Molecular Mechanisms Underlying the Regulation of Na^+/H^+ Exchanger Isoform 1 by Calcineurin B Homologous Protein 3

by

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Abstract

Restoration of cardiac intracellular pH (pH_i) following acidification is of crucial importance for maintenance of myocardial contractility. The major mechanism responsible for this is the function of the sodium/hydrogen exchanger isoform 1 (NHE1) which is the primary isoform of mammalian myocardium. However, elevated activity and expression of NHE1 is also detrimental in numerous diseases of the heart and studies have documented its role in cardiac hypertrophy and heart failure as well as exacerbation of myocardial injury during periods of ischemia/reperfusion. Furthermore, a vast amount of research over the past decade has demonstrated that inhibition of NHE1 by pharmacological antagonists attenuates both ischemic/reperfusion damage as well as hypertrophy. Regulation of the exchanger occurs primarily through its interaction with numerous proteins and biomolecules. One such novel partner is the Ca^{2+}-binding protein CHP3/tescalcin, a member of the calcineurin B homologous protein family, which, unlike isoforms CHP1 and 2, is predominantly restricted to the heart in adult human tissue.

By utilizing both *in vitro* and *in vivo* binding assays as well as confocal fluorescent microscopy in concert with mutational analysis of the regulatory C-terminal motif of NHE1, we determined that CHP3 binds the NHE1 at the juxtamembrane region of the exchanger that is identical to the region that interacts with the other CHP isoforms. Furthermore, functional analysis of the exchanger expressed in NHE-deficient Chinese hamster ovary AP-1 cells, determined that CHP3 upregulates NHE1 by accelerating both biosynthetic maturation and cell surface stability of the exchanger.

The CHP proteins have been shown to be N-myristoylated, and belong to the EF-hand superfamily of Ca^{2+}-binding proteins. By mutating the N-myristoylation domain as well as the sole functioning EF-hand motif, both the upregulation of NHE1 activity was ablated along with the cell surface stability of the exchanger. We determined that although neither site is required for the interaction with NHE1 or for promoting the maturation of the exchanger, both are necessary to stabilize NHE1 at the cell surface, thereby optimizing its

plasmalemmal expression and activity. Furthermore, our results suggest that CHP3 is a member of the Ca^{2+}-myristoyl switch protein family since mutation of either motif by itself resulted in identical regulation of the exchanger, but mutation of both sites in concert does not compound the decrease in exchanger activity or stability.

NHE1 maintains a distinct distribution within the myocardium where it is localized predominantly to the intercalated disks and transverse t-tubules, but not the sarcolemmal membrane. However, upon low flow ischemia/reperfusion or depletion of cellular ATP, NHE1 rapidly redistributes to the lateral sarcolemmal membranes. Furthermore ATP-depletion in AP-1 cells expressing NHE1 results in a decrease in Na^+/H^+ exchange activity which correlates partially to a dephosphorylation and depletion of the plasma membrane phosphoinositide, phosphatidylinositol-4,5-bisphosphate. Our results demonstrate ATP-depletion also causes in a rapid decrease in NHE1 at the cell surface of exchanger expressing AP-1 cells that correlates with the rapid inhibition of exchange activity. Furthermore, CHP3 over-expression is unable to stabilize the exchanger at the cell surface during episodes of ATP-depletion.

Résumé

La récupération du pH intracellulaire (pHi) des cellules cardiaques suivant une acidification est d'importance cruciale pour le maintien de la contractilité myocardique. L'isoforme de l'échangeur sodium/proton (NHE1) qui est l'isoforme principal dans le myocarde des mammifères, en est le principal acteur. Cependant, une activité et une expression élevées de NHE1 est nuisible dans plusieurs pathologies cardiaques. En effet, de nombreuses études ont démontré son rôle au niveau de l'hypertrophie et de la défaillance cardiaques de même que dans l'aggravation des dommages myocardiques durant les périodes d'ischémie/reperfusion. En outre, un grand nombre de recherches effectuées durant la dernière décennie ont montré que l'inhibition de NHE1 par des agents pharmacologiques atténue les dommages causés par l'ischémie/reperfusion de même que via l'hypertrophie. La régulation de l'échangeur s'effectue principalement via son interaction avec de nombreuses protéines et biomolécules. La protéine liant le Ca^{2+} soit CHP3/Tescalcine, un membre de la famille des protéines homologues à la calcineurin B chez l'humain adulte est une de ces nouvelles molécules. Contrairement aux isoformes CHP1 et 2, l'espression CHP3/Tescalcine est principalement limitée au coeur.

Via des essais *in vitro* et *in vivo* d'ancrage, de même qu'à l'aide de la microscopie confocale à fluorescence, de concert avec des analyses par mutations de la partie régulatrice C-terminale de NHE1, nous avons déterminé que CHP3 se lie à la région juxtamembranaire de l'échangeur (NHE1) qui est identique à celle qui interagit avec les autres isoformes CHP. De plus, des analyses fonctionnelles de l'échangeur exprimé dans les cellules AP-1 d'ovaires de hamster chinois déficientes en NHE, ont déterminé que CHP3 module positivement NHE1 en accélérant à la fois la synthèse et la maturation de la protéine, et en stabilisant l'échangeur à la membrane plasmique.

Les protéines CHP sont directement modifiées par myristoylation N-terminale. Elles appartiennent à la superfamille *EF-hand* des protéines liant le Ca^{2+}. En modifiant le domaine N-myristoylation ainsi que l'unique mode de fonctionnement du motif *EF-hand*,les effets de CHP3 sur l'augmentation de l'activité de NHE1 de même que sur la stabilité accrue de l'échangeur à la surface de la cellule, sont diminués. Nous avons déterminé que bien qu' aucun des sites n'est requis pour l'interaction avec NHE1 ou pour la promotion de la maturation de l'échangeur, les deux sont nécessaires pour la stabilisation de NHE1 à la surface de la cellule, ce qui de cette façon optimise son expression plasmalemmale ainsi que son activité. Par ailleurs, nos résultats suggèrent que CHP3 est un membre de la famille des protéines Ca^{2+} -myristoyl interruptrices puisque la mutation d'un motif ou l'autre résulte en une régulation identique i.e. une diminution de l'activité de l'échangeur mais que la mutation des deux sites de concert n'atténue pas davantage la stabilité de l'échangeur à la membrane.

NHE1 est distinctement distribué dans le myocarde où il est principalement localisé aux disques intercalaires ainsi qu'aux tubules-t transversaux mais pas à la membrane sarcolemmale. Toutefois, lors d'un lent débit de reperfusion post-ischémie ou d'un épuisement de l'ATP cellulaire, NHE1 se redistribue rapidement aux membranes sarcolemmales latérales. De plus, l'épuisement d'ATP dans les cellules AP-1 exprimant NHE1 résulte en une diminution de l'activité d'échange Na^+/H^+ , ce qui est partiellement associé à une déphosphorylation et à une réduction des phosphoinositides et phosphatidylinositol-4,5- bisphosphates de la membrane plasmique. Nos résultats démontrent que la réduction en ATP conduit aussi à une diminution précipitée de NHE1 à la surface des cellules AP-1 exprimant l'échangeur, ce qui est en relation avec une inhibition rapide de l'activité de l'échangeur. De plus, une sur-expression de CHP3 est incapable de stabiliser l'échangeur à la surface cellulaire lors d'épisodes d'épuisement d'ATP.

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Preface[*]

As an alternative to the traditional thesis format, the thesis can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: 1) a table of contents; 2) a brief abstract in both English and French; 3) an introduction which clearly states the rationale and objectives of the research; 4) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); 5) a final conclusion and summary; 6) a thorough bibliography;

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis, the candidate must be the primary author (the author who has made the most substantial contribution) for all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

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* Quoted from the “Guidelines for Thesis Preparation”, Faculty of Graduate Studies and Research.

Contribution of the Authors

All the experiments in the three chapters of this thesis were designed and carried out by H.C. Zaun under the supervision of Dr. John Orlowski. In the preamble to Chapter 3, Langendorffer preparations and confocal microscopy images (*Figs. 1 and 2*) were performed by Pascal Lamare, a previous graduate student in our lab, and the GST-pull down assay of the juxtamembrane region of the plasmalemmal NHEs (NHE1 thru 5) with *in vitro* translated CHP1 and 3 (*Fig. 3*) was performed by H.C. Zaun. HL-1 mouse myocyte cells were kindly provided by Roxana Atanasiu of Dr. Shrier's Lab.

Statement of Contribution to Original Knowledge

CHAPTER 1:

- CHP3/Tescalcin interacts with the cytoplasmic tail of NHE1 at the same location as the other CHP isoforms (1 and 2)
- Mutating the four hydrophobic amino acids within the juxtamembrane region of the C-terminal tail of NHE1 that were shown by others to be crucial for direct binding to CHP1 and 2, completely abolishes binding to CHP3.
- CHP3 is not essential for the targeting of NHE1 to the plasma membrane, but in fact requires the interaction with NHE1 to target to the membrane.
- The nature of the mutation to NHE1 that result in abrogation of the binding of the CHP proteins, but not necessary the binding itself effects the kinetic properties of the transporters (*i.e.* V_{max} alone or V_{max} and H^+-affinity).
- CHP3 promotes the maturation and cell surface activity of NHE1, which correlates to an elevation in its transport activity without affecting the exchanger affinity for intracellular H^+.
- CHP3 enhances the stability of NHE1 and the plasma membrane.

CHAPTER 2:

- Neither N-myristoylation nor Ca^{2+}-binding of CHP3 is crucial for its interaction with NHE1.
- The interaction of NHE1 and CHP3 is influenced by the binding of Ca^{2+} to CHP3.
- N-myristoylation and Ca^{2+}-binding of CHP3 are required for optimal NHE1 activity through accumulation of the exchanger at the cell surface:
- N-myristoylation and Ca^{2+}-binding of CHP3 does not influence the cell surface maturation of NHE1.

- Both N-myristoylation and Ca^{2+}-binding of CHP3 are required for cell surface stability of NHE1.
- N-myristoylation and Ca^{2+}-binding of CHP3 appear to work synergistically as a Ca^{2+}-myristoyl switch protein, in relation to the effect on NHE1 maturation and stability.

CHAPTER 3

- NHE1 and CHP3 co-localize to the plasma membrane when over-expressed in the mouse atrial cardiomyocyte cell line HL-1, particularly in regions of cell-cell interaction.
- Metabolic depletion of ATP reduces the amount of cell surface NHE1 abundance without effecting total cell NHE protein level and targeting of both NHE1 and CHP3 to the plasma membrane is reduced after 15 min of ATP depletion.
- Cellular depletion of ATP does not affect the interaction between NHE1 and CHP3.
- Over-expression of CHP3 does not affect the sensitivity of NHE1 activity (V_{max} or H^+-affinity) to depletion of cellular ATP.

