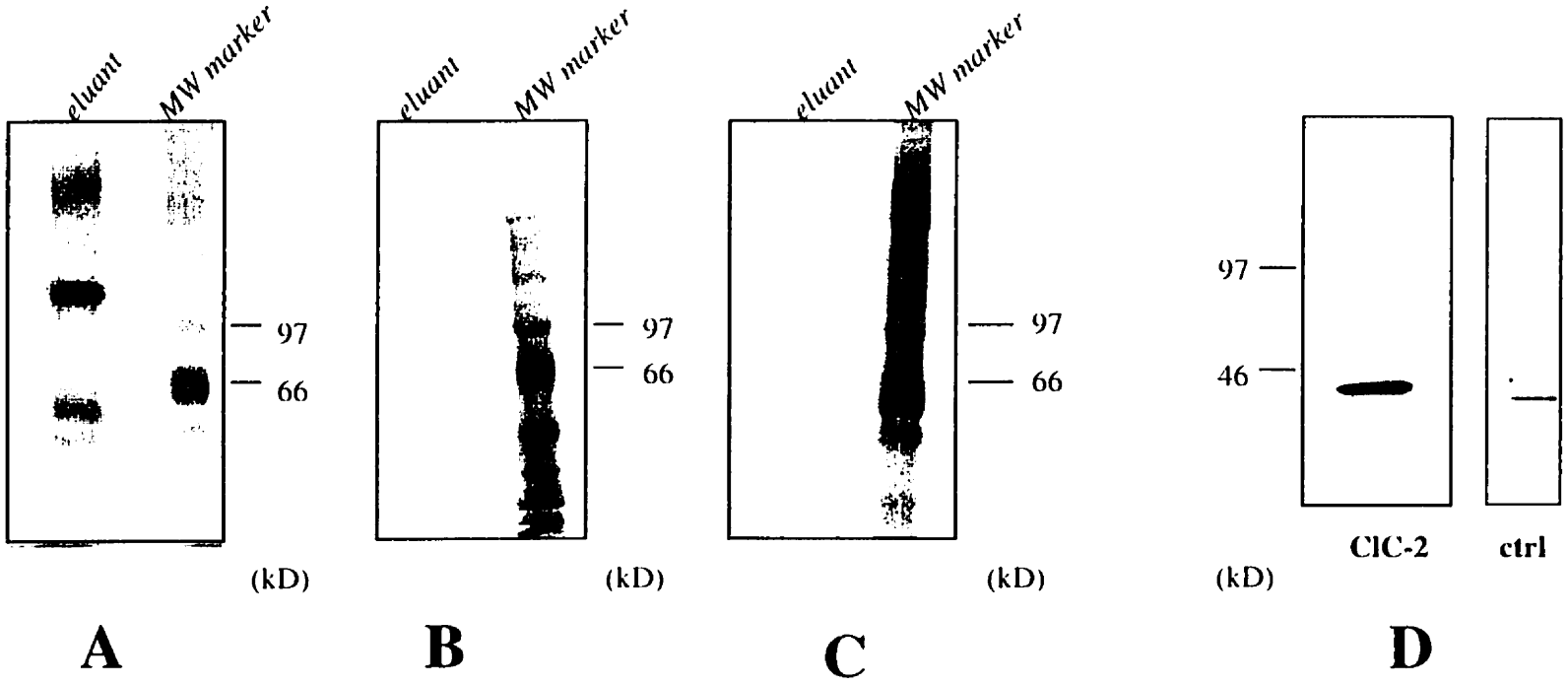
activation of ClC-2 currents, suggesting that this channel is functionally coupled to the cytoskeleton. Furthermore, studies using jasplakinolide, a compound which stabilizes F-actin, had no effect on ClC-2 currents. This suggests that it is the integrity of the actin cytoskeleton that is important in the regulation of ClC-2 function.

Similar experiments in uninjected oocytes did not result in similar changes in hyperpolarization-activated current. Some change was noted at the positive voltages but this was of a much smaller magnitude than that observed in ClC-2 injected oocytes.

3.3.2 Actin binds to a purified ClC-2 column

Proteins which may interact with ClC-2 were identified using a ClC-2 Hi-Trap column and mouse brain lysate. This tissue was chosen as ClC-2 is abundantly expressed in brain, and therefore it is appropriate for identifying potential interacting proteins. Proteins from the tissue lysate which bound to the column were eluted using a pH gradient and then studied by Silver stain analysis. Similar experiments were done using an amylase conjugated column and a control column containing no bound protein. Silver staining revealed the presence of multiple bands in the ClC-2 column and a few fainter bands in the control column (Figure 3.3a,b). No bands were seen in proteins eluted from the amylase column (Figure 3.3c). One band from the ClC-2 column corresponded to a molecular weight in the region of 46kD. Given that the molecular weight of actin is 42kD, immunoblotting was done to determine if this band corresponded to actin. Western blotting confirmed the presence of actin bound to the ClC-2 column, and a small amount was also bound to the control column (Figure 3.3d). However, given that actin is a protein which is prone to bind non-specifically, these results suggested but did not confirm that actin was interacting with ClC-2.

Figure 3.3. Actin binds to a ClC-2 affinity column



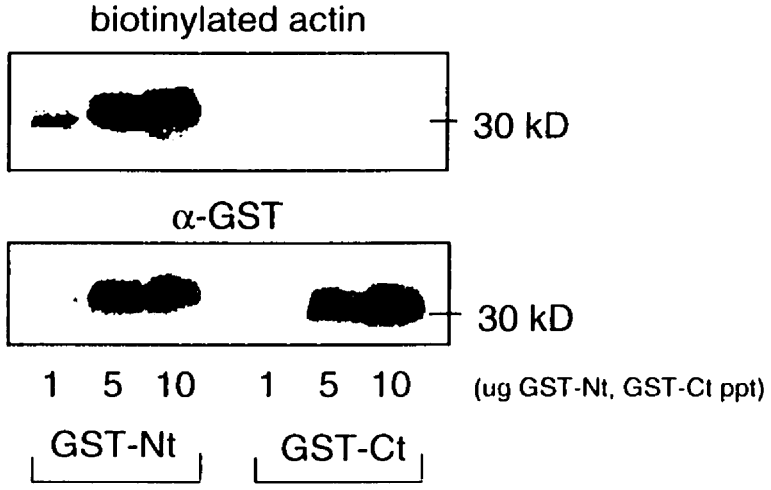
Eluted proteins from a ClC-2 (A), control (B) and amylase (C) column were analyzed by silver staining. Several bands were seen specifically in the eluant from the ClC-2 column (A). Western blotting with anti B-actin confirmed the presence of actin in the eluant from the ClC-2 column (D, left panel). A small amount of actin was also observed in the eluant from the control column (D, right panel).

3.3.3 The N terminus of ClC-2 interacts with actin

It has previously been shown that the amino terminus plays an important role in ClC-2 channel regulation (Grunder *et al*, 1992). In order to determine if this region of the protein is important in the interaction with the cytoskeleton, we performed several biochemical studies experiments using GST fusion proteins for the N and C peptide and purified actin. Firstly, an overlay assay was carried out to identify whether actin was interacting with either the N or C terminus of the protein. 1, 5 and 10μg of the fusion proteins for the N and C peptides were run on SDS-PAGE on precast 12% gels. Following transfer to nitrocellulose, the blots were blocked and incubated with biotinylated actin. Any actin bound would be therefore detectable using streptavidin. As shown in Figure 3.4, actin bound preferentially to the N-terminus of ClC-2 and this binding increased with increasing amounts of N-peptide. Some faint binding was seen with the C-terminal fusion peptide but this was much reduced. These results confirmed an interaction between ClC-2 and actin and suggested that the N-terminus was responsible for the interaction.

To confirm the interaction of the N-terminus of ClC-2 with actin, pull-down studies were performed using the GST-fusion proteins for the N and C peptides and using GST alone. Glutathione agarose bound GST-fusion proteins were incubated with increasing concentrations of F-actin and the amount of F-actin bound was then determined following SDS-PAGE on 12% precast gels and Coomassie blue staining (Figure 3.5a). The amount of actin bound in relation to the amount of GST fusion protein

Figure 3.4 The N-terminus of ClC-2 interacts with actin in an overlay assay

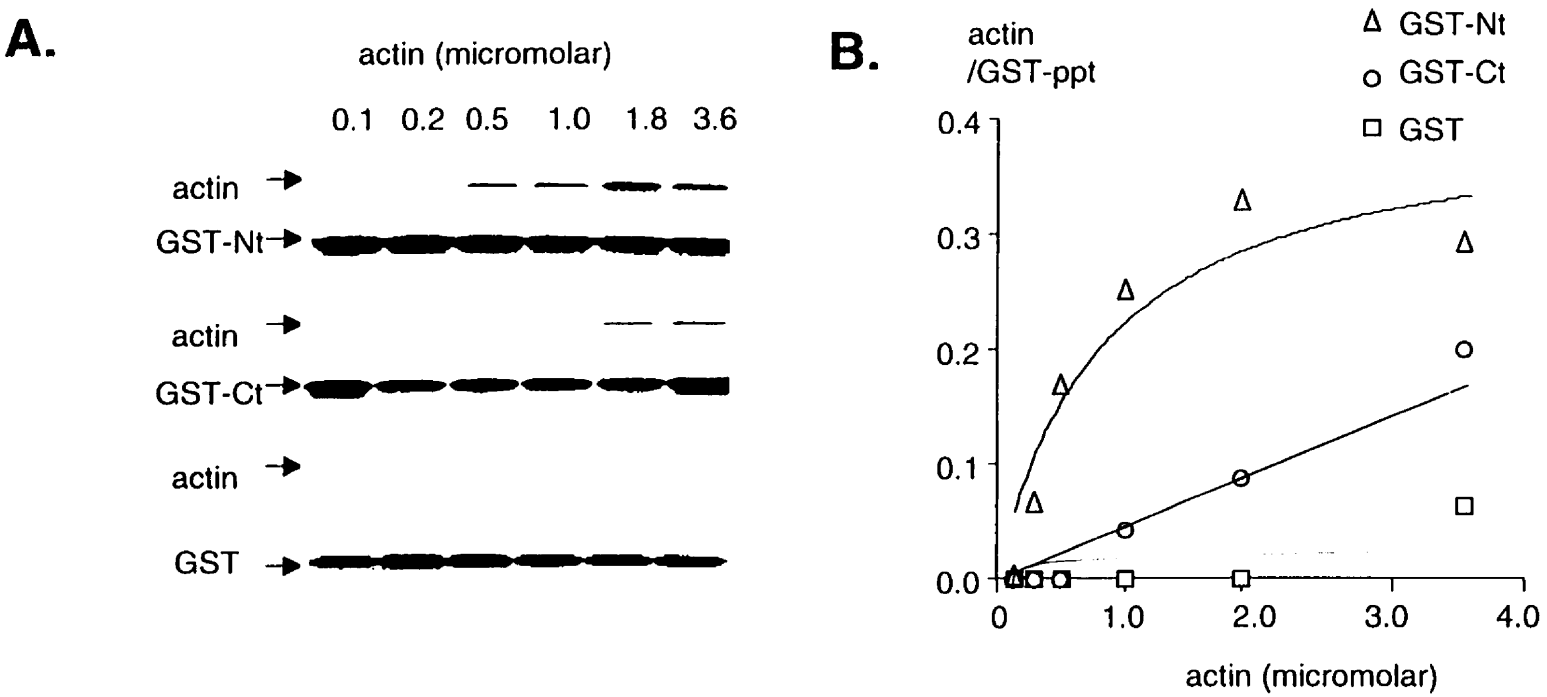


Different quantities (1, 5 and 10 ug) of either GST-Nt or the GST-Ct were run on SDS-PAGE (12%). The predicted molecular mass of GST-Nt is 33kD and for the GST-Ct is 32 kD. Binding of biotinylated actin to each fusion protein was assessed using an overlay assay and detected using streptavidin. Only the GST-Nt was found to bind actin in this assay.

was quantified using NIH Image and Prism software. Using a single site binding algorithm, the amino terminus fusion peptide was found to bind preferentially to F-actin and this binding was saturable with an apparent Km of 0.8μM (Figure 3.5b). Over the same range of actin concentrations, we did not observe saturable binding to the C-terminus fusion protein or to GST alone. These results further confirmed the interaction of ClC-2 with actin and again suggested that the N-terminus of the protein is important in channel regulation.