Abbreviations

AE	Anion exchanger
AP-1	"Antiporter-1" – Plasmalemmal Na^+/H^+ exchanger deficient Chinese hamster ovary cell line
BSA	Bovine serum albumin
CA	Carbonic anhydrase
CaM	Calmodulin
CHE	Cl^--organic anion exchanger
CHO	Chinese hamster ovary cells
CHP	Calcineurin B homologous protein
CMV	Cytomegalovirus
DMA	5-N,N-dimethyl-amiloride
EIPA	5-N-Ethyl-N-isopropyl-amiloride
ERK	Extracellular signal-regulated protein kinase
ERM	Ezrin/Radixin/Moesin family of cytoskeletal associated proteins
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HA	Hemaglutinin epitope of the influenza virus
HRP	Horseradish peroxidase
I/R	Ischemia/Reperfusion
IPTG	Isopropyl-1-thio-β-galactopyranoside
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate co-transporter
MEM	Minimum essential medium
MPA	N5-methyl-N5-propylamiloride
NBC	Na^+/HCO_3^- co-transporter
NCX	Na^+/Ca^{2+} exchanger
NDCBE	Na^+-dependent / Na^+-driven Cl^--HCO_3 exchanger
NHE	Na^+/H^+ exchanger

NIK	Nck-interacting kinase
$p90^{RSK}$	p90 ribosomal S6 kinase
p160ROCK	Rho-associated coiled coil containing kinase
PBS	Phosphate Buffered Saline
PFK	Phosphofructokinase
PiP_2	Phosphatidylinositol-4.5-bisphophase
PKC	Protein Kinase C
RHAG	Rhesus-associated glycoprotein
SDS-PAGE	Sodium dodecyl-sulphate – Polyacrylamide gel electrophoresis
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
TM	Membrane-spanning segment

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Introduction:

1.1.1 Intracellular pH (pH_i)

Assuming protons remain at equilibrium across a biological membrane with an extracellular pH of 7.4, an inside negative membrane potential of -60mV, and with H^+ ions passively distributed across the membrane (Putman, 2001; Boron, 2004), the Donan equilibrium will yield an intracellular pH (pH_i) of roughly 6.3-6.4. This value does not take into consideration acidic stress due to proton production by cellular metabolism (glycolysis) as well as other processes also contribute to acidification of the cell. These include passive flux of acid equivalents (H^+ in and OH^-/HCO_3^- out) across the plasma membrane, leakage of protons from acidic internal organelles, and the pumping of protons from organelles that require an alkaline internal environment, either by active cytosolic directed flux of protons or cytosolic extrusion of bases. All these factors submit the cell to a chronic and considerable acid load which can impair both cellular metabolism as well as many other cellular functions (Busa and Nuccitelli, 1984; Hochachka and Mommsen, 1983; Roos and Boron, 1981). In fact, it requires only a slight variance below 6.85 or above 7.65 to result in symptoms of disease, and below 6.8 or above 8.0 to result in death (Nowak and Handford, 1994). However, cells have developed the ability to vigorously regulate their internal pH in order to tightly control and maintain it between 6.8 and 7.2.

Intracellular pH (pH_i) homeostasis is of crucial importance for cell survival, and the modulation of internal cytosolic conditions is vital for stable protein activity and interactions as well as for the modulation of many biomolecules that are sensitive to subtle changes in pH (Orlowski and Grinstein, 2004). Indirectly, almost all cellular processes are in some way affected by changes in intracellular pH, including cellular metabolism, cell growth and proliferation (Putney and Barber, 2003; Kapus *et al,* 1994; Pouysségur *et al*, 1984), cell volume regulation (Smets *et al,* 2002), as well as muscle contraction, cell-cell coupling and membrane potential and conductance (Putman, 2001).

Proteins are particularly susceptible to changes in pH because the ionization state of proteins strongly influences their tertiary and quaternary structures, and any changes can easily disrupt a protein's function (Antosiewics *et al*, 1996). This in turn disrupts metabolic reactions which are dependent upon protein structure (Whitten *et al*, 2005) causing reactions to fall sharply when pH is outside the optimal range. For example, phosphofructokinase (PFK), a well-known glycolytic enzyme that catalyzes the irreversible transfer of a phosphate from ATP to fructose-6-phosphate, is inhibited by acidic pH, resulting in the abrogation of glycolysis (Erecinska *et al,* 1995).

Intracellular pH also strongly affects the cytoskeleton, and plays a major modulatory role in cytoskeletal alterations (Denker *et al,* 2000) since both actin filaments cross linking and tubulin polymerization are influenced by pH_i. Furthermore, actin binding proteins are influenced by pH and alkalinisation results in tubulin depolymerization and disaggregation of microtubules (Casey *et al*, 2010; Pope *et al,* 2004; Putman, 2001; Edmonds *et al,* 1995; Zimmerle and Frieden 1988). However, it is not clear whether this is an indirect effect of pH change, since changes in pH are often accompanied by changes in intracellular calcium. For example, elevated intracellular H^+ activates the mitochondrial Ca^{2+}/H^+ exchanger, resulting in elevated levels of Ca^{2+} within the cytoplasm (Baysal *et al,* 1991). Further physiological disruption may also occurs when pH changes affect the electrolyte balance, particularly Na^+, Cl^- and K^+ ions. (Putman, 2001).

Ion selective channels require optimal intra- and extra-cellular pH for ion conductance and channel gating, and deviations can alter the membrane potential and affect the excitability of nerve and muscle cells (Casey *et al*, 2010; Putman, 2001; Tombaugh and Somjen, 1997, 1996). Many intracellular messenger and signalling pathways are also affected by changes in pH_i including the pH sensitive enzymes, adenylate cyclase, which synthesizes cAMP, and cyclic nucleotide phosphodiesterase, which hydrolyzes cAMP; thus affecting pathways involving cAMP (Johnson, 1982). The calcium-binding protein calmodulin is also pH

dependent as is the binding of Ca^{2+}-calmodulin complexes to other proteins (Wakabayashi *et al*, 1997a) creating the potential for pH changes to affect Calcium signalling both indirectly and directly.

The role of intracellular pH in promoting cell proliferation has been an active area of research. However, pH alone does not promote cell growth and its signal for cell growth may depend on the cell type and stimulus (Putman, 2001). Additionally, increased cytosolic pH has been linked to the transformation of cells from many different tissues, making it a common factor and a distinct characteristic of cancers. Finally, acidic pH at 0.3-0.4 pH units below normal has been shown to trigger apoptosis (Srivastava *et al*, 2007).

1.1.2 Organellar pH.

Within the cells, there are many different organelles that must maintain an internal pH that may deviate from that of the cytoplasm by over 2 pH units. This difference in pH is essential for the proper operation of these compartments and is essential for cell survival, function, and proliferation (Casey *et al,* 2010). For example, acidification of early/sorting endosomes (~ pH 6.0-6.2) is crucial for ligand-receptor dissociation and recycling, and for the accumulation of macromolecules and in secretion from secretory granules (Orlowski and Grinstein, 2007). Lysosomes are involved in biochemical degradation and maintain a pH as low as 4.7, since the hydrolytic enzymes found within lysosomes have an optimal pH of 5.0 and are inactive at a neutral pH. By contrast, the mitochondrial matrix is considerably alkaline due to H^+ extrusion across the inner mitochondrial membrane by proteins involved in the electron transport chain (Orlowski and Grinstein, 2007; Abad *et al*, 2004; Llopis *et al,* 1998). Additionally, changes in mitochondrial pH have been linked to early events in mitochondrial-dependent apoptosis (Casey *et al*, 2010; Matsuyama and Reed, 2000), although the precise role of pH and the underlying mechanisms remain obscure.

1.2: Mechanisms of pH Regulation.

Regulation of intracellular pH (pH_i) is quite intricate, involving not only physiological buffers and acid-base transporters, but also an increasing number of signal transduction pathways and interacting ancillary proteins that modulate the pH-regulating transporters (Roos and Boron, 1981; Boron, 2004).

1.2.1: Physiochemical Buffering.

Buffering is based on the principle that protons can bind and unbind macromolecules such as proteins, and in doing so, these weak acids will remain in a reversible equilibrium that helps to minimize changes in the free proton concentration [H^+] within a solution or the cytoplasm. By constantly taking up or releasing H^+ ions when the concentration changes, these buffers can respond to shifts in pH in a manner of seconds (Putman, 2001).

The buffering power (ß) of a cell can be defined generally as the ability of the cell to resist changes in pH_i that result from acidic or alkaline loads. A more specific definition is the amount of strong base (e.g., NaOH) or strong acid (e.g. HCl) that would have to be added to 1 litre of solution to raise the pH one unit (Boron, 2004).

ß = dn / dpH, where n = the number of acid or base equivalents needed to alter pH

Buffers can be classified in two ways based on characteristics; a closed buffer, or intrinsic buffer, is one in which the buffer concentration remains constant, which includes the actions of weak acids and bases such as proteins. An open buffer, also termed extrinsic buffer, is one in which the uncharged form of the buffer remains constant. The most common example of an open buffer is the CO_2/HCO_3^--buffering system. As acid accumulates the protons combine with bicarbonate to form carbonic acid which is subsequently converted to carbon dioxide and water.

$$H^+ + HCO_3^- \leftrightarrow H_2CO_3 \leftrightarrow CO_2 + H_2O$$

Since carbon dioxide is both small and electroneutral, it can easily be removed through the cell membrane (Casey *et al*, 2010; Putman, 2001). However, whether removal of CO_2 is merely due to dissipation through the plasma membrane or additionally through aquaporin water channels or the Rhesus-associated glycoprotein (RHAG) of erythrocytes has not been fully clarified and remains controversial (Musa-Aziz *et al*, 2009; Missner *et al*, 2008). A closed buffer system is at maximal buffering capacity when the pH equals the pKa of the buffer in question, whereas the buffering power of an open buffer increases exponentially with pH, so the cells become increasingly resistant to pH_i variation as pH_i changes (Boron, 2004).

Three major biological buffering systems exist within the body; bicarbonate, phosphate and proteins (Madias and Cohen, 1982).

The **bicarbonate buffer system** plays an important role in pH_i regulation for several reasons. First of all, the extracellular milieu can act as an infinite supply of CO_2 (Boron, 2004) and both constituents (CO_2 and HCO_3^-) of this system are regulated. Where CO_2 is regulated by the respiratory system and can respond within several minutes, HCO_3^- is regulated by the kidneys and can respond within a matter of a few hours (Nowak and Handford, 1994). Both responses can continue for extended periods of time as well as maintain the buffering capacity of the cell.

However, the buffering capacity of the bicarbonate buffering system is not very strong, since the pK_a is roughly 6.1 whereas the extracellular pH is roughly 7.4 at a bicarbonate concentration of 28 mM and a pCO_2 of 46 mmHg. However, the reaction of the bicarbonate buffer system is accelerated to a great degree by the enzyme carbonic anhydrase (CA), which catalyzes the conversion of carbon dioxide and water to carbonic acid that subsequently dissociates to bicarbonate and protons over. Even though this reaction occurs spontaneously, this accelerates the catalytic rate up to 10^6/sec which allows for rapid pH dissipation from minutes to milliseconds (Pastorekova *et al*, 2004; Chegwiden and Carter 2000; Stewart *et al*, 1999).

The **phosphate buffering system** is a major intracellular buffer system due to fact that concentrations of phosphates are almost a dozen times higher intracellularly than in the extracellular fluid (75 mM vs. 4 mM). The reaction is at equilibrium between dihydrogen phosphate ions ($H_2PO_4^-$) and hydrogen phosphate ions (HPO_4^{2-}) plus protons (H^+).

$$\mathbf{H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+}$$

Furthermore, the phosphate buffer system operates close to its maximal buffering capacity at physiological pH, as opposed to the bicarbonate system, because its pK_a is roughly 6.8, which is much closer to physiological pH (Fernandez *et al*, 1989).

Finally, the **protein buffer system** is responsible for up to 75 percent of all intracellular physiochemical buffering capacity due to the abundance of proteins within the cell (Greenbaum and Nirmalan, 2005). Furthermore, proteins contain a great number of ionizable groups, particularly the imidazole groups of histidine whose pK_a value is very close to the physiological pH. (Abe, 2000; Madias and Cohen, 1982)

1.2.2 Ion Transport Mechanism Involved in pH Regulation

Although acute changes in pH can be minimized by various buffers, the buffering capacity of cells will be quickly overcome by large or chronic changes in acid-base equivalents. To address this challenge, all cells have evolved complex response mechanisms to control acid-base concentrations in order to maintain pH homeostasis (Casey *et al*, 2010). This is done through the interplay of multiple transporter systems that either extrude or import protons (H^+) or acid equivalents and bases such as bicarbonate (HCO_3^-) across the plasma membrane (Casey *et al,* 2010; Boron, 2004)

These acid extruding and acid loading transporters can be classified into five groups:

1) Cation/proton exchangers that move H^+ in exchange for monovalent or divalent cations.
2) Bicarbonate-dependent transporters that couple the movement of Na^+ and/or Cl^- to propel HCO_3^- across the membrane.
3) Sodium-organic ion co-transporters that move weak bases along with sodium.
4) Chloride-organic ion exchangers that are involved in the counter-transport of weak bases and chloride.
5) Proton pumps of the H^+-ATPase family that utilize energy from ATP hydrolysis to actively transport protons.

The prominent family of **cation/proton exchangers** that responds to cellular acidification are the alkali cation/proton exchangers that extrude H^+ in exchange for monovalent cations such as Na^+, but in some cases also K^+ or Li^+, and are simply referred to as Na^+/H^+ exchangers (NHEs) (see Rev. Orlowski and Grinstein, 2004). These exchangers have been studied functionally for several decades (Murer *et al*, 1976) and first identified molecularly by Pouysségur and colleagues in 1989 (Sardat *et al,* 1989). As these exchangers are the major topic of this thesis, they will be discussed in much greater detail in section 1.4.