The mechanism by which the N-terminus of ClC-2 may interact with actin is not clear, however, one possibility is that the interaction is electrostatic. Analysis of the sequence of ClC-2 (ExPASy-Compute pI/Mw tool), indicated that the amino terminus peptide is quite basic with respect to the carboxy terminus peptide (pI 8.43 vs pI 4.7). To evaluate this possibility, the salt-dependence of the ClC-2 and F-actin binding was studied. 10μM ClC-2 N-peptide was incubated with 10μM F-actin and following a high speed centrifugation to pellet the actin, the amount of N-peptide in the pellet was quantified. In the absence of actin, all the N-peptide remained in the supernatant. However, in the presence of actin, the N-peptide was detectable in the pellet (Figure 3.6a). Furthermore, the ratio of the amount of N-peptide in the pellet to the amount of actin in the pellet, varied with increasing concentrations of NaCl. At a NaCl concentration of 400mM, virtually no N-peptide was present in the pellet. The salt-dependence of this interaction is illustrated in a one-phase exponential decay analysis of the data. Using this analysis, half maximal binding would be predicted to occur at 0.18M

Figure 3.5 F-actin binding to the N-terminus of ClC-2 is saturable



The F-actin concentration dependence for association with GST-Nt, GST-Ct or GST alone was assessed in pulldown studies. Bound F-actin and fusion proteins were analyzed by SDS-PAGE and Coomassie Blue staining. The amount of bound actin was quantified using NIH Image and Prism software. (B) The concentration dependence of F-actin binding to the N-terminus peptide was fit using a single site binding algorithm (R2=0.9) to yield an apparent Km of 0.8uM and Bmax of 0.407. Over the same range of concentrations , we observed no saturable binding to either GST-Ct or GST alone

NaCl (Figure 3.6b). These results indicate that ClC-2 interacts with F-actin, and that this interaction is mediated by the N-terminus of the protein and is likely electrostatic.

3.3.4 ClC-2 and actin colocalize in Caco-2 cells

Immunofluorescence studies were carried out in Caco-2 cells to determine if the cellular distribution of ClC-2 and actin overlap and therefore support a biological interaction. ClC-2 and actin were observed to share a similar membrane distribution, and to colocalize, confirming that these proteins are in a position to interact with each other (Figure 3.7).

Figure 3.7 Colocalization of actin with ClC-2 in Caco-2 cells

A

B

Immunofluorescence staining of Caco-2 cells with ClC-2 (A) and phalloidin (B) to label actin indicates that ClC-2 and actin colocalize.

3.4 DISCUSSION

The ClC-2 chloride channel has been previously shown to be activated by cell swelling and suggested to play a role in mediating regulatory volume decrease. Studies have proposed that the regulation of the channel is via a ball-and-chain mechanism, with the N-terminus serving as the ball region and the D7-D8 intracellular loop as the docking region (Grunder *et al*, 1992). However, the mechanism for this regulation has not been fully established.

The present studies indicate that ClC-2 interacts with the actin cytoskeleton and that this interaction is functionally important. Disruption of the cytoskeleton by the fungal metabolite, cytochalasin D, in *Xenopus* oocytes resulted in an increase in chloride conductance and a change in gating properties, similar to that seen with cell swelling. Cytochalasin D depolymerizes F-actin (Cooper, 1987), and has been shown to inhibit regulatory volume decrease in several cell types (Foskett and Spring, 1985, Cornet *et al*, 1988, Linshaw *et al*, 1991, Downey *et al*, 1995), and to stimulate swelling-activated chloride channels in others (Park *et al*, 1998, Levitan *et al*, 1995). The effect of cytochalasin D on absolute F-actin content is variable depending on the cell type and concentration and therefore, it is difficult to draw any general conclusions regarding the exact effect on F-actin (Schweibert *et al*, 1994, Smith and Clayton, 1995, Franki *et al*, 1992, Pedersen *et al*, 1999). However, studies in *Xenopus* oocytes have used concentrations in the range of 2-20μM, similar to that employed in the current studies, to assess the effects of actin disruption on various membrane transporters (Honore *et al*, 1992, Pajor *et al*, 1999). The similarity in the current profile obtained with cytochalasin

D treatment, to that seen by activation by cell swelling, suggests that the cytoskeleton is likely involved in mediating the channel activation observed with hypotonic shock.

Grunder *et al.*, have studied mutations within the amino terminus of ClC-2 and have identified putative "essential" and "modulating" domains that result in constitutive channel function. The currents we observed in *Xenopus* oocytes expressing ClC-2 following the disruption of the cytoskeleton were similar to the "open" phenotype observed in these mutants. The biochemical studies presented implicate the amino-terminus of the channel in the interaction with actin, which is in keeping with this model of ClC-2 regulation (Jordt and Jentsch, 1997, Grunder *et al*, 1992). Both our cosedimentation experiments and the overlay assay confirm that the N-terminus is important for mediating the interaction with actin. Previous studies have shown that transplantation of the amino-terminus to the carboxyl terminus of the protein result in normal gating, and this may be mediated by the ability of the N-terminus to still interact with actin (Jordt and Jentsch, 1997). Complete deletion of the "essential" region within the N-terminus would be expected to prevent any interaction with actin, and therefore result in a loss of normal gating behavior.

Furthermore, we have shown that the interaction between the amino terminus and F-actin is salt dependent, suggesting that the interaction is electrostatic in nature. Similar observations have been made for the membrane protein dystrophin that is defective in Duchenne Muscular Dystrophy (Rybakova *et al*, 1996, Amann *et al*, 1999). This protein has been shown to interact with actin via its amino-terminal domain and its rod domain, and that this interaction is extremely sensitive to increasing ionic strength. This interaction is thought to be mediated by a series of basic repeats within this region. While

ClC-2 does not possess any typical actin binding motifs or protein-binding motifs such as PDZ, SH2 or SH3 domains, the amino-terminus of ClC-2 contains a region of basic residues, as in dystrophin, which may be important in mediating the interaction with actin. Electrostatic interactions have been shown to mediate other physiologically important interactions including that between band 3 and aldolase, phosphofructokinase or glyceraldehyde (Yu and Steck, 1975, Murthy *et al*, 1981, Higashi *et al*, 1979). Thus, there is existing evidence of electrostatic interactions of membrane proteins with the cytoskeleton. While this *in vitro* study of ClC-2 and F-actin suggests a direct interaction, further study will be required to assess whether this holds true *in vivo*.

ClC-2 also colocalizes with actin, thereby confirming that these two proteins are in close proximity allowing for a direct interaction. Attempts to visualize the effect of actin disruption by cytochalasin D on the localization were unsuccessful, as the abundance of actin did not allow for the visualization of any minor changes in ClC-2 distribution.

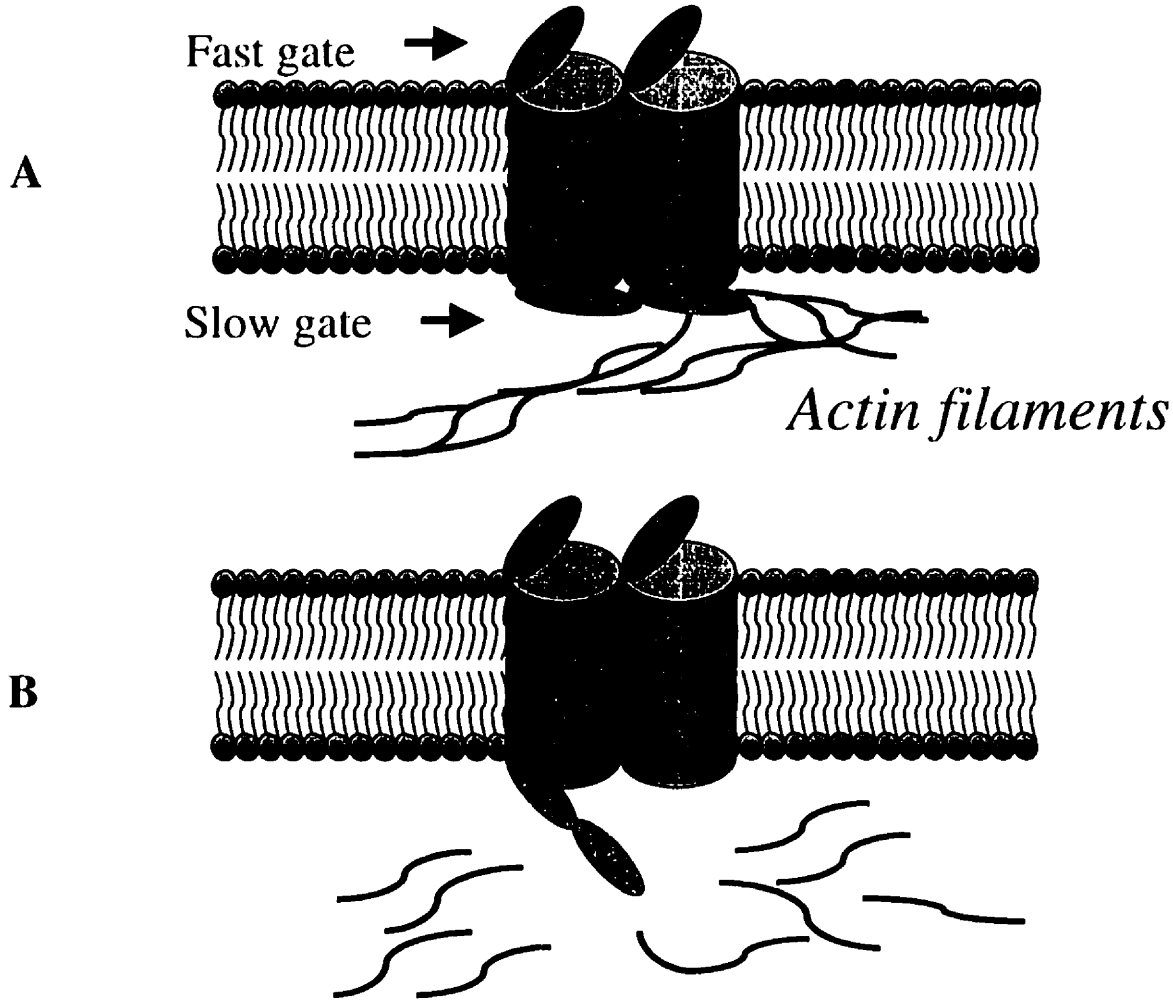
As stated previously a number of membrane transporters involved in volume regulation have been shown to be associated with the cytoskeleton, however, the steps by which this regulation occurs is not clear. A number of mechanisms have been suggested to be important in the regulation of specific ion transporters by the cytoskeleton. These include i)the direct regulation of membrane transporters (Guharay and Sachs, 1984, Sackin, 1989), ii) activation of signal transduction pathways leading to volume regulation and iii) effects on trafficking of transporters to the membrane (Lewis and De Moura, 1982). The argument for direct regulation of membrane transporters comes from studies in which changes in actin filaments affect transporter activity, such as that seen with the