The **HCO_3^--dependent transporters** are a super-family of transporters, characterized by the ability to co-transport bicarbonate (HCO_3^-) and their inhibition by stilbene derivatives. This super-family can be further subdivided into three major types; The Cl^-/HCO_3^--exchanger, also called the anion exchanger (AE), the (Na^+/HCO_3^-)-Cl^--exchanger, also referred to as the Na^+-dependent- or Na^+-driven-Cl^-/HCO_3^--exchanger (NDCBE), and finally, the Na^+/HCO_3^--cotransporter (NBC).

The *chloride-bicarbonate exchanger*, or *anion exchanger* (AE), was one of the first transporters of this class to be studied and exchanges bicarbonate for chloride in a reversible manner with a stoichiometry of 1:1, although other ions can be transported. Its many functions include pH homeostasis in numerous tissues (Tanner, 1997; Alper, 1991; Lee *et al*, 1991), regulation of cell volume

(Motais *et al*, 1997), cell shape (Jay *et al*, 1996; Peters *et al*, 1996), glycolysis (Low *et al*, 1993), and generation of senescent cell antigen (Kay *et al*, 1991, 1981).

There are several isoforms of the anion exchanger, designated as AE1, AE2 and AE3, though numerous splice-variant transcripts give rise to more isoforms. All share similar architecture with amino- and carboxy-termini in the cytoplasm and a transmembrane domain containing 12-14 membrane spanning segments (Bonar and Casey, 2008). Although there is also a transporter designated as AE4, it shares greater identity to the Na^+/HCO_3^--cotransporters and exhibits Na^+-independent, DIDS insensitive Cl^-/HCO_3^- exchange (Tsuganezawa *et al*, 2001).

AE1, originally called the erythrocyte band 3 protein, was the first and smallest isoform to be cloned (Kopito *et al*, 1985) and exists as two splice-variants (eAE1 and kAE1) that are transcribed in a tissue-specific manner. The eAE1 variant is expressed in erythrocytes where it fulfills a major role in the CO_2/HCO_3^- buffer system in the blood as well as anchoring to the cytoskeleton to give erythrocytes flexibility. The second variant, kAE1 is expressed on the basolateral surface of α-intercalated cells of the renal collecting duct of the kidneys (Bonar and Casey, 2008). AE2 is widely distributed and appears to act as the housekeeping anion exchanger since it works in concert with the Na^+/H^+ exchanger isoform 1 (NHE1) to maintain stringent pH_i control (Casey *et al*, 2010). AE2 contains several allosteric regulatory sites that activate the exchanger at alkaline values and inhibits activity as much as 80-90% when pH_i drops to 6.8 (Alper, 2009). Finally, AE3 has a much more restricted tissue distribution, being found in excitable tissue of the brain and heart where it plays a role in regulating pH.

The *electroneutral (Na^+-HCO_3^-)/Cl^--exchanger*, or *Na-dependent Cl^-/HCO_3^--exchanger* (NDCBE) is found in a wide variety of cells where it exchanges one external Na^+ and HCO_3^- for one internal Cl^- in a reversible manner and is inhibited by the stilbene drugs DIDS and SITS. One of the first transporters

to be studied as a pH regulator (Russel *et al*, 1976; Thomas *et al,* 1977), it is as nearly widespread as the Na^+/H^+ exchanger (Roos and Boron, 1991). In many cell types, such as cultured astrocytes and glial cells, it is the most important pH regulator (Alpern *et al,*1990; Schwiening *et al*, 1994, Bevensee *et al*, 1997; Faff *et al*, 1996; Mellergard *et al*, 1993; Schlue *et al,*1988; Shrode *et al,* 1994).

The third subfamily of the HCO_3^--dependent transporters is the *Na^+/HCO_3^--cotransporter* (NBC) which consists of six mammalian isoforms that mediate either electroneutral or electrogenic cotransport of Na^+ and HCO_3^- across cell membranes independently of chloride. Transport is driven by three independent variables: Na^+ concentration, HCO_3^- concentration and, in the case of the electrogenic variants, membrane potential. Originally the NBC was described in renal proximal tubule epithelia, where it is involved in HCO_3^--(re)absorption, but has since been characterized in numerous non-epithelial cell types, where it is involved in intracellular pH homeostasis (Soleimani and Burnham, 2001; Boron and Boulpaep *et al*, 1983). As a pH regulator, it acts predominantly as an acid extruder, by mediating influx of bicarbonate during acid load (Boron and Boulpaep, 1983). However, the NBC has several modes of transport depending on the cell type and a variety of factors that includes phosphorylation state (Gross *et al,* 2001a), intracellular Ca^{2+} concentration (Muller-Berger *et al*, 2001) and possibly changes in resting pH_i (Gross *et al,* 2001b).

As mentioned above, the NBC translocates Na^+ and HCO_3^- in the same direction, but with varying stoichiometries of one sodium ion for one, two, or three bicarbonate molecules. The electroneutral NBCs, termed NBCn1 (SLC4A7) and NBCn2, have a stoichiometry of 1:1 (Na^+:HCO_3^-). Transport is unaffected by membrane potential and is driven solely by the combined chemical concentration gradient (Cordat and Casey, 2009). Since the Na^+ concentration is highest extracellularly, this favours the inward co-transport of ions, which is equivalent to acid extrusion (alkalinizing). The electrogenic NBCs, which includes NBCe1 (SLC4A4) and NBCe2 (SLC4A5), have a stoichiometry of one Na^+ for either two or three HCO_3^- molecules, respectively (Gross *et al*, 2001a). In this case, the

driving force for the co-transport of ions is both the chemical gradient and electric potential of the cell membrane. In the case of NBCe1, which carries Na^+ and HCO_3^- into the blood across the basolateral surface of the proximal tubule, the electric potential trumps the concentration gradient and transporter acts as an acid loader (Romero *et al*, 1997).

The **Na^+-organic anion co-transporter** consists of the sodium-monocarboxylate co-transporters (SMCT) family of transporters found in the renal proximal tubule and in a variety of organisms. They are part of the mechanism for trans-epithelial movement of organic molecules by mediating the influx of sodium with an organic weak base such as lactate or acetate. Although the co-transporters do not directly regulate pH, the movement of the base results in cellular alkalinisation due to H^+-binding, thereby affecting pH_i (Coady *et al,* 2004; Putman, 2001).

The lactate-proton cotransporter is a member of the monocarboxylate co-transporter (MCT) family that mediates the co-transport of monocarboxylic acids, predominantly lactate, with protons (Halestrap *et al*, 2004a). However, it is not a widespread pH regulatory mechanism, and is largely restricted to tissues undergoing anaerobic metabolism, such as skeletal muscle and tumours. In the heart, this transporter accounts for up to 40% of the acid recovery during ischemic acid load (Vandenberg *et al,* 1993), where it utilizes the outward gradient of lactate to expel protons.

The **chloride-organic anion exchanger** (CHE) has been proposed to be involved in the electroneutral exchange of organic ion for inorganic ions such as chloride (Cl^-) or hydroxide (OH^-) in both the renal proximal tubules as well as in guinea-pig ventricular myocytes, where it mediates intracellular pH recovery from an alkali load (Niederer *et al,* 2008, Putney, 2002, Leem and Vaughan-Jones, 1998). Though it has yet to be properly characterized, it involves exchange that is electroneutral, voltage-insensitive and tightly regulated by both internal and external pH (Niederer *et al* 2008). Although it is insensitive to the stilbene

derivative DIDS, it is inhibited by another derivative, DBDS (dibenzamidostilbene-disulphonic acid) (Leem and Vaughan-Jones, 1998).

The **H^+-ATPases** or proton pumps are a family of ATP-dependent proton pumps that may have an indirect role in pH homeostasis. The proton-pumps can be divided into three known variants that include the mitochondrial *F_0F_1-type H^+-ATP synthase (F-ATPase)*, the *E_1-E_2* or *P-type ATP hydrolases (P-ATPase)*, which includes the H^+/K^+-ATPase, and finally the vacuolar or *V_0V_1-type H^+-ATP hydrolase (V-ATPase)*. The V-type ATPase are found in endomembrane compartments (Golgi, endosomes, lysosomes, secretory granules) of all eukaryotic cells as well as at the plasma membrane of specialized cells that include macrophages, osteoclasts and α-intercalated cells of the kidney (Putman, 2001).

The V-type ATPase is primarily responsible for the acidification of intracellular compartments in eukaryotic cells (Forgac *et al*, 1999). In addition, in specialized cells where it is active at the plasma membrane, such as tumourigenic cells (Martinez-Zaguilan *et al*, 1994), glial cells (Volk *et al*, 1998), macrophages (Swallow *et al*, 1990), osteoclasts (Nordstrom *et al*, 1997) and hepatocytes (Wadsworth and van Rossum, 1994), it plays important roles not only in the acidification of the extracellular milieu, but also in the regulation of intracellular pH. The latter is often observed in mitochondria-rich cells, where CO_2-induced cellular acidification results in an increase in the intracellular Ca^{2+} concentration, which in turn results in exocytosis of the V-type ATPase-containing vesicles to the plasma membrane to assist in pH_i restoration (Wadsworth and van Rossum, 1994).

The involvement of carbonic anhydrase in ion-transport mediated pH_i regulation.

It has been proposed that carbonic anhydrases (CA) may also assist in pH regulation by functionally coupling their activity to that the bicarbonate transporters. As previously mentioned, CAs are a family of enzymes that accelerate the equilibrium reaction of carbon dioxide and water to bicarbonate and

protons ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$) over 5000-fold (turnover rate ~ 10^6/sec). It has been suggested that HCO_3^- transport may be enhanced by the presence of extra- and intra-cellular CAs directly bound to bicarbonate transporters which act to provide a substantial amount of substrate for the transporter (Morgan *et al,* 2007; Li *et al*, 2006; Loiselle *et al,* 2004; Sterling *et al*, 2001; Chegwidden and Carter, 2000; Vince and Reitmeier, 1998). This transport metabolon, as it has been termed, is proposed to work in two ways. First, extracellular CA (CA4 or CA14) catalyzes the hydration of extracellular CO_2 and supplies bicarbonate ions to the extracellular transport sites (Sterling *et al*, 2002). Secondly, intracellular CAII catalyzes the conversion of transported HCO_3^- back to CO_2, which then diffuses back across the membrane (Loiselle *et al*, 2004). However, while the notion of a transport metabolon is very attractive, other studies (Boron, 2010) have challenged this view based on theoretical and technical considerations. Further studies will be required to resolve these discrepancies.

1.3 pH_i in the Myocardium:

The heart is an extremely metabolically active organ and cardiomyocyte activity results in significant acid production that must be effectively managed to prevent tissue damage. Aerobic metabolism generates carbon dioxide which is subsequently hydrated to release H^+ ions, whereas anaerobic metabolism generates H^+ ions in the form of lactic acid (Vaughan-Jones, 2009). Excess acidification can severely disrupt sodium and calcium homeostasis as well as gap junctional coupling, ultimately compromising contractile function (Vaughan-Jones, 2009: Poole-Wilson, 1989; Steenberger *et al*, 1977; Swietach *et al*, 2007).

Contractility of the heart is extremely sensitive to small physiological changes to either intracellular or extracellular pH (pH_i or pH_o, respectively). pH_i is particularly important due to its modulatory effects on excitation and contraction and, if unregulated, can act as a potent trigger of cardiac arrhythmias. A significant decrease in pH_i from the physiological level of 7.2 leads to disruption of intracellular calcium signalling and contraction depression (Fabiato *et al*, 1978; Allen *et al*, 1983; Bountra and Vaughan-Jones, 1989, Orchard and Kentish, 1990, Harrison *et al*, 1992; Choi *et al,* 2000). Many key proteins involved in calcium handling are inhibited to varying degrees by acidosis. These include the sodium/calcium exchanger (NCX) (Philipson *et al*, 1982), L-type Ca^{2+}-channels (Komukai *et al*, 2001), sarcoplasmic reticulum Ca^{2+}-release channel (Ryanodine receptor) (Rousseau and Pinkos, 1990; Balnave *et al,* 2000) and the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Fabiato *et al*, 1978; Mandel *et al*, 1992).