epithelial sodium channel (Cantiello *et al.* 1991). Interaction may also be mediated by actin binding proteins. For example, the human melanoma cell line which lacks an actin-binding protein, is unable to undergo regulatory volume decrease, but this could be restored if the protein was expressed (Cantiello *et al.* 1993). Similarly, the epithelial sodium channel ENaC has also been shown to interact with the cytoskeleton via the acting binding protein, spectrin. This interaction is mediated by an SH3 domain in the C-terminal region (Rotin *et al.* 1994). Signal transduction pathways involved in actin reorganization involve the Rho family of GTPases, and studies have shown that inhibition of Rho resulted in decreased swelling-induced anion efflux (Hall, 1998). Thus, the effect of cell swelling on specific transporters may also be mediated by this pathway. The mechanism by which the cytoskeleton regulates membrane transporters is likely complex and may comprise an interplay of several pathways. With respect to ClC-2, it appears that this channel interacts directly with actin based on studies in a purified system. However, further study is required to determine if ClC-2 interacts directly or indirectly with actin .

ClC-2 has been implicated in volume regulation and the mechanism by which this occurred had not yet been elucidated. These studies suggest that the actin cytoskeleton is important in the regulation of channel activity and that this regulation is mediated by the N-terminus of the protein. Furthermore, the interaction between actin and ClC-2 is sensitive to ionic strength, suggesting that the interaction is electrostatic. Disruption of the actin cytoskeleton, cell swelling or hyperpolarization may interfere with this interaction, resulting in channel activation (Figure 3.8). Understanding of the details of the regulation of this channel will allow for the correlation between channel function and

physiologic processes. It has already been suggested that ClC-2 may be a candidate to compensate for CFTR (Murray *et al*, 1995, Joo *et al*, 1999), and it is therefore necessary to understand its regulation, in order to consider it as a potential therapeutic target in cystic fibrosis.

Figure 3.8 Model of Double-barreled channel gating for ClC-2



Actin interacts with the amino terminus of ClC-2 and may regulate the channel's slow gate (A).Disruption of the actin cytoskeleton would presumably result in an interference with this and lead to a constitutively open channel (B).

CHAPTER 4

ClC-2 INTERACTS WITH THE MOLECULAR MOTOR, DYNEIN

4.1 INTRODUCTION

Molecular motors have been shown to play an important role in vesicle trafficking in epithelial cells (Vallee and Sheetz, 1996, Hirokawa, 1998). The microtubule network serves as the track for the movement of the dynein and kinesin family of motors. Kinesin was initially thought to be an anterograde motor that moved vesicles and organelles towards the growing (+) end of microtubules, while cytoplasmic dynein was a retrograde motor that moved its cargo to the minus end of these structures. However, subsequent studies have shown that some forms of kinesin are capable of retrograde transport (Saito et al, 1997). Since the discovery of cytoplasmic dynein in 1987 (Vallee et al, 1998), it has been shown that this motor protein moves in the opposite direction to conventional kinesin, however, dynein has not been as well characterized, and at this time it is not clear whether it is also capable of bidirectional movement. In epithelial cells including enterocytes (Sandoz and Laine, 1985, Gilbert et al, 1991), pancreatic acinar cells (Marlowe et al, 1998) and hepatocytes (Ihrke et al, 1993), microtubules are oriented with their plus ends towards the nucleus and their minus ends at the apical surfaces. Thus dynein is thought to play a role in the movement of cargo between cellular compartments, towards the apical surface. It should be noted that another form of dynein, flagellar or axonemal dynein, exists, and plays an important role in cellular movement.

Dynein exists in a complex of two heavy chains, each containing a globular and stalk domain, two to four intermediate chains and several light chains, and associates with the dynactin complex of proteins (Vallee and Sheetz, 1996, Hirokawa, 1998). Three genes have been identified which encode for the dynein heavy chains (DHC1-DHC3) (Vaisberg et al, 1996). The role of DHC3 has yet to be defined, however, DHC1 has been

localized to endocytic organelles and mitotic structures, while DHC2 appears to associate with the Golgi complex (Lin and Collins, 1992, Oda et al, 1995). Dynein has been implicated in the distribution of late endosomes and lysosomes, (Lin and Collins, 1992). trafficking between early and late endosomes (Aniento et al, 1993), movement of phagosomes (Blocker et al, 1997) and the localization of the Golgi complex (Corthesy-Thelauz et al, 1992) as well as the transport of Golgi derived membranes to the apical surface of intestinal epithelial cells (Fath et al, 1994). Vesicles containing proteins from the endoplasmic reticulum may interact with dynein for transport to the Golgi apparatus (Presley et al, 1997). In neurons, dynein plays a role in fast axonal transport, as well as in neuronal growth and development (Waterman-Storer et al, 1997, Ahmad et al, 1998). More recently dynein, has been shown to translocate the membrane protein rhodopsin within photoreceptors of the eye (Tai et al, 1999). That study is one of the first to demonstrate that dynein is involved in the movement of specific membrane proteins to their destination, and implicated the light chain tctex-1 in binding to the cargo. Similarly, dynein has been implicated in the sorting of ligand-receptor complexes in the hepatocyte for targeting to specific endocytic compartments (Oda et al, 1995, Goltz et al, 1992). The elaborate microtubular and motor protein system is likely to be important in the transport of multiple membrane proteins, and this remains an active area of ongoing study.

The ClC-2 chloride channel has been shown to be regulated by cell swelling, extracellular pH and hyperpolarization (Grunder et al, 1992). However, the mechanisms for channel regulation remain unclear. Mutagenesis studies have suggested that the amino terminus is important for channel regulation. The authors of these studies have proposed that an interaction between the amino-terminal region and the D7-D8

intracellular loop may function as a ball and chain model of channel regulation (Thiemann et al, 1992, Jordt and Jentsch, 1997). However, other mechanisms for regulating channel activity have not been explored. ClC-2 trafficking has not been characterized, and it is possible that the movement of ClC-2 to and from the membrane may also play a role in channel regulation. The ClC-5 chloride channel has been localized to endosomal compartments, but the channel can also be detected at the apical surface, suggesting that there may be a role for trafficking between cellular compartments in channel regulation (Friedrich et al, 1999, Gunther et al, 1999). Stimulation of ClC-2 by hypotonic shock, changes in pH or hyperpolarization, may directly or indirectly stimulate an increase in ClC-2 trafficking to the membrane, and thereby result in increased currents. While molecular motors have not yet been shown to play a role in volume regulation, in some studies the microtubule cytoskeleton has been shown to undergo changes in response to cell volume changes (Haussinger et al, 1994, Downey et al, 1995, Shen et al, 1999).

Regulation of activity by the modulation of vesicle trafficking has been proposed for other membrane transporters. Studies of the Na/H antiporter, NHE3, have shown that it is mainly localized within the endosomal compartment (D'Souza et al, 1998), but may be recruited to the plasma membrane with specific stimuli (Hensley et al, 1989, Zhang et al, 1996). This form of regulation has also been proposed for the regulation of H+/K+ ATPases and aquaporin-2 water channels (Brown and Sabolic, 1993, Courtois-Coutry et al, 1997). Trafficking of these transporters to the membrane surface may involve the activity of molecular motors, given the role of these proteins in the movement between cellular compartments.

The regulation of ClC-2 is not yet well established, and while the ball and chain model of channel regulation is one form of modulation, it is possible that other mechanisms exist. The microtubule and molecular motor network is another plausible method by which this channel could be regulated, through the modulation of movement between cellular compartments.

4.2 METHODS

4.2.1 *Mass Spectroscopy Analysis*

Mass spectroscopy analysis was performed at the University of Toronto using the Maldi-TOF technique.

4.2.2 Density Centrifugation

Caco-2 cells were grown to confluent monolayers and cells were harvested to obtain a 0.5mL cell pellet. For dynein inhibition studies, cells were pretreated with 0.5mM EHNA (Sigma). Cells were then homogenized in 0.25M sucrose, 1mM EDTA, 10mM Tris, pH 7.4 using a Dounce homogenizer. The homogenate was centrifuged at 3000g using a JA17 rotor (Beckman Inc.) for 10 minutes to obtain a post-nuclear supernatant. The supernatant was mixed with an equal volume of 60% iodixanol (Nycomed Diagnostics), and centrifuged at 100 000g for 60 minutes in a VTi 65 rotor (Beckman Inc.). At this speed iodixanol forms a steep gradient between 1.1 g/mL and 1.25 g/mL. One mL fractions were then collected and protein concentrations for each fractions were determined. The fractions were then analyzed by SDS-PAGE and Western blotting to identify proteins of interest.

4.2.3 Dynein Cosedimentation Experiments

Purified ClC-2 proteoliposomes or Sf9 membranes containing ClC-2 were incubated with 3.5ug of purified dynein (gift from T. Schroer) in PEM buffer (80mM PIPES, pH6.8, 1mM EGTA, 1mM MgSO4) at room temperature for 1 hour. 125ug of Taxol-stabilized microtubules (Cytoskeleton Inc.) were then added to the samples for 10 minutes at 30 degrees. The vesicle-dynein-microtubule mixture was then layered over 1M sucrose in

PEM/Taxol buffer and centrifuged at 40 000g for 40 minutes at 30 degrees in a SW55 rotor. Pellets were resuspended in 2% SDS and analyzed by SDS-PAGE and immunoblotting.

4.2.4 Antibodies

Polyclonal antibodies to ClC-2 were obtained by protein A purification as described previously. Monoclonal antibodies to cytoplasmic dynein intermediate chain were purchased from Sigma and Chemicon (California, USA). Monoclonal antibodies to E-cadherin were obtained from Zymed Inc (California, USA), and a polyclonal antibody to rab 5b was from Santa Cruz Biotechnology (California, USA).

4.3 RESULTS

4.3.1 Mass spectroscopy analysis indicates that the dynein heavy chain interacts with ClC-2

A ClC-2 affinity column was prepared as described previously (chapter 2), and a mouse brain lysate was passed through the column. Bound proteins were eluted using a pH gradient and analyzed by silver staining. This technique revealed the presence of a prominent band at approximately 100kD. This band was sent for analysis by mass spectroscopy and determined to correspond to cytoplasmic dynein heavy chain. This result indicated that ClC-2 was interacting with dynein, and was confirmed using western blotting on the column eluant with an antibody to dynein (Figure 4.1). An antibody to the dynein intermediate chain (Chemicon) was used as dynein is known to exist in a complex of heavy, light and intermediate chains. No dynein was detected in the control column or amylase column eluants.

4.3.2 Dynein inhibition results in the activation of ClC-2 currents in Xenopus oocytes

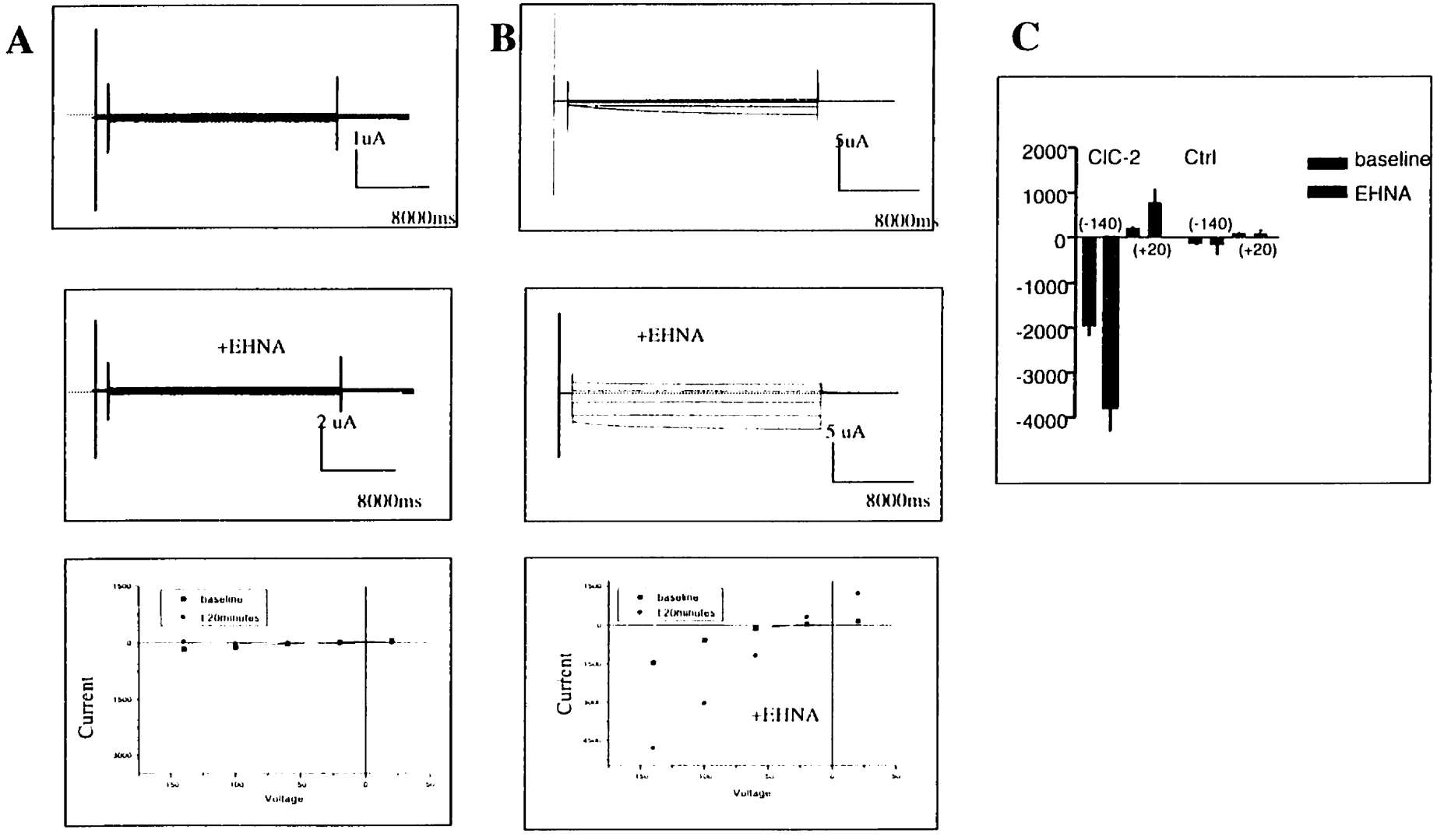
To determine if the interaction between ClC-2 and dynein was functionally important, the two-electrode voltage clamp technique was used to study ClC-2 function. *Xenopus* oocytes expressing ClC-2 were treated with dynein inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride(EHNA) (Sigma) at a final bath concentration of 0.125 mM. EHNA is an ATPase inhibitor which specifically inhibits dynein activity. ClC-2 activation by hyperpolarization increased beginning at 5 minutes after drug addition and

continued to increase up to 20 minutes. Uninjected oocytes did not exhibit any response to the drug (Figure 4.2). These results suggested that the inhibition of the dynein molecular motor, increased ClC-2 activity, and thus the interaction between these proteins was functionally important.

4.3.3 Density gradient centrifugation indicates that ClC-2 and dynein have an overlapping distribution.

To determine if ClC-2 and dynein were located in similar membrane compartments, and would therefore be capable of interacting, density gradient centrifugation was carried out. Post-nuclear supernatants from a 0.5mL Caco-2 cell pellet were fractionated in 30% iodixanol (Nycomed Inc). At this concentration, this medium forms self generating gradients with a density of 1.1g/mL to 1.25g/mL (Nycomed Centrifugation Technical Bulletin VII: gradient formation). Plasma membrane proteins are identified at the top of the gradient and cytosolic proteins at the bottom (Grindstaff et al, 1998). One ml fractions were collected and western blotting was performed to identify the plasma membrane and endosomal compartments, using antibodies to E-cadherin and rab5 respectively (Figure 4.3a and 4.3b). The distribution of E-cadherin was consistent with other studies employing this technique, confirming that fractionation was occurring appropriately (Figure 4.3a) (Grindstaff et al, 1998). Furthermore, initial studies suggest that ClC-2 was also present in compartments labelled by the early endosomal marker rab5 as well as the plasma membrane compartment (Figure 4.3a,b). This suggests that ClC-2 can be found in early endosomes, however, these preliminary findings require further confirmation.

Figure 4.2 Inhibition of dynein results in an increase in ClC-2 mediated current in Xenopus oocytes



Immunoblotting of the fractions with an antibody to dynein intermediate chain, indicated that most of this protein was also located in the upper fractions (Figure 4.3c).

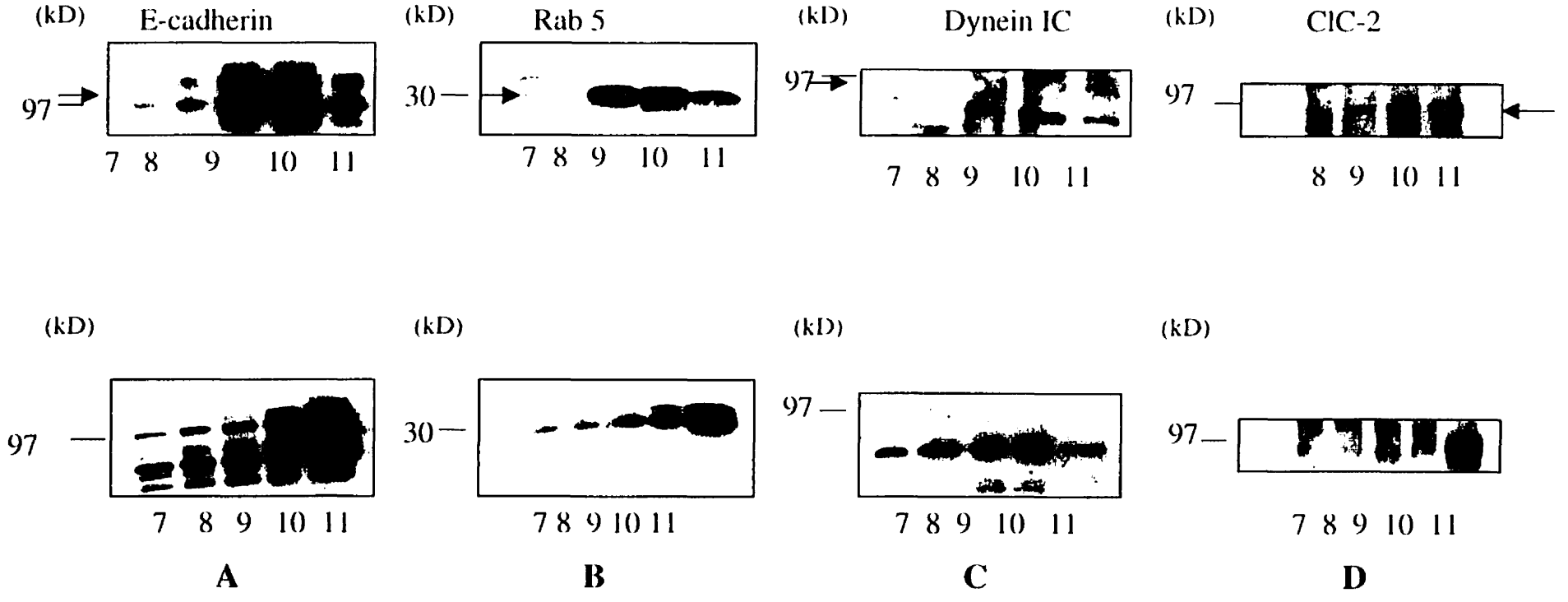
To evaluate whether the dynein inhibitor, EHNA, had an effect on the distribution of ClC-2, Caco-2 cells were pretreated with 0.5mM EHNA prior to harvest. The cells were then fractionated as above. Initial results suggest that the distribution of ClC-2 was changed by treatment with this drug. With EHNA treatment, more ClC-2 appeared fraction 11, suggesting that inhibiton of dynein resulted in a redistribution to the plasma membrane compartment. Furthermore, EHNA was observed to result in increased E-cadherin in fraction 11.

4.3.4 *ClC-2 cosediments with dynein in a microtubule dependent manner*

Preliminary studies were carried out to evaluate the *in vitro* interaction between dynein and ClC-2, using a microtubule cosedimentation assay. Membranes from Sf9 cells expressing ClC-2 were prepared. The membranes were then incubated with purified dynein and then incubated with Taxol-stabilized microtubules. Following centrifugation of this mixture through a sucrose cushion, the pellet containing microtubule bound vesicles was analyzed by SDS-PAGE and western blotting (Figure 4.4).