The proposed mechanism for the rise of intracellular calcium involves a functional coupling between the Na^+/H^+ exchanger (NHE), the Na^+-HCO_3^--co-transporter (NBC) and the Na^+/Ca^{2+} exchanger (NCX). A drop in intracellular pH stimulates NHE and NBC to increase the influx of Na^+ (Bountra *et al*, 1998; Harrison *et al*, 1992; Yamamoto *et al*, 2005). Increase in intracellular Na^+ results in an exchange for extracellular Ca^{2+} by the sarcolemmal NCX working in reverse, and this Ca^{2+} intake is then pumped back into the sarcoplasmic reticulum by the SERCA pumps. This, in turn, enhances the subsequent release of Ca^{2+}

from SR-stores, further raising intracellular Ca^{2+} (Harrison *et al*, 1992; Choi *et al*, 2000). The involvement of these acid extrusion transporters was demonstrated through the inhibition of either NHE or NBC, either by pharmacological inhibition or by the inhibition of carbonic anhydrase to inactivate NBC (Ch'en *et al*, 2008; Alvarez *et al*, 2007; Scholz *et al*, 1995).

Gap junction permeability is also exquisitely sensitive to inhibition by protons (Spray *et al*, 1981; Noma and Tsuboi, 1987). Studies have shown that decreases in pH_i uncouple gap junctions, thereby blocking cell-cell communication. This was further demonstrated by expression of a connexin-43 mutant that is insensitive to H^+ (Liu *et al*, 1993) as well as the observation that chronic exposure to H^+ results in a withdrawal of organized connexin-channels form the membrane, thereby reducing the number of channels available (Duffy *et al*, 2004; Beardslee *et al*, 2000). It also appears that acidification can act indirectly by raising Ca_i^{2+}, which in turn can inhibit gap junctional coupling of cells (Noma and Tsuboi, 1987, White *et al*, 1990).

1.3.1 pH_i Regulation in the Myocardium.

The heart has developed a sophisticated system of rapidly coping with metabolic fluctuations of acids and bases to maintain intracellular pH near neutral (i.e. ~pH 7.2) (Vaughan-Jones, 2009; Ellis *et al*, 1976; Liu *et al,* 1990; Lagadic-Gossmann *et al*, 1992a, b; Xu *et al*, 1994; Leem *et al*, 1999). The first line of defence against fluctuations in intracellular pH is intracellular buffering. This is accomplished mainly by histidine residues on proteins (intrinsic buffering) and by the CO_2/HCO_3^--buffer system which responds in a manner of subseconds. Sarcolemmal ion transport proteins mediate pH recovery by moving H^+ equivalent ions (H^+, OH^-, and HCO_3^-) across the membrane. These transporters include the Na^+/H^+-exchanger (NHE) and the Na^+-HCO_3^- co-transporter (NBC) to mediate acid extrusion. Acid loading is mediated by the Cl^-/HCO_3^- exchanger (AE) and the Cl^-/OH^- exchanger (CHE). A fifth transporter, the lactate-H^+-, or monocarboxylate co-transporter (MCT) can operate in either direction (Halestrap *et al*, 2004). However, due to the low levels of lactate during normoxic and non-exercise

condition, it is largely inactive but can significantly alter pH_i during hypoxia when lactate levels increase (Vaughan-Jones *et* al, 2009; Swietach *et al,* 2010; Vandenberg *et al*, 1993; Halestap *et al*, 1997). (***Fig. 1***)

Intracellular H^+ mobility is physiologically mediated by both mobile and fixed buffers. Proteins have a very low intracellular mobility due to their molecular weight, which causes proton diffusion to be severely restricted (Junge and McLaughlin, 1987; Swietach *et al*, 2003). This mobility within the cell is close to two orders of magnitude slower than in water (Vaughan-Jones *et al,* 2002, Spitzer *et al*, 2002) which creates pH heterogeneity across the cell, particularly when local generation of acid/base are high. Proton mobility is an essential component of pH regulation in order to ensure efficient coupling of cytoplasmic pH with pH-regulating transporters at the sarcolemma (Vaughan-Jones and Spitzer, 2006). This is facilitated by low molecular weight cytoplasmic H^+-carriers that act as mobile buffers in order to shuttle protons within the cell. The most abundant of these intrinsic mobile buffers in the heart are histidyl dipeptides (HDP) or histidyl-containing dipeptides. These small molecules that include aceteycarnosine, anserine, and homocarnosine, have pK_a values near the physiological range and shuttle H^+-ions between the cytoplasm and the sarcolemma, in order to establish adequate coupling for membrane H^+-transport while attenuating spatial variations of pH within the cell (Vaughan-Jones *et al*, 2002; Zaniboni *et al,* 2003a; Swietach *et al*, 2007a, b). (***Fig. 1***)

In addition to proton transport across the sarcolemmal membrane, another novel form of spatial pH regulation exists within the myocardium. Protons can also diffuse through connexin channels at gap junctions which provide a direct conduit between the cytoplasmic compartments of adjacent cells (Zaniboni *et al,* 2003b; Swietach *et al*, 2007a, b; Kanno and Loewentstein, 1966). The predominant connexin channel in mammalian ventricular myocytes is the connexin-43 (Cx43) channel, though the connexin-45 (Cx45) channel is expressed at lower levels (Vaughan-Jones *et al*, 2006). This type of cell-cell acid flux has been shown to be as large as that estimated for H^+ flux by Na^+/H^+ exchange

during similar acid loads (Zaniboni *et al*, 2003b). Communication between cells allows for an equalization of local differences of pH among cardiac cells and assists in maintaining a H^+ continuum within the myocardium (Vaughan-Jones, 2009). This cell-cell flux of H^+ is inhibited by pharmacological agents that inhibit gap junction conductance (α-glycerrhetic acid) (Zainiboni *et al*, 2003b). However, while modest intracellular acid loads increase H^+-permeability of these junctions to promote pH equalization, extreme acid loads ($pH_i < 6.2$) will greatly inhibit H^+-permeability. It is likely that this provides a protective measure by isolating local disturbances and preventing potentially damaging effects on neighbouring tissue (Vaughan-Jones, 2009). As with pH heterogeneity within the cytoplasm that is dissipated spatially by mobile H^+-buffers, these buffers are also required for H^+-flux across gap-junction channels (Swietach *et al,* 2010) (***Fig. 1***).

The carbonic anhydrases (CAs) also play an important role in both H^+ and metabolic CO_2 dissipation. Intracellular H^+ are buffered by HCO_3^-, which generates CO_2 by the intracellular carbonic anhydrase II (CAII). CO_2 then permeates across the sarcolemmal membrane, where it is then converted back to H^+ and HCO_3^- by CA4 and CA14 that are tethered on the outer leaflet of sarcolemma (Scheibe *et al*, 2006), and the ions then diffuse through capillaries for extrusion. It has been shown that inhibition of carbonic anhydrase by pharmacological antagonists leads to intracellular acidosis in the deeper cell layers (Vaughan-Jones *et al*, 2009). CA activity has also been shown to accelerate global post-ischemia and post-hypercarbic pH recovery in rat heart in vitro (Vandenberg *et al*, 1996).

1.3.2 Myocardial pH_i Pathophysiology

Although fluctuations of intracellular pH occur continuously in the heart, major acidosis can occur during pathophysiological events such as myocardial ischemia that can reduce pH_i to roughly 6.5 (Vaughan-Jones, 2009). Occlusion of the coronary artery results in a dramatic decrease in intracellular pH during periods of myocardial ischemia (Poole-Wilson, 1989; Garlick *et al*, 1979;

Fig. 1.

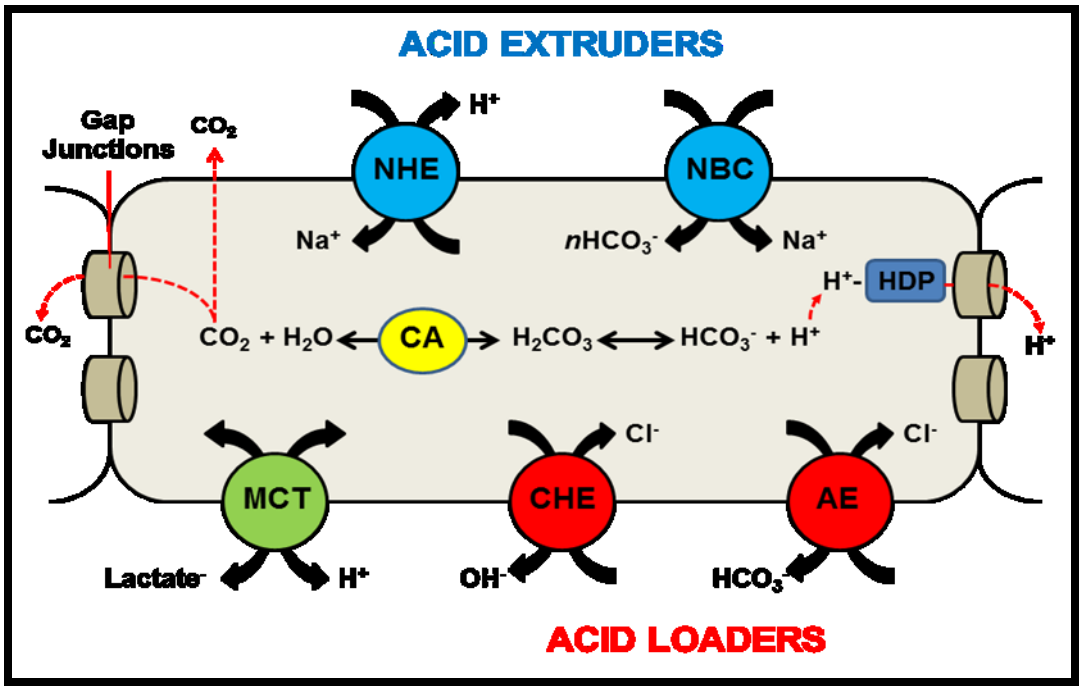

Figure 1. **Schematic diagram of pH_i regulation in cardiac myocyte:** The sarcolemmal acid equivalent transporters include the acid extruders (in blue) consisting of the Na^+/H^+ exchanger (**NHE**) and the Na^+/HCO_3^--cotransporter (**NBC**). Whereas the NHE has a stoichiometry of 1:1 (2:2 based on homo-dimerization), the NBC has a stoichiometry of 1:1 or 1:2 (Na^+:***n***HCO_3^-) based on the expressed isoform. Note that NBC expression in the heart is a combination of electrogenic and electroneutral isoforms. Acid-loaders (red) consist of the Cl^-/OH^- exchanger (**CHE**) and the Cl^-/HCO_3^--anion exchanger (**AE**). Although not strictly a pH_i-regulating transporter, the monocarboxylic acid transporter (**MCT**), is a reversible transporter whose direction is driven by the amount of molecules on either side of the membrane. Intracellular acidification can also be dissipated across the gap junctions by intrinsic mobile buffers such as histidyl dipeptides (**HDP**), or by combining with HCO_3^- and being converted to CO_2 and H_2O by carbonic anhydrase (**CA**) which can then either diffuse from the cell or pass through the gap junction.
(Adapted from Vaughan-Jones et al, 2008; Swietach et al, 2010)

Steenbergen *et al*, 1977) which accounts for a majority of observed contraction failures in the heart (Katz and Hecht, 1969).

Interestingly, mild acidosis during periods of ischemia has been suggested to be advantageous through the inhibition of calcium fluxes across the membrane along with a possible reduced mitochondrial calcium overload. Calcium uptake upon reperfusion or reoxygenation has been linked to an inward movement of sodium by the Na^+/H^+ exchanger (NHE) and subsequent reverse exchange of sodium and calcium by the Na^+/Ca^{2+} exchanger (NCX) resulting in a $Ca^{2+}{}_i$ overload. This calcium overload causes the sarcoplasmic reticulum to spontaneously initiate a propagating Ca^{+2}-wave release (Cheng *et al*, 1996) which provides a substrate for delayed after-repolarizing (DAD) (Leader *et al*, 1976), ectopic beats and possible triggering of cardiac arrhythmias (Janse *et al*, 1998). Furthermore, pH_i-induced Ca^{2+} overload has also been implicated in the disruption of mitochondrial function (Halestrap *et al,* 2004b; Duchen *et al*, 2008; Murphy *et al*, 2008). The effects of acidosis on the cell-cell connexin channels may also alter the dynamics of electrical propagation in the heart, by either increasing junction permeability during modest local H_i^+ load, or decreasing permeability during more severe $H^+{}_i$ overload (Swietach *et al*, 2007).