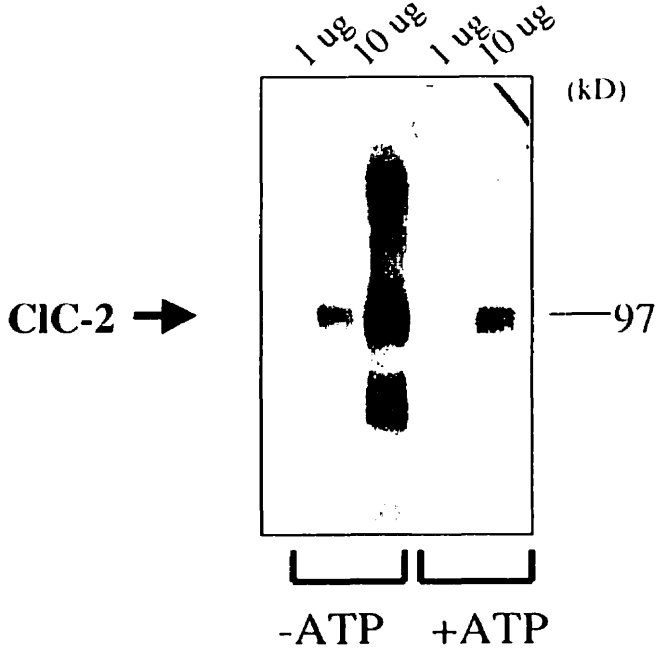
ClC-2 was found to cosediment with microtubules in the presence of dynein, indicating that there was an interaction between these three proteins. In the presence of 10mM MgATP which results in the dissociation of dynein from microtubules, there was a reduction in the amount of ClC-2 in the pellet. Initial studies performed in the absence of microtubules suggest that there is also a decrease in the amount of ClC-2 in the pellet. Taken together, theses results suggest that ClC-2 cosediments with microtubules in the

Figure 4.3 Identification of the plasma membrane and endosomal compartments following Optiprep fractionation of Caco-2 cells



Following Optiprep density centrifugation, fractions were analyzed for the presence of the plasma membrane marker,E-cadherin and the early endosomal marker, rab5 (A and B). Western blotting identified dynein in fractions 9-11before EHNA treatment, and in 7-11 after EHNA treatment (C). ClC-2 was identified in fractions 9-11 prior to EHNA treatment and redistributed to fraction 11 following EHNA (D).

Figure 4.4 ClC-2 cosediments with dynein in the presence of microtubules



Membranes from Sf9 cells expressing ClC-2 were incubated with dynein and microtubules in the absence or presence of 10 mM ATP. In the presence of ATP there was as decrease in the amount of ClC-2 in the pellet.

presence of dynein, and confirm the interaction between this membrane protein and the molecular motor.

4.4 DISCUSSION

Dynein has been implicated in the movement of cargo between cellular compartments and vesicle trafficking. More recently, it has been implicated in the trafficking of the membrane protein rhodopsin (Tai et al, 1999). Here we have shown that dynein also interacts with the chloride channel ClC-2 and likely plays a role in the regulation of this membrane protein. This interaction appears to be specific and is functionally important.

Our data indicate that the chloride channel ClC-2 interacts with dynein both biochemically and functionally in epithelial cells. In *Xenopus* oocytes, dynein inhibition by EHNA increased ClC-2 mediated currents. At the concentration used, this drug is thought to specifically affect dynein activity. At higher concentrations, above 1mM, EHNA can also affect the basolateral Na/K ATPase, however, in our experiments in which we used a much lower concentration we assume that this effect is minimal (Penningroth et al, 1985). *Xenopus* oocytes have complex microtubular arrays which differ with the stage of the cell. In stage IV-VI oocytes, there is an increase in the amount of tubulin and the microtubular array becomes polarized along the animal-vegetal axis, while in stage III oocytes the microtubules exhibit a radial distribution (Gard et al, 1995). The directionality of microtubules in this system was established only recently by electron microscopy. In the stage III-VI *Xenopus* oocyte, most microtubules radiate from the nucleus which is located in the animal pole in stage VI cells, towards the cortex in both animal and vegetal poles, with their minus ends directed towards the cortex (Pfeiffer and Gard, 1999). However, there is a population of microtubules that have the opposite orientation. It has been established that dynein plays an important role in forming and

maintaining the endoplasmic reticulum in the oocyte (Allan and Vale, 1994), and it has been suggested that dynein may also be important in movement to the membrane while kinesin is important for transport away from the membrane (Allan and Vale, 1994). However, given that dynein inhibition increased ClC-2 currents, it would be plausible that a normal process of ClC-2 recycling from the membrane is being disrupted suggesting that in this system, dynein may be involved in the movement of ClC-2 away from the apical surface. Studies of ClC-2 function in Caco-2 cells have shown that dynein inhibition also results in increased ClC-2 mediated current (Mohammad-Panah R, Bear CE, unpublished findings). In these cells, which model the human small intestine, there are two populations of microtubules. The first and most abundant group consists of microtubules oriented with their minus ends at the apical surface and there plus ends at the basolateral surface. The second population consists of microtubules that run parallel to the apical surface and form a dense network in this area, and also form a sparse network just beneath the basolateral surface (Gilbert et al, 1991). Inhibition of dynein would be expected to affect the trafficking of ClC-2 towards the apical surface via the longitudinal microtubules, however, it is not clear how this would affect movement along the subapical horizontal microtubular network. It is notable that the distribution of microtubules varies between fully differentiated cells and undifferentiated cells. In the undifferentiated cell, microtubules have a radial orientation with their minus ends towards the center of the cell (Hamm-Alvarez and Sheetz, 1998). Consequently, the effect of dynein inhibition will vary according to the maturity of the cell. In the patch clamp studies mentioned previously, single cells were studied (Mohammad-Panah R and Bear CE, unpublished findings), and consequently, dynein inhibition would be expected to

result in decreased movement of ClC-2 away from the plasma membrane. However, in fully differentiated cells, dynein inhibition would be expected to result in decreased movement of ClC-2 towards the membrane.

As stated previously, dynein is thought to be exclusivey a minus-end directed motor, but it is possible that like its counterpart kinesin, it will be found to have the capacity for movement in the opposite direction. It has similarly been difficult to reconcile some of the activities of kinesin with its predicted function. In pancreatic acinar cells, microtubules are oriented with their minus ends towards the apical surface, and in these cells inhibition of kinesin inhibits the movement of zymogen granules to this surface, and furthermore agonist induced secretion results in an increase in the association of these granules with kinesin (Marlowe et al, 1998). Thus, conventional kinesin may be important in movement away from the apical surface. While there is now an isoform of kinesin, KIFC2, that moves towards the minus ends of microtubules in neurons, it is not clear whether this occurs in other cell types (Saito et al, 1997). Furthermore, the role of dynein in movement along the horizontal subapical microtubular network is not clear. Therefore, it is likely that the movement of proteins along microtubules by specific motors is more complex than initially predicted, and further study is needed to determine how these motor enzymes affect protein trafficking.

The above studies confirm that dynein and ClC-2 are capable of interacting biochemically. Using density gradient fractionation, dynein and ClC-2 are shown to have an overlapping distribution which can be manipulated by dynein inhibition. While this method does not confirm a direct interaction, it lays the groundwork for the more detailed cosedimentation experiments. ClC-2 and dynein are shown to be present in both

membrane and endosomal compartments, and therefore are likely capable of interacting given their overlapping distribution. With dynein inhibition prior to harvesting of the Caco-2 cells, there is a much more diffuse pattern of dynein distribution, while ClC-2 appears to be more concentrated in the plasma membrane compartment. This suggests that dynein inhibition is altering the distribution of ClC-2, consistent with the increased currents observed in both the *Xenopus* oocyte and Caco-2 system.

The cosedimentation experiments presented confirm that membranes containing ClC-2 are capable of interacting with dynein, and cosediment with this motor in the presence of microtubules. While these studies do not identify the precise binding site of ClC-2 with dynein, it lays the groundwork for future studies. The dynein light chains and intermediate chains have been suggested to play an important role in the binding of dynein to its cargo. Indeed, the tctex-1 light chain has been implicated in the interaction with rhodopsin (Tai et al, 1999). However, given the diversity of both light and intermediate chains, it is likely that different chains mediate different dynein functions. The intermediate chain is proposed to mediate binding of cargo via an interaction with the dynactin subunit p150Glued (Vaughan and Vallee, 1995), however the dynactin complex was not necessary for the binding of rhodopsin to dynein (Tai et al, 1999). Therefore, the mechanism by which this motor complex interacts with its cargo is likely complex and this may be important in dynein function.

ClC-2 is a chloride channel that is ubiquitously expressed, and that may be a potential target for therapy in cystic fibrosis. As such, understanding its regulation is critical. While a ball and chain model of internal channel regulation has been proposed, this is likely important for rapid control of channel activity (Grunder et al, 1992,

Thiemann et al. 1992, Jordt and Jentsch, 1997). Regulation by movement of the channel between endosomal and plasma membrane compartments is another mechanism that is now supported by the functional and biochemical interaction of this channel with the molecular motor, dynein. It is likely that both these mechanisms are important in the control of ClC-2 function, and may occur simultaneously or in response to different stimuli.

The role of the microtubular network in vesicle trafficking has been well described. Disruption of this network results in the delay of protein delivery to the apical membrane as well as a missorting of proteins (Gilbert et al. 1991, Breitfeld et al. 1990). Molecular motors have also been implicated in the trafficking of a number of membrane proteins, including the Na/H exchanger, aquaporins and the H/K ATPase (D'Souza et al. 1998, Hensley et al. 1989, Zhang et al 1996, Brown and Sabolic, 1993, Courtois-Coutry et al. 1997). This study presents new evidence for a role of these proteins in the movement of specific membrane transporters and suggests that molecular motors may be important in the regulation of such proteins directly or indirectly. It has already been shown that dynein plays a role in Na channel activity in A6 renal epithelial cells (Rehn et al. 1998). In these cells, dynein inhibition by EHNA resulted in a decrease in Na current in response to hyponicity. Our results also suggest that there may be a role for molecular motors in channels involved in volume regulation, given that ClC-2 is implicated in regulatory volume decrease (Xiong et al. 1999). The microtubule network has been suggested to play a role in volume regulation, as the disruption of microtubule structure affects the cell's ability to volume regulate in some cell types (Downey et al. 1995, Shen et al. 1999). Many membrane transporters have been suggested to play a role in volume

regulation, and it is plausible that the movement of such proteins to the membrane in response to cell swelling or shrinkage plays an important role in mediating regulatory volume change.

The role of molecular motors in cellular processes is an area of ongoing research activity. Indeed, this study presents the first evidence of a direct interaction between a chloride channel and the dynein molecular motor. Further study is required to determine the functional significance of this interaction and the cellular mechanisms by which this occurs.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

CHAPTER 5. DISCUSSION AND CONCLUSIONS

The ClC-2 chloride channel is a ubiquitously expressed protein whose role in physiology remains under investigation. The channel has been identified in cells of the pancreas, kidney, gastrointestinal tract as well as in neurons (Thiemann *et al*, 1992). To date it has been implicated in the maintenance of intracellular chloride concentrations in neurons (Clayton *et al*, 1998, Smith *et al*, 1995), chloride secretion in gastric tissue (Murray *et al*, 1996) and has been localized to the apical surface of lung epithelia (Murray *et al*, 1995). ClC-2 has also been found to be present at the apical surface of the intestinal epithelium, located uniquely at the tight junction (Gyomorey *et al*, 2000 Mohammad-Panah *et al*, 2000). While unlike ClC-1, ClC-2 has not been associated with any clinical disease process, the localization of the channel at the epithelial surface in the lung and the apical surface of the intestine allows this channel to be well suited as a possible alternate chloride conductance in cystic fibrosis. In this inherited disease, there is a defect in the Cystic fibrosis transmembrane conductance regulator (CFTR) protein as a result of mutations in the encoding gene. This defect results in absent or impaired chloride conductance in many tissues including the lung, pancreas, sweat gland and reproductive tract (Quinton, 1983). Clinically, patients with this disease develop a spectrum of symptoms which may include lung disease, pancreatic dysfunction, intestinal obstruction and infertility. At the present time, therapy for this disease remains symptomatic, and for those who develop end-stage lung disease, lung transplantation is a possible intervention. Recent research has focussed on the possibility of trying to bypass the chloride conductance defect that exists in cystic fibrosis, and the other chloride

channels that may possibly be used as therapeutic targets. For this reason, the ClC-2 chloride channel is of interest and an understanding of its regulation is important especially if this channel is in the future a therapeutic tool. Indeed, ClC-2 has been found to be present in the intestine of CFTR knockout mice (Joo *et al*, 1999).