1.4 Na^+/H^+ Exchangers:

The sodium/proton exchangers, or NHEs, are a family of functionally diverse, ubiquitously expressed secondary active membrane transporters that are responsible for the transport of protons (H^+) across a biological membrane in exchange for sodium (Na^+). Under physiological conditions the exchanger utilizes the electrochemical gradient for sodium established by the Na^+-K^+-ATPase to provide the energy needed to extrude intracellular protons (H^+) (reviewed in Orlowski and Grinstein, 2004). The exchange is electroneutral with a stoichiometry of 1:1 or 2:2 based on evidence that the exchangers exist as a homodimer (Aronson, 1985; Fuster *et al*, 2009).

As mentioned previously, the NHEs have evolved as a major group of membrane transport proteins that protect cells against excess acid accumulation. Currently there are eleven isoforms of the NHE family, consisting of 5 plasma membrane–type isoforms, named NHE1 to NHE5 (SLC9A1 – SCL9A5), four intracellular organellar-type isoforms, NHE6 to NHE9 (SLC9A6 – SCL9A9), and two related genes, designated NHA1 and NHA2 due to similarity to the bacterial cation/proton exchangers (Brett *et al*, 2005) (***Fig. 2***). However, apart from their crucial role in intracellular pH regulation, the exchangers also participate in a variety of other cellular functions (Putney *et al,* 2002; Orlowski and Grinstein, 1997; Wakabayashi *et al*, 1997b). These include cell volume regulation (Alexander and Grinstein, 2006), Na^+ (re)absorption across the epithelia (which indirectly results in HCO_3^- (re)absorption), cell proliferation, as well as organellar ion and volume homeostasis (Counillon and Pouysségur, 2000; Numata *et al*, 1998; Numata and Orlowski, 2001; Orlowski and Grinstein, 2004, Wakabayashi *et al*, 1997; Putney *et al*, 2002). Furthermore, the NHEs have also been shown to function as a scaffold through physical interactions with the actin cytoskeleton, implicating them in cell adhesion and motility (Denker and Barber, 2002).

Fig. 2.

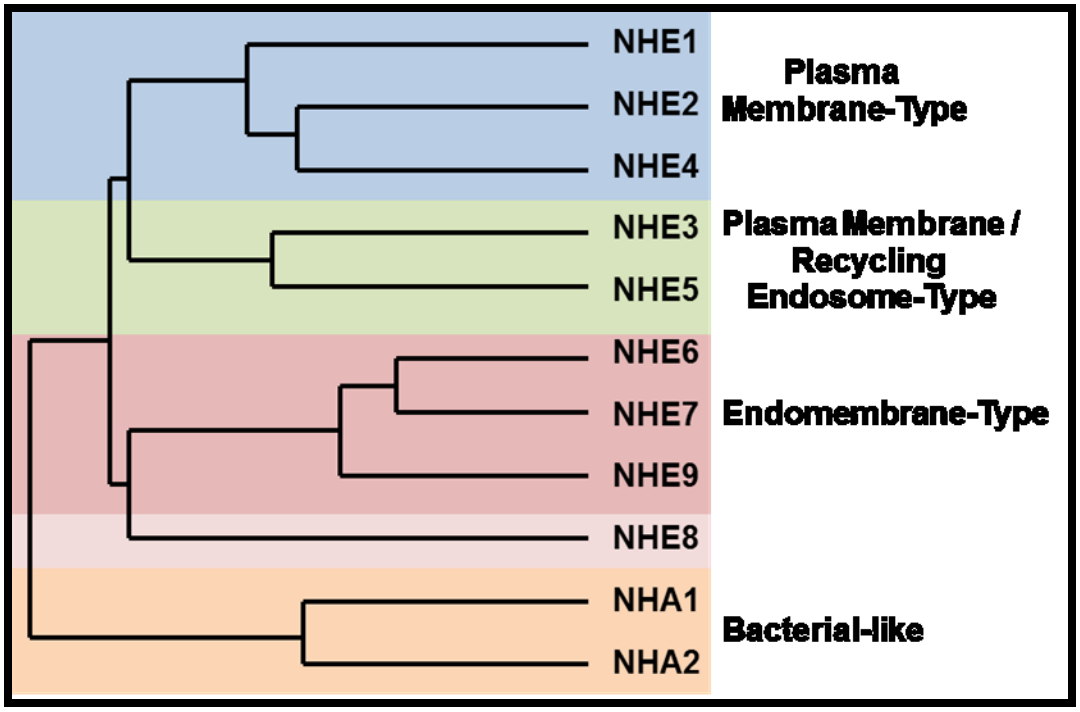

Figure 2. **Phylogenetic tree displaying the genetic diversity and membrane distribution of the mammalian Na^+/H^+ Exchanger family of transporters.** The genetic relationships between the 11 mammalian alkali cation/proton exchangers were determined by sequence alignment as described previously (Orlowski and Grinstein, 2007; Brett *et al*, 2005). Relationships were further organized according to membrane diversity.

(Adapted from Orlowski and Grinstein, 2007; Brett, et al, 2005)

1.4.1. Structural Features

All the Na^+/H^+ exchangers share a similar structure that consists of two major domains, a hydrophobic transmembrane domain and a hydrophilic cytosolic tail. The amino-terminal transmembrane domain is approximately 500 amino acids and is made up of 12 membrane spanning segments (TMs). This large hydrophobic segment is much more similar among the NHE isoforms, sharing roughly 45-65% amino acid identity, and is responsible for both ion translocation and binding of pharmacological inhibitors. The second domain is a hydrophilic cytosolic carboxy-terminal domain of varying size depending on the isoform. Unlike the N-terminal domain, the primary sequence is much more diverse with only 25-35% identity among the isoforms and contains numerous motifs that are recognized by various proteins and other regulatory factors (Numata *et al*, 1998; Numata and Orlowski, 2001; Orlowski and Grinstein, 2004, 1997).

Currently structural studies on the mammalian NHE isoforms have been obtained through the elucidation of the hydropathy plots as well biochemical topological studies, which has led to a better understanding of the tertiary and quaternary structure of the exchanger (Sardet et al, 1989). Cysteine scanning accessibility mutagenesis (SCAM) analyses have allowed for a more detailed model of the topological organization of NHE1 (Wakabayashi *et al*, 2000a). Originally, hydropathy analysis predicted that twelve transmembrane domains make up the N-terminal region (Sardet *et al*, 1989), which was subsequently supported by SCAM (Wakabayashi *et al*, 2000a). However, numerous differences between the hydropathy and mutagenesis analysis were noted. It was found that parts of the original second and forth intracellular loops were accessible to external thiol-modifying reagents suggesting that this region may be a part of the ion translocation pore. Furthermore, the original region that was designated as TM10 seemed to be embedded within the lipid bilayer. In order to accommodate these discrepancies, the last extracellular loop in the original hydropathy model forms an intracellular loop and a TM domain, now designated TM11 (Wakabayashi *et al*, 2000a). Although a three-dimensional structure has yet to be obtained for any of the mammalian NHEs, extensive studies the individual TMs

using SCAM as well as NMR spectroscopy of purified peptides of numerous transmembrane domains has increased our understanding of the structure function relationship of the exchanger (Lee *et al*, 2009a; 2009b; Reddy *et al*, 2008; Slepkov *et al*, 2007, 2005), It should be noted that a 3D structure has been obtained for the *E.coli*. exchanger, NhaA (Williams *et al*, 2000; Hunte *et al* 2005) and although the bacterial homolog shares little sequence identity with NHE1, the structure will provide new insights into the structure/function relationship of the mammalian exchanger as well as the underlying mechanisms of ion translocation.

The membrane orientation of the cytoplasmic C-terminal domain is controversial. Studies of the NHE3 isoform located in brush border membrane vesicles, found the extreme C-terminus to be accessible to the extracellular milieu when utilizing antibodies to the final 131 amino acids of the exchanger (Biemerfsderfer *et al*, 1998). However a study by Wakabayashi et al, indicated that the entire C-terminal domain is cytosolic (Wakabayashi *et al*, 2000a). Furthermore, numerous studies in our lab using an extreme C-terminal epitope tag of various NHE isoforms affirms the generally accepted view that this region is indeed cytosolic.

As mentioned at the beginning of this section, there is evidence that Na^+/H^+ exchangers exist as homodimers. However since the basic functional unit of the exchanger seems to be a monomer, the functional significance of dimerism is unclear (Fafournoux *et al,* 1994). Evidence for homodimerism includes cryo-microscopic analysis of the NhaA (William *et al*, 2000), functional analysis in relation to coupled dimer transport models (Fuster *et al*, 2009), immunological data of NHE1 and 3, as well as the fact there are no dominant/negative effects when a non-functional mutant of NHE1 was co-expressed with wild type in plasmalemmal NHE-deficient PS120 cells (Fafounoux *et al*, 1994). This dimerization could possible form through disulfide bond formation within the N-terminal transmembrane domain (Fafounoux *et al,* 1994).

1.4.2. NHE Diversity, Tissue Distribution, and Characteristics

Na^+/H^+-exchangers are present in all mammalian cells, though the tissue distribution and function of each member varies. NHE1 is found in virtually all cells where it is primary responsible for pH_i and cell volume homeostasis (Orlowski and Grinstein, 1997; Wakabayashi *et al*, 1997) and for this reason, NHE1 has often been termed the housekeeping isoform. The intricacies of function and regulation of NHE1 will be discussed in detail in section 1.5 of this thesis.

The NHE2 isoform shares roughly 50% homology with NHE1 is found primarily in the gastrointestinal tract as well as in kidney, brain, uterus, as well as the lungs, although to a lesser extent (Bookstein *et al,* 1997; Malakooti *et al,* 1999) where it's cellular distribution implies a role in sodium absorption in the colon. Localization is predominantly restricted to the apical membrane of epithelial cells in which two proline-rich SH3-like domains are implicated (Chow *et al,* 1999a).

NHE3 is predominantly expressed in epithelial cells of the gastrointestinal tracts and kidney, where is it exclusively localized to the apical membrane. Studies utilizing pharmacological inhibition of this isoform have shown it to be important in mediating Na^+ and HCO_3^--(re)absorption (Orlowski *et al,* 1992, 1997; Wakabayashi *et al*, 1997).

NHE4 is most abundant in the epithelium of the stomach and the inner medullary collecting duct of the kidney (Chambrey *et al,* 2001; Peti-Peterdi *et al,* 2001; Pizzonia *et al,* 1998), but is also expressed in low levels skeletal muscle, brain and in the basolateral membrane of cells in the uterus and pancreas (Bookstein *et al*, 1997; Orlowski *et al*, 1992). Though the physiological role is unclear, NHE4 is thought to mediate transport of NH_4^+ across the basolateral membrane of the distal nephron segments (Chambery *et al,* 2001).

The last of the plasma membrane isoforms is NHE5, which shares the most similarity with NHE3 (62% homology). Though its functional significance has not yet been elucidated, it is expressed primarily in multiple regions of the

brain, particularly in neuronal cell bodies (Attaphitaya *et al*, 1999; Baird *et al,* 1999).

Gene disruption has been a very powerful tool in studying the physiological effects of particular proteins. NHE1 knockout mice (*nhe1*$^{-/-}$), although viable at birth, usually die before weaning. They exhibit slowed post-natal development as well as neurological symptoms by 14 days that includes gait problems, seizures and slow-wave epilepsy (Bell *et al*, 1999; Cox *et al*, 1997). NHE2 knockout mice (*nhe2*$^{-/-}$), exhibit long-term gastric changes that include decreased acid secretion and parietal cell degradation after weaning (Schultheis *et al,* 1998a). NHE3 knockout mice (*nhe3*$^{-/-}$) have severe absorptive deficiencies in both the intestines and proximal convoluted tubules, as manifested by mild acidosis, diarrhea and lowered blood pressure (Schulthise *et al*, 1998b).

Compared to the plasmalemmal NHEs, much less is known about the organellar NHE subfamily, both in terms of transport kinetics, regulation and trafficking. This is mainly because of the highly dynamic nature of the endomembrane compartments and the fact that they are not easily accessible for study. Much of what we do know has been deduced from the study of the plasma membrane isoforms, such as NHE1. The organellar NHEs (NHE6 through 9) share only 20-25% amino acid identity with the plasmalemmal isoforms, but are more closely related to each other, sharing about 70% homology. They reside primarily, but not exclusively in the endomembrane compartments, though there is substantial overlap in their organellar expression. Of these isoforms NHE8 is the least similar, with only 25% identity to the other isoforms (Orlowski and Grinstein, 2007).

NHE6 is found primarily in the recycling endosome pathway where it overlaps considerably with NHE9, although they tend to be distributed differently to early and recycling endosomes (Nakamura *et al*, 2005). NHE7 is found primarily in the trans-Golgi network and neighbouring vesicles (Numata and Orlowski, 2001; Lin *et al*, 2005) and to a considerably lesser extent at the cell surface (Lin *et al*, 2007). In Cos-7 cells, NHE8 is found primarily within the mid- to trans-Golgi network (Nakamura *et al,* 2005) , although native NHE8 has been

detected in microvillar surface, as well as a lower degree in intermicrovillar-coated pits and subapical vesicles of epithelial cells (Goyal *et al*, 2004) which suggests that NHE8 may also be recycled from the apical surface.

Though the roles for endomembrane NHEs have not yet been elucidated, their capacity as a luminal pH regulator is supported by studies of the yeast ortholog, Nhx1. It has been shown that Nhx1 serves as an alkalinizing mechanism to control pH of the prevacuolar compartment (Brett *et al*, 2005).

Although NHE3 and NHE5 are classified as plasma membrane-type isoforms, both are also sorted to recycling endosomes in a clathrin-dependent manner (Kocinsky *et al,* 2005; Szabo *et al*, 2005; Szazi *et al*, 2002). It is postulated that endosomal recycling of these transporters may serve as a rapid means of down- or upreglating their activity at the cell surface in response to various stimuli (Orlowski and Grinstein, 2007). Another functional role would involve the initial acidification of the lumen of the endosomal compartment due to the Na^+-rich fluid engulfed from the extracellular milieu during vesicle formation (D`souza *et al*, 1998; Gekle *et al,* 1999; Akhter *et al*, 2000, Orlowski and Grinstein, 2007). However, the acidifying action of these transporters is likely to be transient as the outward Na^+ concentration gradient would be rapidly depleted. Further acidification would require the activity of the V-type ATPase and the electrogenic $2Cl^-/1H^+$ exchanger which together are the primary means of organellar acidification (Inou *et al,* 2005; Jentsch *et al*, 2007; Orlowski and Grinstein, 2007). This acidification is critical for the proper trafficking of internalized cargo (Maxfield and McGraw, 2004), ligand-receptor dissociation and recycling of receptors (Orlowski and Grinstein, 2007).

1.4.3 Biochemistry

The dependence of the exchanger for external Na^+ exhibits simple Michaelis-Menten kinetics with different apparent affinities for external sodium for different isoforms ranging from 3 to 50 mM, which is well below the physiological values for external Na^+ concentration (145 mM) as a regulatory substrate (Counillon and Pouysségur, 2000; Orlowski *et al*, 1997; Orlowski and

Grinstein, 2003; Yu *et al*, 1993). However this external cation-binding site is not exclusive to Na^+, but also binds other monovalent cations that include H^+, Li^+ and NH_4^+, that compete for binding and occasionally bind with higher affinity (Aronson *et al*, 1983; Paris and Pouysségur, 1983). For example, NHE1 has an affinity for extracellular Li^+ that is much higher than that for Na^+ (2.2 mM vs. 50 mM respectively), yet the turnover rate for Li^+ is much slower that it is for sodium. Furthermore, although K^+ is not transported by NHE1 or NHE2, potassium does compete with sodium for the external binding site (Orlowski *et al*, 1993; Yu *et al*, 1993).

The endomembrane transporters (NHE6-9) are thought to operate not only as Na^+/H^+ exchangers, but also as K^+/H^+ exchangers under physiological conditions; thereby suggesting more diverse roles (Orlowski and Grinstein, 2007; Numata and Orlowski, 2001; Nakamura *et al*, 2005; Hill *et al*, 2006). Although detailed cation selectivity and kinetic analyses have yet to be undertaken, these exchangers most likely utilize the cytosolic chemical gradient of potassium to transport luminal protons out of the organelles, thereby serving as an electroneutral alkalinizing mechanism (Orlowski and Grinstein, 2007).

NHEs are activated by a variety of physiological stimuli that are thought to enhance the binding of H^+ to an allosteric site within the N-terminal segment of the exchanger that is different from the translocation site (Aronson *et al,* 1985, Otsu *et al*, 1992). Although quiescent at near neutral intracellular pH (7.2), the activity increases sharply as $[H^+]_i$ increases with a Hill coefficient greater than 1.

Support for this hypothesis comes from studies of the bacterial NhaA antiporter by Olkhova and colleagues who have identified a H^+-sensing amino acid cluster at the entry to the cytoplasmic translocation funnel that is coupled electrostatically to conformational changes that expose ionizable residues in another cluster involved in translocation that is separate from the sensing cluster (Olkhova *et al,* 2009, 2007, 2006). However, other studies dispute the allosteric mechanism, and instead propose that the exchanger exists as a symmetrical dimer that is in thermodynamic equilibrium between two conformations characterized by their affinity for protons at the transport site. As $[H^+]_i$ increases, high affinity

site become activated, thereby shifting that balance of the two conformation states from low to high affinity resulting in activation of the transporter and at resting pH, transition to low affinity conformation inactivates the transporter (Lacroix *et al*, 2004, Monod *et al*, 1965).

Apart from numerous signalling pathways that promote the phosphorylation of the exchanger and thereby change its affinity for intracellular H^+, a decrease in cell volume also activates

The exchanger is also activated by a decrease in cell volume that does not seem to involve direct phosphorylation, but a unique pathway that involved ATP- and GTP-binding pathways. This is apart from the many signalling pathways that promote the phosphorylation of the exchanger thereby changing the exchanger affinity for intracellular H^+ (Grinstein *et al*, 1992).

Numerous isoforms have been shown to be glycosylated, either through N-linked, O-linked glycosylation or both. NHE1 contains an N-linked glycosylation site in the first extracellular loop, as well as a putative site for O-linked glycosylation (Counillon *et al*, 1994). NHE2 has been shown to be O-linked glycosylated (Tse *et al*, 1994), whereas NHE3 is not glycosylated (Counillon *et al,* 1994). It is not yet known what role glycosylation of the exchanger plays since the first two transmembrane domains of NHE1 have been shown to be nonessential for the activity of the exchanger (Shrode *et al*, 1998).

1.4.4 Pharmacological Inhibition.

The NHE isoforms are often characterized by their varying sensitivity to pharmacological agents, particularly amiloride and its derivatives. NHE1 is the most sensitive to these agents, being inhibited by a variety of amiloride compounds. NHE2 is less sensitive to amiloride, whereas NHE3, 4 and 5 are the least sensitive to amiloride analogs (Kapus *et al*, 1993, Orlowski and Grinstein, 2004)

Amiloride and its 4-N alkyl substituted derivatives are a class a diuretic compounds that also inhibit other Na^+-transport proteins such as the epithelial sodium channel (ENaC). Amiloride derivatives such as 5-(N-Ethyl-N-isopropyl)-

amiloride **(**EIPA), 5-(*N*,*N*-dimethyl)-amiloride (DMA), and N5-methyl-N5-propylamiloride (MPA) have been shown to be more potent and more selective (Khadilkar *et al* 2001, Orlowski *et al*, 1993, Bookstein *et al*, 1996; Counillon *et al*, 1993a; Kleyman and Cragoe, 1998; Orlowski *et al*, 1996). Although several studies have shown amiloride to be a competitive inhibitor of translocation, suggesting a common binding site (Aronson *et al*, 1985; Counillon *et al*, 1993a), more recent studies utilizing site directed mutagenesis and chimerical studies have demonstrated drug sensitivity without obstruction of the Na^+-affinity of the exchanger, implementing both transmembrane segment 4 (TM4) and 9 (TM9) (Counillon *et al*, 1997, 1993b; Wang *et al*, 1995, Orlowski *et al*, 1996). Interestingly, TM4 and 9 have been shown to be involved in both drug sensitivity and Na^+ affinity. Touret and colleagues showed that a single mutation (F162S) of TM4 that had low drug and Na^+-affinity could be reverted to high Na^+-affinity without affecting the drug sensitivity of the exchanger through separate mutations on the same segment (I169C, I170T) (Touret *et al*, 2001).

Other pharmacological inhibitors of the NHEs are the acylguanidine compounds that include HOE694 and HOE642 (cariporide). These compounds have been shown to bind to NHE1 with much greater selectivity and have potential as effective therapeutic agents in blocking ischemia/reperfusion injuries of the heart (Karmazyn *et al*, 1999, Scholz *et al*, 1995). Due to pharmacological interest, many other types of inhibitors have also become available (Masereel *et al*, 2003)

1.5. Na^+/H^+ Exchanger Isoform 1 (NHE1)

The sodium/proton exchanger isoform-1 (NHE1) is the most extensively studied isoform of the NHEs. It is widely expressed and resides in the plasma membrane of almost all cell types and tissues studied to date. NHE1 is of vital importance in numerous physiological processes, including maintenance of intracellular pH, in concert with the bicarbonate transporter, as well as regulation of cell volume through transmembrane influx of Na^+ accompanied by chloride uptake through anion exchangers and fluid osmosis (Rotin and Grinstein, 1989).

NHE1 was first identified molecularly in 1989 by Sardet and colleagues (Sardet *et al*, 1989). With the availability of the cloned cDNA for transfection and mutagenesis studies, new roles have been ascribed to NHE1, include serving as a scaffolding platform for certain cell signalling pathways and for influencing cell shape that are independent of its role in cation translocation (Denker *et al*, 2000). In some cell types, NHE1 resides in distinct domains which suggest it may fulfill specialized roles in the function of these cells. For example, in cardiac myocytes, the exchanger is localized exclusively at the intercalated disk and t-tubules, but not along the lateral sarcolemma (Petrecca *et al*, 1999), and along the basolateral membrane of polarized epithelial cells (Beimesderfer *et al,* 1992).

NHE1 function is also associated with cell proliferation and differentiation. In fibroblasts from NHE1-null mice (*Nhe*$^{-/-}$) as well as NHE1-deficient cells generated by chemical mutagenesis, both proliferation (Kapus *et al*, 1994; Pouysségur *et al*, 1984) and differentiation pathways are impaired (Wang *et al*, 1997) by delayed transit through the G2-M checkpoint (Putney *et al*, 2003). Another important physiological role for NHE1 apart from proliferation is that of modulating cell death by apoptosis and necrosis (Putney *et al*, 2002), though this appears to be a result of secondary changes to the internal pH (pHi) as well as osmotic stress directly affecting the components of the cell death pathways (Pedersen, 2006). In this aspect, evidence suggests that this regulation or modulation occurs through mitogen activated protein kinase (MAPK), Akt/protein kinase B (PKB) as well as other proteins with important roles in the control of cell proliferation and cell death (Pedersen, 2006; Harguindey *et al*, 2005).

Apart from the roles mentioned above, there is other data demonstrating that NHE1, which is localized to the lamellipodia of many cell types, regulates cell morphology, adhesion and migration (Putney *et al*, 2002; Schwab, 2001). Denker and colleagues demonstrated a structural role for NHE1 in the remodelling of the cortical actin cytoskeleton and cell shape of fibroblasts through its direct interaction with the Ezrin/radixin/moesin (ERM) family of cytoskeletal associated proteins (Denker *et al*, 2000). Though remodelling of the cytoskeleton appears to be independent of ion translocation of the exchanger, the same group showed that in relation to cell proliferation, both cytoskeletal anchorage and cation translocation of the exchanger are both necessary for remodelling of focal adhesions at both frontal and trailing edge of the cells required for guided movement (Denker and Barber, 2002). Furthermore, mutations of the amino acids that are crucial to ERM anchorage to NHE1 prevents the formation of focal adhesions and inhibits cell migration (Denker and Barber 2002; Putney *et al*, 2002). This role of NHE1 in cytoskeletal remodelling and motility has been reinforced with other studies showing that pharmacological inhibitors of NHE1 in virally transformed MDCK cells results in disassembly of filamentous actin, retardation of pseudopodia formation, as well as reduced cell adhesion and motility (Lagana *et al,* 2000).