The results presented in this thesis provide insight into the regulation of ClC-2 by identifying interacting proteins. The regulation of this channel is likely complex, and other mechanisms of regulation have already been established, including the protein kinase C pathway (Staley *et al*, 1996). The activation of ClC-2 by acidic extracellular pH, hypotonic stress and hyperpolarization is also well established, however the mechanism by which these factors lead to channel activation remained unclear. The work presented provides evidence that the chloride channel ClC-2 interacts with the cytoskeleton, and provides novel insight into the regulation of its activity.

The actin cytoskeleton is a complex network of actin filaments complexed with numerous binding proteins. This network which is important for the maintenance of cellular architecture is also implicated in the regulation of a number of membrane proteins including ion channels. It has been linked directly or indirectly to such proteins as the cystic fibrosis transmembrane conductance regulator (CFTR), epithelial sodium channel and the Na/H exchanger (Prat *et al*, 1995, Cantiello *et al*, 1991, Henson, 1999). The cytoskeleton is thought to play a role in volume regulation by sensing volume changes and relaying this to membrane proteins, however, the details of this relationship remain unclear (Cantiello *et al*, 1997).

We have shown that ClC-2 interacts with actin and that this interaction is functionally significant as demonstrated by the increase in ClC-2 mediated current with

disruption of the actin cytoskeleton. The interaction with actin appears to be direct in the *in vitro* studies performed, however, it remains to be determined if this is the same *in situ*. The salt dependence of the interaction between ClC-2 and actin suggest that electrostatic forces play a role in mediating this relationship, however, it is likely that this is a complex relationship in which many other pathways may be involved.

It is well established that ClC-2 is activated by hypotonic shock (Schmidt-Rose and Jentsch, 1994, Jordt and Jentsch, 1997), and given the results presented, it is possible that this activation may be mediated by changes in the cytoskeleton. Cellular changes in actin are known to occur with cell swelling and shrinkage (Foskett and Spring, 1985, Linshaw *et al*, 1991, Cornet *et al*, 1988). In most cells, cell swelling results in the disruption of actin bundles (reviewed by Henson, 1999), and this effect may in turn result in ClC-2 activation. Previous studies have shown that ClC-2 plays a role in regulatory volume decrease in Sf9 cells (Xiong *et al*, 1999). In response to hypotonicity, these cells, when transfected with ClC-2, swell and then return to their previous volume, however, the mechanism of the ClC-2 activation had not been determined. The results of the current study suggest that this is mediated by changes in the actin cytoskeleton that occur with cellular volume changes. This is consistent with the effect of actin disruption on other membrane transporters that have been implicated in volume regulation. In renal cells, the swelling activated chloride conductance is activated by disruption of the actin cytoskeleton, and this has been confirmed in other cell types (Henson, 1999). Furthermore, the Na/K/2Cl cotransporter, which plays a role in regulatory volume increase, has also been functionally linked to the cytoskeleton. Therefore, the biochemical and functional interaction between the actin cytoskeleton and ClC-2 is

consistent with that of other proteins involved in volume regulation. However, the final pathway by which cellular volume changes lead to channel activation is not clear. The Rho family of GTPases, the phosphoinositide kinase pathway and the phospholipase C pathway have all been implicated in actin reorganization (Schmidt and Hall, 1998). The Rho family of GTPases have been shown to play a role in the formation of actin stress fibers in response to specific stimuli, as well as modulate actin's role in the formation of adherens junctions, axonal growth and endocytosis (Schmidt and Hall, 1998, van Aelst and D'Souza-Schorey, 1997). The phosphoinositide kinase and phospholipase C pathways are also important regulators of the actin cytoskeleton (Toker and Cantley, 1997). Indeed Rho GTPases have been shown to interact with phosphoinositide kinases (Ren *et al*, 1996, Tolias *et al*, 1985, Schmidt and Hall, 1998). Phospholipase C has been implicated in the regulation of cytoskeletal rearrangement in phagocytosis and cell migration (Toker and Cantley, 1997). The role of these pathways needs to be explored to determine the sequence of events that lead to the coupling of volume change to actin rearrangement and finally to ClC-2 channel activation.

Furthermore, as ClC-2 has been localized to the tight junction in intestinal cells, it is likely that cytoskeletal proteins are involved in either trafficking or retaining it at this location. A number of proteins come together to form this cellular barrier, including ZO-1, occludin, and claudins (Mitic and Anderson, 1998). The tight junction exists in close proximity to the actin cytoskeleton, and it has been proposed that actin plays an important role in regulating paracellular permeability (Anderson and Van Itallie, 1995). Actin filaments have been shown to enter the tight junction and terminate at the cell surface, however the exact relationship between actin and the tight junction remains under

investigation (Hirokawa and Tilney, 1982). It is likely that actin plays an important role in the regulation of ClC-2 at this location, and it may also be hypothesized that the ClC-2-actin interaction may be a pathway for modulating paracellular permeability.

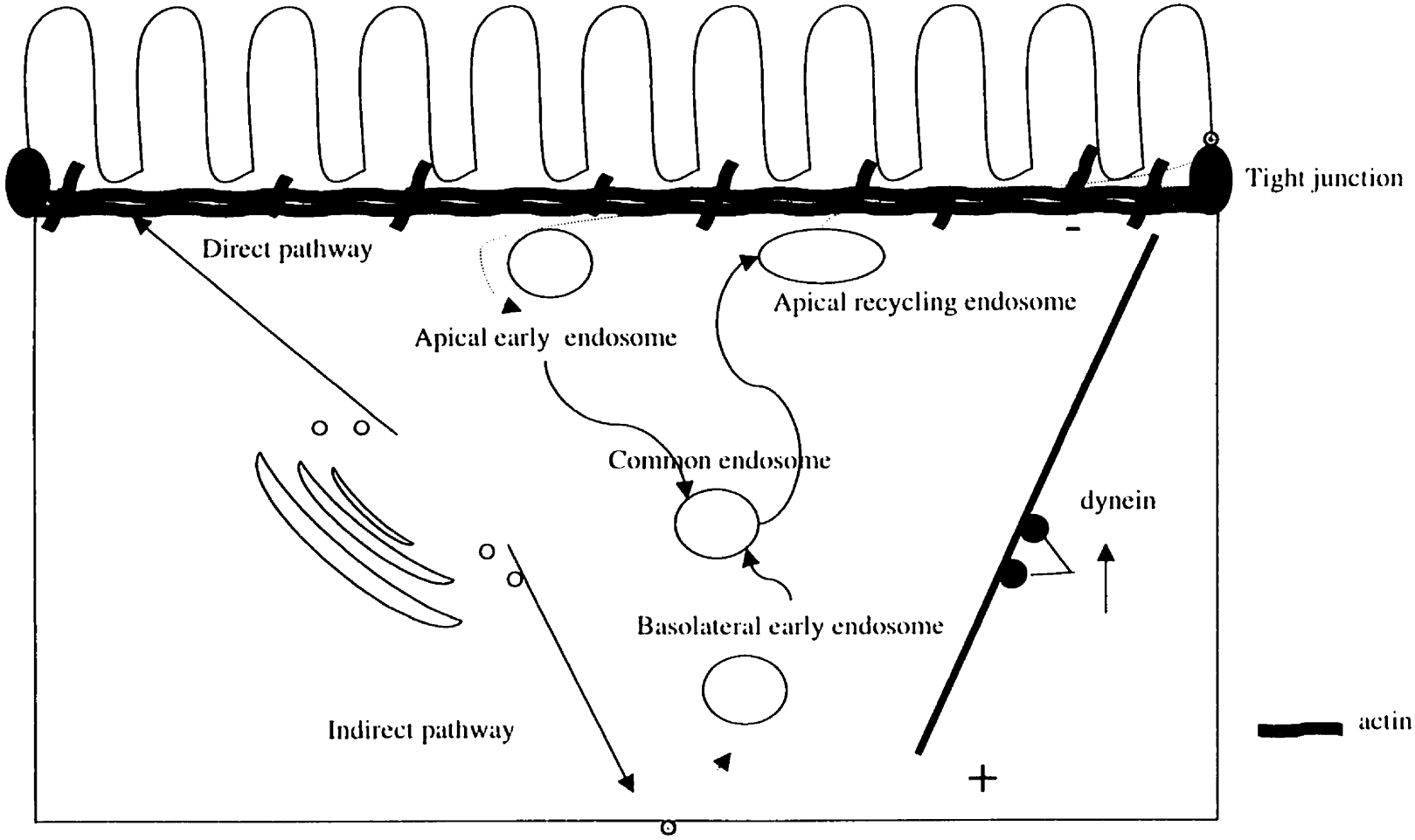
It has been shown that ClC-2 exists as an oligomer and that the channel exhibits double-barreled channel activity (Ramjeesingh *et al*, 2000). In a double-barreled channel, there are individual gates for the individual pores and a common gate, termed the "slow" gate. The actin cytoskeleton may be important in the regulation of the slow gate of ClC-2, as the addition of actin to purified ClC-2 in single channel studies, results in a partial restoration of the channel's slow gate (unpublished findings, Li, C and Bear CE).

We have shown that there is a biochemical interaction between ClC-2 and actin and that this interaction is important for channel regulation. The results presented in this work also show that ClC-2 interacts with the molecular motor dynein, and that this interaction is functionally important. Inhibition of dynein by the compound EHNA resulted in activation of ClC-2 mediated currents in *Xenopus* oocytes. In epithelial cells, dynein moves its cargo towards the apical surface, however, the distribution of microtubules in the oocyte is much more complex and differs with the maturity of the oocyte. In the mature, stage VI oocyte used for voltage-clamp studies, most microtubules are oriented with their minus ends towards the cortex, however, some microtubules have the opposite orientation (Gard *et al*, 1995, Pfeiffer and Gard, 1999). Since dynein moves along microtubules towards their minus end, in the oocyte, this involves movement mainly towards the cortex. Consequently, disruption of dynein activity would be expected to result in decreased movement of ClC-2 towards the membrane and a

subsequent decrease in current. However, this is contrary to what has been observed in the current studies. The reasons for this remain to be determined, however several possibilities exist. Firstly, dynein may, like kinesin, be involved in movement in both directions along microtubules, and may therefore be involved in recycling ClC-2 from the membrane. Another possibility is that the complex distribution of microtubules in oocytes may make it difficult to determine the direction of movement in these cells. In many cell types, in which the orientation of microtubules is such that dynein moves cargo towards the interior of the cell, this motor has been implicated in the movement of endocytic vesicles from early to late endosomes (Bomsel *et al*, 1990, Aniento *et al*, 1993). If a large population of microtubules are oriented with their minus ends towards the interior of the cell, inhibition of dynein would impair trafficking away from the plasma membrane and would result in an increase in ClC-2 current.