1.5.1 Regulation

NHE1 is regulated by numerous hormones, growth factors, as well as by mechanical stimuli (e.g., osmotic cell shrinkage, cell spreading or stretch) to facilitate particular biological processes (Pedersen, 2006;Orlowski and Grinstein, 2004; Wakabayashi *et al*, 1997; Tominaga and Barber, 1998; Yamasaki *et al*, 2001). (***Fig. 3***).

Fig. 3

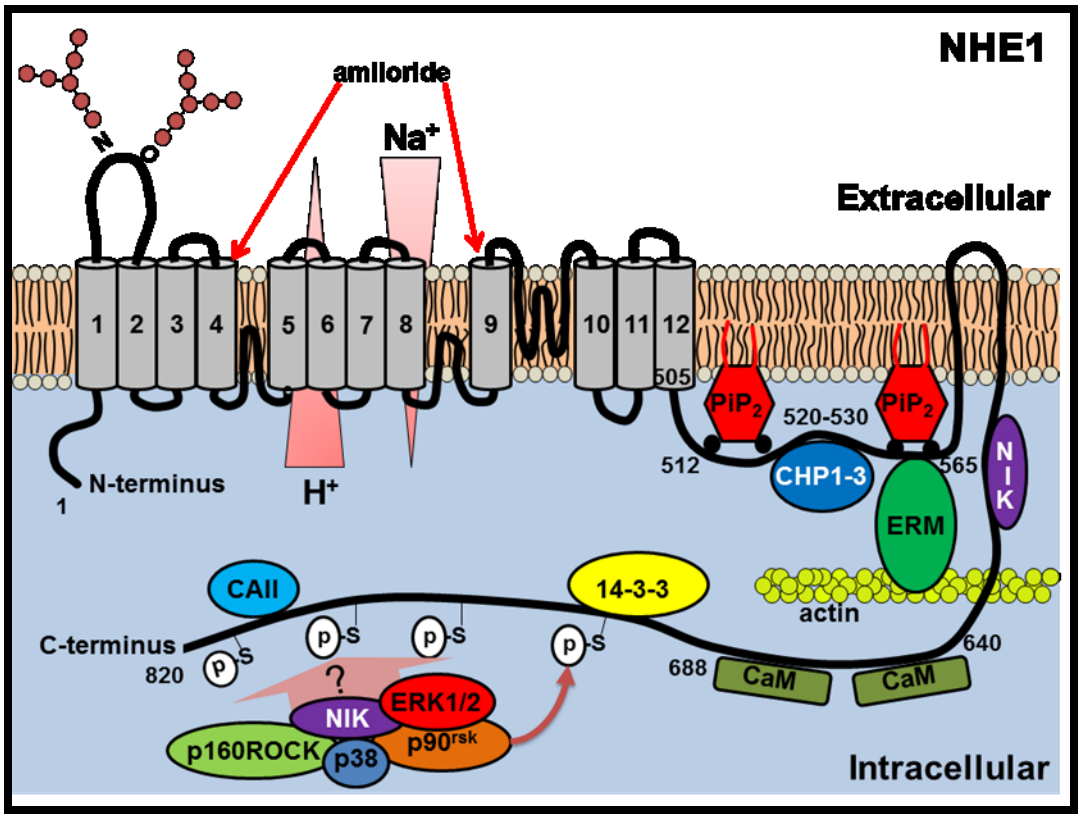

Figure 3. **Schematic representation of the Na^+/H^+ exchanger isoform 1 (NHE1).** Transmembrane organization of the N-terminal translocation domain consisting of 12 transmembrane spanning α-helices, and a large cytoplasmic C-terminal domain that interacts with numerous proteins and biomolecules. Phosphatidylinositol-4,5-bisphosphate (**PIP_2**), Calcineurin B homologous protein isoforms 1 to 3 (**CHP1-3**), Ezrin/radixin/moesin family of actin binding proteins (**ERM**), Ca^{2+}/calmodulin (**CaM**) and carbonic anhydrase II (**CAII**). The 14-3-3 scaffolding protein binds upon phosphorylation of NHE1 tail (**14-3-3**). Numerous protein kinases are involved in phosphorylation of the NHE1; Nck-interacting kinase (**NIK**), extracellular signal regulated protein kinase 1 and 2 (**ERK1/2**), p38 mitogen-activated protein kinase (**p38**), p90 ribosomal S6 kinase (**$p90^{rsk}$**), and Rho-associated coiled coil containing kinase I (**p160ROCK**).

(Adapted from Orlowski and Grinstein, 2004)

1.5.1.1: Phosphorylation:

Although the exact mechanisms by which different stimuli activate NHE1 activity still remains obscure, several studies indicate that many growth factors and hormones exert their influence by transducing their signals through a common pathway involving mitogen-activated, extracellular signal related kinase (MEK-ERK)p90RSK (Lehoux *et al*, 2001; Takahashi *et al,*1999). Phosphorylation of the exchanger by p90RSK results in binding of the scaffolding protein 14-3-3 (Lehoux *et al*, 2001; Takahashi *et al*, 1999) that does not appear to directly affect activity, but rather acts as a scaffold for interactions with other signalling molecules (Fanger *et al,* 1998). Other kinases also phosphorylate NHE1 directly, resulting in an increase in the H^+_i affinity and transport activity of the exchanger (Khaled *et al*, 2001; Moor *et al*, 2001; Tominaga *et al*, 1998) (Sardet *et al* 1990).

With respect to ERK1/2 (Moor and Fliegel, 1999; Wang *et al*, 1997a; Wei *et al*, 2001), overexpression of a dominant-negative mutant of ERK1/2 in CHO cells blocks serum-stimulated alkalinisation mediated by NHE1 (Wang *et al*, 1997a). p160Rho associated kinase (p160ROCK) mediates NHE1 activation in non-myocardial tissue and has been shown to play a key role in cytoskeletal reorganization and mediation of signals from integrin receptors that modulate cell adhesion and spreading (Tominaga *et al*, 1998). p38 kinase phosphorylates and stimulates NHE1 in progenitor B (pro-b) cells and is important in activation that mediates apoptosis (Khaled *et al*, 2001) and Nck-interacting kinase (NIK) phosphorylates NHE1 in fibroblasts and HEK293 cells in response to platelet derived growth factor (PDGF) independent of ERK-p90RSK pathway (Yan *et al*, 2001).

Other protein kinases also activate NHE1, but through an indirect mechanism involving other regulatory proteins. Protein kinase A (PKA) is known to mediate NHE1 activity in a cell-specific manner, even though the exchanger does not contain a consensus sequence site for phosphorylation. When NHE1 is expressed in the plasma membrane of the plasmalemmal NHE deficient cell line, PS120 fibroblasts, there is no response to cAMP analogs (Borgese *et al*, 1992). However, when expressed in opossum kidney cells (OK), NHE1 has been shown

to be inhibited by PKA (Helme-Kolb *et al*, 1993), yet activated in other cell lines (Moule and McGivan, 1990; Gupta *et al,* 1994) including the plasma-membrane exchanger deficient Chinese hamster ovary AP-1 cell line expressing NHE1 (Kandasamy *et al*, 1995). As with PKA, protein kinase C (PKC) also does not bind NHE1 directly, nor does it phosphorylate the exchanger (Fliegel *et al*, 1992; Wang *et al*, 1997b), yet stimulates NHE1 activity when activated by phorbol esters (Orlowski and Shull, 1996).

Just as phosphorylation of NHE1 activates Na^+/H^+ exchange, dephosphorylation has been shown to inhibit activity. Purified protein phosphatase PP1 was shown to completely dephosphorylate the C-terminal tail of NHE1 and subsequently, decrease the activity of the exchanger (Misik *et al*, 2005). Type 2A protein phosphatase (PP2Ac) was demonstrated to dephosphorylate RSK-mediated phosphorylation of NHE1 *in vitro*, resulting in a counter-activation of α1-adrenoreceptor-mediated stimulation of NHE1 (Snabiatis *et al*, 2006).

Apart from activation of NHE1 by phosphorylation of residues in the cytoplasmic tail of the exchanger, there are numerous interacting proteins and molecules that regulate the exchanger.

1.5.1.2: Phosphatidylinositol-4,5-bisphosphate (PIP_2)/ ATP-dependence:

The ubiquitous plasmalemmal phosphoinositide, phosphatidylinositol 4.5-bisphosphate (PIP_2) binds to two positively charged amino acid clusters in the juxtamembrane region of the carboxy-terminal tail of NHE1 and confers the inhibitory effect of ATP-depletion on the exchanger (Aharonovitz *et al* 2000). Though ion transport by NHE1 is a passive process across an electrochemical gradient and therefore NHE1 does not bind, nor directly consume ATP, the exchanger does require physiological levels of ATP for optimal function. The absence of ATP has been shown to reduce the function of both NHE1 and NHE2 significantly, and totally abolishes the activity of NHE3 and NHE5 (Kapus *et al*, 1994; Szabo *et al*, 2000; Wakabayashi *et al*, 1992). Although the mechanisms involved for the requirement of ATP are not fully resolved, ATP sensitivity may

be accounted for partially by the dephosphorylation of PIP_2, though the exact role of its association with NHE1 has yet to be determined. Interestingly, PIP_2 has been shown to play a significant role in the regulation of other ion transporters including the cardiac Na^+/Ca^{2+} exchanger (NCX) and the K^+-ATP channels (Hilgemann and Ball, 1996).

1.5.1.3: Ca^{2+}/Calmodulin (CaM):

Ca^{2+}/calmodulin binds to the C-terminal tail of NHE1 at two sites between amino acids 637 and 700. The first of these sites from residues 637 to 656 binds calmodulin with a much higher affinity that the second site between residues 657 to 700 (kd ~ 20 mM vs ~350 mM respectively). Ca^{2+}/calmodulin is thought to activate NHE1 by interacting with an autoinhibitory site, resulting in an increase in its affinity for intracellular protons (H^+_i). This was demonstrated by the hyperactivity of the exchanger when calmodulin sites are mutated (Wakabayashi *et al*, 1994a).

1.5.1.4: Calcineurin B homologous Protein (CHP):

The calcineurin B homologous proteins (CHPs) are a group of EF-hand calcium binding proteins that share homology to the regulatory B subunit of the serine/threonine phosphatase calcineurin. Of the three isoforms identified to date, the first two isoforms (CHP1 and 2) have been shown to interact with several members of the plasma membrane sodium/proton exchangers, whereas the third isoform (CHP3), also named tescalcin, has been shown to interact with NHE1 both in vitro and in vivo (Mailander *et al*, 2001). The first two isoforms interact with NHE1 at the juxtamembrane region of the cytosolic tail and seem to constitutively activate the exchanger (Lin and Barber, 1996; Pang *et al*, 2001, 2002). However, a study by Li et al, demonstrated that CHP3 binds the extreme C-terminus of the NHE1 tail *in vitro*, and inhibits the activity of the exchanger when overexpressed in Chinese hamster ovary fibroblasts (Li *et al*, 2003).

The tissue distribution varies among the three CHP isoforms, with CHP1 ubiquitously expressed in all adult tissue (Lin and Barber, 1996), CHP2 expressed

in intestinal epithelia (Inoue, *et al*, 2003), but also substantially upregulated in malignant cells where it seems to function in the protection of these cells from serum-deprivation-induced cell death (Pang *et al*, 2002). CHP3 was first detected in developing mouse testis (Perera *et al*, 2001), and is predominantly expressed in the heart, brain and stomach of adult tissue (Mailander *et al*, 2001, Gutierrez-Ford *et al*, 2003).

1.5.2.5: Ezrin/Radixin/Moesin (ERM) Proteins:

The Ezrin/Radixin/Moesin (ERM) family of cytoskeletal binding proteins play important roles in the linking of actin filaments of the cytoskeleton to integral membrane proteins, thereby acting as scaffolding in order to maintain cell shape, as well as the mechanical properties of the plasma membrane (Malo and Fliegel, 2006; Vaheri *et al*, 1997). The cytosolic tail of NHE1 contains two motifs for binding ERM proteins between residues 553 and 564 (Denker *et al*, 2000) that corresponds to the distal site of PIP2 interaction (Aharonovitz *et al*, 2000). This interaction is important for the function of the exchanger as a scaffolding protein in a number of important cellular events that include cell migration, signalling complex formation, as well as resistance to apoptosis (Denker and Barber, 2002; Denker *et al*, 2000; Wu *et al*, 2004).