During biosynthesis, plasma membrane proteins such as ClC-2 are normally transported from the endoplasmic reticulum to the Golgi complex and are packaged into vesicles for targeting to the cell surface. This process is known to involve molecular motors to enable specific targeting of proteins to the membrane surface. There are two proposed pathways of membrane traffic in polarized epithelial cells (Figure 5.1). One pathway involves direct trafficking of proteins to the apical or basolateral surface. In the second "indirect" pathway, proteins are sent to one surface first, usually the basolateral surface, and are then endocytosed and targeted to the appropriate cell surface. The pathway used depends on the cell type with intestinal cells using the "indirect" route more frequently and MDCK cells using the direct pathway (Mostov *et al*, 2000). The mode of sorting has important implications for the role of dynein in protein trafficking.

Figure 5.1 Potential pathways for ClC-2 trafficking



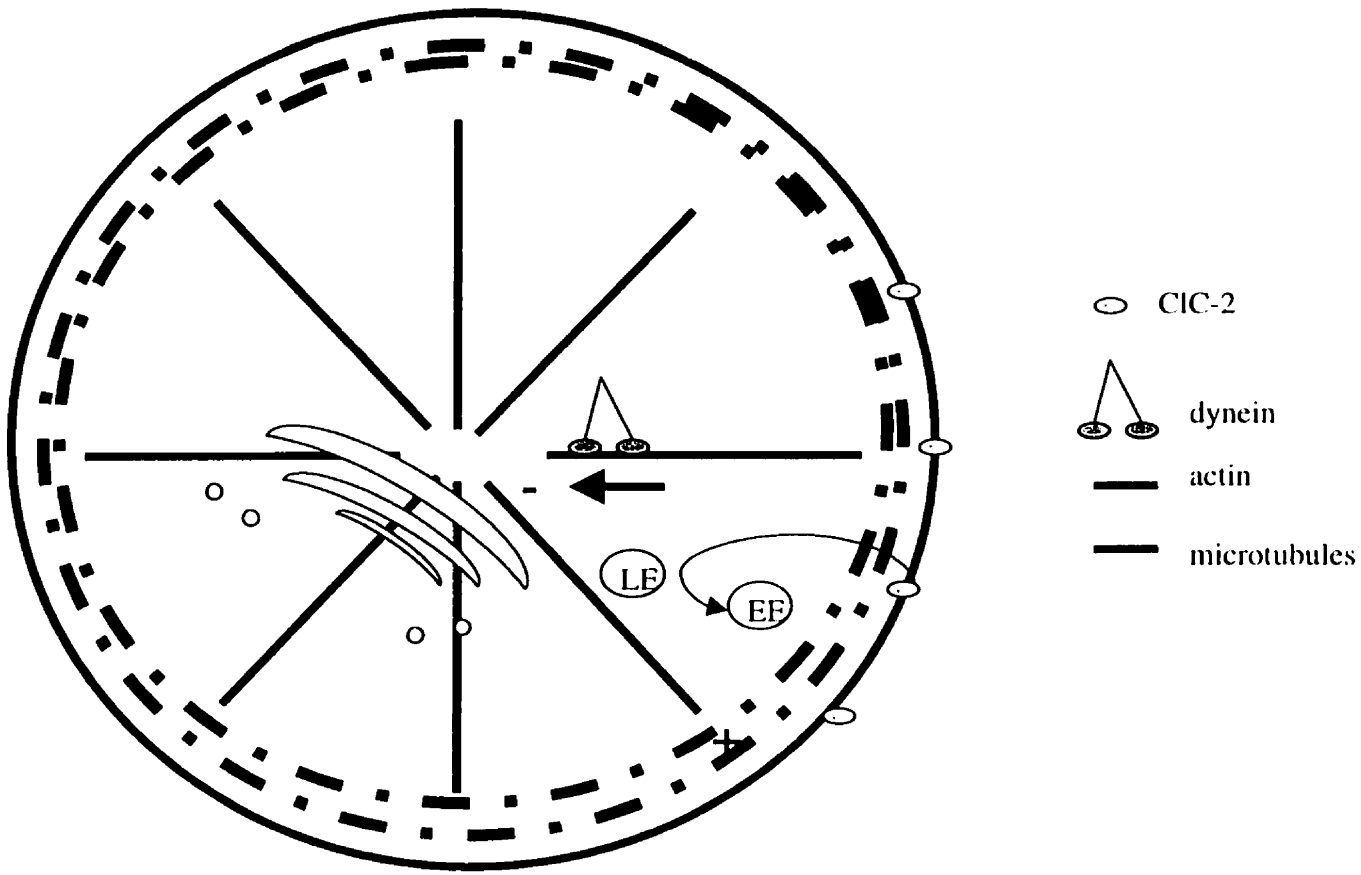
ClC-2 (green) can be targeted from the Golgi apparatus to A) the apical membrane directly or B) to the apical membrane via initial targeting to the basolateral membrane followed by sorting. ClC-2 may also be trafficked between various endosomal compartments.

For example, for ClC-2, if a polarized epithelial cell uses the indirect method, dynein may be important in the movement of the channel away from the basolateral plasma membrane to the endosomal compartment for targeting to the apical surface. If this is true, inhibition of dynein activity would result in impaired re-distribution of ClC-2 from the basolateral surface to the apical surface. In contrast, if the direct route is used, dynein would likely be involved in the movement between the golgi network and the plasma membrane. If dynein activity were to be inhibited, there would be a decrease in ClC-2 delivery to the apical surface. Since the trafficking of many membrane proteins, including ClC-2, is poorly understood, it is difficult to make any definitive conclusions about the movement of this channel by dynein.

It is also possible that dynein plays a role in ClC-2 regulation via the endocytic pathway. Following endocytosis, proteins are sorted into compartments destined for recycling to the plasma membrane or degradation. Both the microtubule network and actin cytoskeleton have been implicated in the movement of endocytic vesicles (Bomsel *et al*, 1990, Lamaze *et al*, 1997). ClC-2 may recycle between the membrane and an endosomal compartment as this has already been observed for ClC-5 in renal cells, where the channel has been localized to apical early endosomes (Gunther *et al*, 1998). Patch-clamp studies in Caco-2 cells, have confirmed that an increase in ClC-2 current occurs with dynein inhibition by EHNA (unpublished findings, Mohammad-Panah, R and Bear, CE). Biochemical studies performed to further investigate the interaction between ClC-2 and dynein, suggest that these proteins share an overlapping distribution. In initial cellular fractionation studies using iodixanol gradients, ClC-2 was localized to the same compartments as E-cadherin, a marker of the plasma membrane, confirming that proper

fractionation was occurring. Furthermore, preliminary results suggest ClC-2 could also be found in cellular fractions containing the early endosomal marker rab5. While this suggests that ClC-2 may be found in the early endosomal compartment, further study is required to confirm this finding. However, these preliminary results suggest that ClC-2 can be found both at the plasma membrane and in the endosomal compartment, and that it may be trafficked between these compartments as a mode of regulation. If dynein is involved in moving ClC-2 from the plasma membrane to the endosomal compartment, this would explain the functional studies which showed that inhibition of dynein activity increases ClC-2 channel activity. Furthermore, when dynein is inhibited, initial studies suggest that there is a re-distribution of ClC-2 to compartments from those identified by rab 5 to the plasma membrane compartment labelled by the marker E-cadherin. These results suggest that the inhibition of dynein results in an increase in ClC-2 at the membrane. It has been previously established that in intestinal epithelia and in differentiated cells of the Caco-2 cell line, that microtubules are oriented with their minus end towards the apical surface, (Gilbert *et al*, 1991, Meads and Schroer, 1995), however, in undifferentiated cells, microtubules are oriented radially with their (+) ends towards the periphery. The Caco-2 cells studied by patch clamp analysis were single cells (unpublished findings, Mohammad-Panah, R and Bear, CE), and consequently would have this radial microtubular orientation. Consequently, if dynein activity is inhibited, it would be expected that there would be a decrease in the movement of vesicles away from the plasma membrane (Figure 5.2). Further studies are required to determine exactly how dynein plays a role in ClC-2 trafficking. However, it is clear that this motor protein is

Figure 5.2 Proposed mechanism for ClC-2 regulation by dynein in single cells



important in ClC-2 regulation as its inhibition affects ClC-2 function and distribution in cellular compartments.

In vitro studies indicate that ClC-2 and dynein are interacting directly or indirectly. Cosedimentation studies confirmed that ClC-2 and dynein are interacting, as evidenced by the presence of detectable ClC-2 in the pellet in the presence of dynein and microtubules. Furthermore, in the presence of ATP, which causes the dissociation of dynein from microtubules, ClC-2 was not detectable in the pellet. This control indicates that ClC-2 is not simply binding to the microtubules, or pelleting non-specifically.

The role of dynein in ClC-2 channel regulation is likely complex. The movement of ClC-2 between cellular compartments may be important for alteration of channel activity either directly or indirectly. As mentioned previously, regulation of channel activity at the level of vesicle trafficking has already been proposed for other transporters such as aquaporin-2 and H/K-ATPase (Brown and Sabolic, 1993, Courtois-Coutry *et al.* 1996). Moreover, in the case of ClC-2, with its proposed localization to the tight junction of epithelial cells, it is likely that trafficking plays an important role in targeting the protein to this location.

It has already been established that ClC-2 is regulated by phosphorylation by PKC and PKA. Dynein is also regulated by phosphorylation, likely via the phosphorylation of dynactin. Thus, regulation of ClC-2 activity via PKC may be mediated in part by dynein. The interplay between phosphorylation and vesicle trafficking in the regulation of ClC-2 will need to be evaluated.

The results presented in this thesis show that ClC-2 interacts with both dynein and actin and that these interactions are important in the regulation of channel function. The

regulation of channel activity by these two distinct proteins may be completely separate modes of regulation or they may interact in a complex model of channel regulation. If the first model is considered, with two distinct modes of regulation, it may be that for quick, short-term channel regulation, actin may be involved in altering ClC-2 activity, such as that observed in response to hypotonic shock. Dynein may play a more important role in determining the density of ClC-2 at the membrane and recycling this pool between the plasma membrane and endosomal compartments for more long-term regulation. If the second model is considered, with the two proteins interacting in a more complex manner of regulation, the actin and microtubular cytoskeleton may function together to regulate ClC-2 channel activity. Actin exists in two distinct pools within the cell, a cortical web at the cell surface as well as microfilaments within the cell. These cellular microfilaments also serve as "tracks" for movement of cellular cargo by the motor protein myosin. It has been suggested previously that microtubular transport by dynein and kinesin is important for long-range vesicle movement while movement along actin filaments by myosin motors is important for short-range transport (Atkinson *et al.* 1992). It has been previously shown that the actin and microtubule cytoskeletons interact, and this may be important for ClC-2 channel regulation. Movement along microtubules is important for movement along longer distances, however movement also occurs along actin filaments, and this is thought to be important for shorter, faster transport. It has also been proposed that dynein moves proteins destined for the apical membrane to the apical cytoplasm where they are then moved via myosin-I to the apical membrane (Fath *et al.* 1994). Thus, the two different motor systems may interact to finely regulate ClC-2 activity.

The physiologic role of ClC-2 is yet to be determined. It has been shown to mediate intestinal chloride secretion (Gyomorey *et al*, 2000, Mohammad-Panah *et al*, 2000), and has also been implicated in the maintenance of intracellular chloride in neurons (Clayton *et al*, 1998, Smith *et al*, 1995), and gastric secretion (Murray *et al*, 1996). While the channel is known to be activated by hyperpolarization, low extracellular pH and cell swelling in *Xenopus* oocytes, the physiologic stimuli for channel activation are still under investigation.

It has been postulated that ClC-2 may be able to compensate for CFTR given its presence at the apical surface of lung and intestinal epithelial cells. CFTR knock-out mice exhibit a variable phenotype of intestinal disease (Rozmahel *et al*, 1996, Snouwaert *et al*, 1995), and can be classified into three groups based on this clinical manifestation. Studies of these mice have not indicated that ClC-2 is a candidate for a modifier gene with respect to the intestinal phenotype. However, it has been shown that ClC-2 can contribute to intestinal chloride secretion (Gyomorey *et al*, 2000, Mohammad-Panah *et al*, 2000), and while ClC-2 may not be a modifier gene, this does not exclude it from being used as a therapeutic target to improve chloride transport across the intestinal and respiratory epithelia. To begin to test the usefulness of ClC-2 in improving chloride transport, it is critical to understand its regulation. The work presented provides evidence that ClC-2 is regulated by the cytoskeleton.

CHAPTER 6

FURTHER DIRECTIONS

6.1. ClC-2 interactions with actin

The studies discussed in this thesis indicate that ClC-2 interacts with actin *in vitro*, however, the nature of this interaction *in vivo* remains to be determined. The *in vitro* studies also suggest that the N-terminus is important in mediating the interaction which involves electrostatic forces. Further study is required to determine:

1) the interaction between actin and ClC-2 *in situ*
2) the role of other actin binding proteins in this interaction
3) the role of actin in the "ball-and chain" model of ClC-2 channel regulation
4) the effect of tonicity on this interaction

To address the first issue, studies need to be performed in cultured cells or in tissue to characterize the interaction between ClC-2 and actin *in vivo*. Several approaches could be used to study this relationship. Firstly, to confirm the functional effects of actin disruption on ClC-2 activity, patch clamp studies could be performed on whole cells, such as Caco-2 cells which endogenously express ClC-2, in the presence and absence of cytochalasin D. This cell line would be useful, as immunofluorescence studies have established the presence of ClC-2 and its distribution in these cells. These cells could also potentially be used to confirm the biochemical interaction by immunoprecipitation studies. However, the latter experiment would require an antibody to ClC-2 that was able

to specifically immunoprecipitate the protein. In order to confirm the interaction, it would be necessary to see that actin could be co-immunoprecipitated with ClC-2 in the presence of a specific ClC-2 antibody but not in the presence of a control antibody. However, because of the tendency of actin to bind to many proteins, this experiment may be technically difficult. Furthermore, the electrostatic nature of the interaction may make it difficult to maintain throughout the various steps of the experiment, such as washing. If this is successful however, the conditions used for immunoprecipitation could be modified to evaluate the electrostatic nature of this interaction. For example, the salt concentration could be manipulated to determine if ClC-2 and actin co-immunoprecipitate at varying salt concentrations.

The interaction between actin and ClC-2 could also be evaluated through the use of ClC-2 mutants that involve the "essential" region that is critical for normal gating. Mutant versions of ClC-2 could be transfected into CHO cells or HEK293 cells to evaluate the ability of these mutants to interact with actin. If the interaction with actin was impaired, it would provide insight into the region of the protein required for this interaction.

To determine if the interaction between ClC-2 and actin is direct or indirect, it is necessary to evaluate the role of other actin-binding proteins. This could be done using some of the techniques employed in the presented work. A purified ClC-2 protein column could be used to look for other proteins which bind when a tissue or cell culture lysate is passed through the column. Proteins which would be of interest include the ezrin/moesin/radixin family as well as other actin binding proteins such as cortactin or actinin. The interaction with other proteins could also be done using the yeast two hybrid

assay, using the N-terminus of ClC-2 as bait to screen a brain cDNA library, since ClC-2 is expressed at a high level in this tissue. One of the drawbacks of this technique is the possibility of both false positive and false negative results. In order to reduce the false positive rate, any interactions found by this technique would need to be confirmed by other biochemical studies such as immunoprecipitation. However, this technique does offer the power to screen a wide range of cellular components to find interacting proteins (Vidal *et al*, 1999).

The interaction between ClC-2 and actin may not be mediated by actin proteins but may involve other pathways such as PKC. It has already been shown that activation of PKC results in an inhibition of ClC-2 activity, however, it is possible that this may also be mediated through the actin cytoskeleton. It would be of interest to determine the effect of phorbol esters, known activators of PKC, on the interaction between actin and ClC-2. This could be done using immunofluorescence studies to directly visualize changes in the actin cytoskeleton in response to these compounds.

Finally, the role of actin in the ball and chain model of ClC-2 channel regulation remains to be determined. The proposed model, which is the result of elegant mutagenesis studies, indicates that the amino terminal plays a critical role in channel regulation, whereby deletion of large regions the N-terminus results in constitutive channel activity (Grunder *et al*, 1992). It would be important to determine if the interaction with actin occurs using constructs containing this region or with the region deleted. If the interaction occurs with peptides containing the critical region, this would suggest that actin is interacting with the N-terminus in this model, and may be responsible for tethering the "ball" to keep the channel in the closed configuration.

6.2 *ClC-2 interaction with dynein*

The interaction between dynein and ClC-2 is of interest, as the interaction between dynein and its cargoes is a novel area of study. The results presented in this thesis serve to identify the presence of an interaction between dynein and ClC-2 however much remains to be determined. Specifically, areas which need to be addressed include:

1) the role of dynein in the trafficking of ClC-2 between cellular compartments
2) the region of ClC-2 involved in binding to dynein
3) the effect of dynein inhibition on ClC-2 membrane localization
4) the effect of dynein inhibition on channel activity

The trafficking of ClC-2 between cellular compartments is a novel area of study. ClC-2 has been localized to the plasma membrane in many cell types, and specifically to the apical surface of epithelial cells. However, the presence of ClC-2 in other cellular compartments has not been evaluated. Recently, ClC-5 was localized to the early endosomal compartment in kidney cells. It is thought that this protein may play a role in the acidification of endosomes (Gunther *et al*, 1998). The work presented in this thesis indicates that ClC-2 shares an overlapping distribution with the early endosomal protein rab5. However, further study is needed to determine if ClC-2 is present in endosomes. Techniques which would be useful to evaluate this would be cellular fractionation methods. The iodixanol gradient method could be used to fractionate the endosomal

compartment and then western blotting could be employed to look for the presence of ClC-2 (Nycomed Technical Bulletin). Other confirmatory studies which would be useful include immunofluorescence studies to look for an overlapping cellular distribution between endosomal markers such as rab5 and ClC-2. Similar techniques could be employed to look for the presence of ClC-2 in other cellular compartment such as recycling endosomes by evaluating colocalization with corresponding protein markers such as rab11. These studies would provide insight into the trafficking of ClC-2 which may be an important mode of regulation of channel activity. Immunofluorescence studies would be a useful method to evaluate the effect of the disruption of endosome recycling on ClC-2 localization. These studies could be done directly on cells expressing ClC-2, such as Caco-2 cells or neuronal cells. However, it may also be useful to use cells transfected with GFP-labelled ClC-2.

The interaction between dynein and its cargo is not well understood. It would be important to attempt to identify the region of ClC-2 involved in binding to dynein. This could be done using pulldown studies with ClC-2 mutants to determine which region is important for binding dynein. This could be done using either purified dynein or a cellular lysate rich in dynein such as brain cytosol. Dynein is a complex macromolecule made up of a number of subunits, including heavy chains, light chains and intermediate chains as well as a complex of other proteins (discussed previously). The heavy chains are thought to be important in binding to microtubules, and both the light chains and intermediate chains have been implicated in the binding of dynein to its cargo. It would be important to evaluate how ClC-2 binds to dynein and to attempt to identify which chain is important for this interaction. This could be done by creating fusion proteins for

the dynein intermediate chains or light chain such as tctex-1 to determine if these proteins are able to bind to ClC-2.

Furthermore, it would be important to determine if the dynactin subunit plays a role in the interaction between dynein and ClC-2. While some studies suggest that this is a crucial entity, more recently it has been shown that dynein can bind to the photoreceptor protein, rhodopsin, in the absence of dynactin (Tai *et al*, 1999). Studies which would be useful to determine the role of dynactin include cosedimentation experiments done in the presence or absence of blocking antibodies to the dynein intermediate chain IC74. These antibodies interfere with the ability of dynein to bind dynactin and therefore would prevent binding of dynein to ClC-2 if dynactin is a necessary cofactor (Steffen *et al*, 1997).

An elegant study to confirm that dynein is indeed able to move ClC-2 along microtubules would be an *in vitro* motility study. It is possible to use microscopy to directly visualize the movement of fluorescently labelled ClC-2 containing vesicles along fluorescent taxol stabilized microtubules (commercially available), in the presence of dynein. This experiment would provide direct evidence that dynein is able to translocate ClC-2 *in vitro*.

ClC-2 trafficking could also be studied through the use of GFP-tagged constructs. By using cells transfected with GFP-ClC-2, it would be possible to directly visualize the location of the protein, and the effect of inhibition of dynein on localization. Furthermore, it would be possible to confirm the presence of ClC-2 in the endosomal compartments by doing colocalization studies with endosomal markers such as rab5.

Finally, it would be important to evaluate the role of dynein with respect to the activation of ClC-2 by hypotonic shock. Using the *Xenopus* oocyte system or patch-clamp methods on Caco-2 cells, it would be possible to evaluate if ClC-2 could be further activated in these cells by hypotonicity, following treatment with the dynein inhibitor. If ClC-2 could not be further activated, this may suggest a role for dynein in the activation of ClC-2 by cell swelling.

It would also be important to confirm the effect of dynein inhibition on ClC-2 function by other methods, given the limitation of pharmacologic agents. EHNA has been shown to inhibit dynein, but may also have other non-specific effects that are not yet understood, and consequently it would be important to confirm these results. One strategy might be to study dynein knock-outs, however, mice which are homozygous mutants for one of the dynein heavy chains do not survive in utero (Harada *et al*, 1998). However, dynein function can also be disrupted by overexpression of the dynactin subunit, dynamitin. Overexpression of this subunit results in disruption of dynein-dependent organelle distribution (Burkhardt *et al*, 1997). Thus, it may be possible to study ClC-2 in cells overexpressing this subunit. Another possible strategy is to use inhibit dynein function by expressing a dominant negative subunit of dynactin (Gaglio *et al*, 1996). Dynein function can also be inhibited by using blocking antibodies to the intermediate chain (Sigma).

Further study of the interaction between dynein and ClC-2 will provide insight into the role of this protein in channel regulation and the trafficking of membrane proteins.

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