1.5.1.6: 14-3-3:

The 14-3-3 scaffolding protein binds directly to NHE1 upon phosphorylation of the exchanger by the protein kinase, p90RSK (Lehous *et al,* 2001). Though the role of 14-3-3 interaction with NHE1 has not yet been elucidated, evidence suggests it could serve for assembly with other signalling molecules (Fanger *et al,* 1998) that may be responsible for regulation of NHE1. However, it has also been suggested that the interaction between 14-3-3 with the exchanger may stimulate serum-dependent activation by reducing phosphorylation and stabilizing active conformation (Lehous *et al*, 2001).

1.5.1.7: Carbonic Anydrase II (CAII)

Carbonic anhydrase II (CAII) binds to several acidic residues in the cytoplasmic tail of NHE, is dependent on the exchanger being phosphorylated at a site upstream from the CAII binding site and increases exchanger activity by serum induced phosphorylation (Li *et al*, 2002). As mentioned previously, CAII catalyzes the hydration of carbon dioxide to HCO_3^- and H^+, thereby elevating the total intracellular proton pool to be extruded by the exchanger, while simultaneously increasing levels of cytosolic bicarbonate (Li *et al*, 2006).

1.5.2 Osmotic Cell Shrinkage:

Acute regulatory volume increase (RVI) is of vital importance in counteracting cell shrinkage-induced programmed cell death and NHE1 plays an important role in this process (Alexander and Grinstein, 2006; Pedersen, 2006). It has been suggested that NHE1 modulates the balance between cell death and proliferation in osmotically shrunken cells by affecting various signalling pathways, independent of its regulation of cell volume (Pedersen, 2006), though the mechanisms remain obscure. NHE1-activation by cell shrinkage operates in parallel with the Cl^-/HCO_3^--exchanger (AE), resulting in an uptake of NaCl, accompanied by H_2O through osmosis, in order to facilitate volume recovery (Orlowski and Grinstein, 2004; Pedersen *et al* 2006). However, other shrinkage activated transporters, such as the $Na^+/K^+/2Cl^-$-cotransporter (NKCC1) also function in concert with NHE1 and AE (Hoffmann and Mills, 1999; Pedersen *et al*, 1996).

Though phosphorylation of the exchanger is not significantly altered (Alexander and Grinstein, 2006; Grinstein *et al*, 1992), evidence suggests that phosphorylation related changes of NHE1-associated effector molecules may be partially responsible for this increased activity (Pedersen and Cala, 2004). This includes phosphatidylinositol 4,5-bisphosphate (PIP_2), which not only is required for optimal activity of NHE1, but also has been shown to increase in levels upon osmotic cell shrinkage (Orlowski and Grinstein, 2004; Pedersen *et al,* 2001). Other effector molecules may include calmodulin, which also modulates NHE1

activity (Bertrand *et al,* 1994; Garnovskaya *et al*, 2003), and the ezrin/radixin/moesin (ERM) proteins that are rapidly phosphorylated upon osmotic cell shrinkage (Darborg *et al*, 2005).

1.5.3 NHE1 and the Heart:

Within the myocardium, NHE1 has a distinct localization at the intercalated disks and t-tubules, but not at the peripheral sarcolemmal membranes (Petrecca *et al*, 1999). This localization suggests that NHE1 may have a potential role in controlling the microenvironmental pH of the cells of the myocardium, thereby controlling the activity of closely associated pH-sensitive proteins such as the gap junction protein, connexin-43 (White *et al*, 1990), or the ryanodine-sensitive Ca^{2+}-release channel (Xu *et al*, 1996). This would allow for the possibility of NHE1 to influence impulse conductance and excitation-contraction coupling (Orlowski and Grinstein, 2004). This hypothesis is supported by the observation that NHE1 inhibition by the pharmacological antagonist, amiloride, decreases conductance of gap junctions of paired cardiomyocytes in culture (Firek and Weingart, 1995).

Regulation in the myocardium:

The myocardial NHE1 is regulated by a variety of receptor-mediated responses that generally involve G-protein coupled receptors (GPCRs). For example, NHE1 activation induced by catecholamines occurs via stimulation of the α1-adrenergic receptor (Wallert and Frohlich, 1992) in order to raise the steady state intracellular pH (pH_i) and enhance NHE1-mediated recovery from acute acid load (Fliegel and Karmazyn, 2004; Terzic *et al*, 1982). Endothelin-1 (ET-1) is another NHE1 stimulating factor shown to both increase resting intracellular pH (Moor and Fliegel, 1991) as well as accelerate pH recovery from acid load in isolated myocytes (Ito *et al*, 1997a) as does thrombin via the PKC dependent pathway (Yasutaki *et al,* 1996). The cardiomyocyte growth stimulating peptide hormone angiotensin II (AngII) activates NHE1 through the AT1 receptor which can be simultaneously antagonized by the AT2 receptor

(Gunasegaram *et al*, 1999). NHE1 expression in the heart is increased with aldosterone treatments after 24 hours and prolonged treatment increases the activity of the exchanger (Karmazyn *et al*, 2003; Kroichneva *et al*, 1995).

1.5.4 Pathophysiology:

Over the past decade there has been much work and study of the role of the sodium/proton exchangers in many different pathophysiological conditions.

1.5.4.1: Hypertension:

Primary hypertension has been associated with an increase in NHE1 activity in both experimental animal models and in human hypertensive subjects (Orlov *et al*, 1991). Though a defect in NHE1 is not the primary cause, the exchanger seems to be chronically active during essential hypertension and this correlates with an altered Ca^{2+} balance (Bobulescu *et al,* 2005; Lifton *et al*, 1991). However, the regulatory mechanism underlying this defect is still poorly understood and the NHE1 locus has been ruled out as a candidate by genetic linkage studies (Lifton *et al*, 1991). It has been suggested by several authors that alterations in signalling by various protein kinase pathways, such as enhanced MAPK activity, results in phosphorylation and activation of NHE1 (Pham *et al,* 1997; Sweeney *et al*, 1997).

Other studies have also proposed that overactivation of NHE1 in vascular smooth muscle results in a net increase in intracellular sodium accumulation. As intracellular Na^+ increases, there is a decrease, or even possibly a reversal, of the action of the Na^+/Ca^{2+}-exchanger (NCX), which in turn increases intracellular calcium levels, causing chronic vascular smooth muscle contraction (Bobulescu *et al*, 2005). This hypothesis is supported by studies showing that inhibition of the NCX by the pharmacological antagonist SEA0400 decreases cytosolic blood pressure in rat models and heterozygous $ncx^{+/-}$ mice are resistant to salt-dependent hypertension (Iwamoto *et al,* 2004). It has also been suggested that the chronic increase of NHE1 activity may also drive abnormal vascular smooth muscle cell growth and proliferation (Bobulescu *et al*, 2005).

1.5.4.2: Ischemia/Reperfusion (I/R) Injury:

Extensive evidence has been presented over the past few decades implicating hyperactivation of NHE1 in myocardial ischemia/reperfusion injury (Karmazyn *et al,* 1999, 2001; Avkiran and Marber, 2002), and that inhibition of the exchanger represents a potentially effective approach toward limiting this injury (Karmazyn, 1988). NHE1 activation is a crucial event in cell damage induced by both ischemia as well as hypoxia followed by subsequent reperfusion not only in heart (Liu *et al*, 1997; Wang *et al* 2003), but also in the central nervous system (Horikawa *et al*, 2001; Jorgensen *et al*, 1999; Phillis *et al*, 1999), liver (Gores *et al*, 1989) and lung (Rios *et al*, 2005). Though this process is complex and incompletely understood, the underlying mechanism involves the inability of the cell to maintain physiological ionic gradients due to energy deprivation during ischemia, followed by inflammatory response triggered by reperfusion that further exacerbates the injury (Bobulescu *et al*, 2005).

Abnormal hyperactivation of NHE1 is thought to occur through a combination of mechanisms. During ischemic episodes, the depletion of ATP inactivates both the Na^+/K^+-ATPase as well as NHE1 (requires the Na^+ gradient produced by the Na^+-pump) resulting in a reduction of intracellular pH. When flow is restored by reperfusion, extracellular Na^+ is normalized, resulting in an increase of sodium influx via hyper-activation of NHE1 which attempts to correct intracellular pH. This dramatic increase of intracellular Na^+ results in the Na^+/Ca^{2+}-exchanger (NCX) functioning in reverse, leading to an influx of Ca^{2+} (Karmazyn and Mofat, 1993; Pulsinelli, 1992). This overload of internal Ca^{2+} triggers a multitude of tissue dysfunctional events such as cardiac arrhythmias and altered synaptic transmission that leads to tissue damage from free radical toxicity, cellular edema as well as apoptosis and necrosis (Bobulescu *et al*, 2005, Fliegel and Karmazyn, 2004).

Numerous studies have shown that pharmacological inhibition of NHE1 protects against a variety of cardiac dysfunctions, including limitation of infarct size, attenuation of Ca^{2+}/Na^+ dyshomeostasis, reduction of arrhythmias and apoptosis, as well as an improvement of functional recovery after reperfusion and

preservation of metabolic energy (Fliegel and Karmazyn, 2004; Karmazyn *et al*, 2001, 1999). This role of NHE1 inhibition-induced protection has also been shown in NHE-null (*Nhe*$^{-/-}$) mice, which are resistant to cardiac ischemia/reperfusion injury comparable to the effect of cariporide inhibition of the exchanger relative to wild-type mice (Wang *et al*, 2003).

1.5.4.3: Cardiac Hypertrophy and Congestive Heart Failure:

Heart failure is a complex clinical syndrome arising from many interrelated intracellular and molecular defects. The underlying components include an initial hypertrophic response following myocardial injury leading to subsequent heart failure (Fliegel and Karmazyn, 2004). Studies using cultured neonatal cardiomyocytes as well as isolated tissue have demonstrated the involvement of NHE1 in cardiac hypertrophy. Furthermore, evidence suggests that NHE1 is a key factor in mediating hypertrophic responses following myocardial infarction and its activation may be an important cellular target in heart failure as well. Several recent studies utilizing transgenic mice that overexpress NHE1 with high activity in hearts indicate that activation of the exchanger is sufficient to initiate cardiac hypertrophy and heart failure (Nakamura *et al,* 2010; Xue *et al*, 2010). As with ischemia/reperfusion injuries, pharmacological inhibition of NHE1 appears to benefit attenuation in response to biochemical stresses involved in cardiac hypertrophy and heart failure (Orlowski and Grinstein, 2004; Karmazyn, 2001), although the underlying mechanisms involved remain incompletely understood.

Evidence suggests the possible role of NHE1 in the activation of various kinases involved in hypertrophy. Excess sodium accumulation is followed by protein kinase C (PKC) activation (Karmazyn, 2003), as well as transcriptional changes that result in hypertrophy (Gu *et al*, 1998; Hayasaki-Kajiwara *et al*, 1999) Inhibition of NHE1 reduces sodium entry into the cell and prevents sodium-induced activation of PKC isoforms, PKCδ and PKCε (Fliegel and Karmazyn, 2004). It was demonstrated that inhibition of PKC results in a reduction in hypertrophic responses (Fleigel and Karmazyn, 2004). Furthermore, the NHE1

inhibitor, HOE642 blocked both raf-1 and MAP kinase activation associated with stretch-induced cardiac hypertrophy (Yamazaki *et al*, 1998). Pharmacological inhibition of NHE1 has been shown to block several downstream factors activated by a variety of hypertrophic stimuli, including stretch-induced stimulation of protein synthesis in neonatal cardiomyocytes (Yamazaki *et al*, 1998), alkalinisation in feline papillary muscles (Cingolani *et al*, 1998), as well as norepinephrine-induced protein synthesis in rat cardiomyocytes (Hori *et al*, 1990).

1.5.5.4: Cancer

There is a growing body of evidence that implicates NHE1 as an important factor in the multiple steps involved in cancer cell proliferation, malignancy, as well as resistance to programmed cell death. This is mainly due to the key role pH plays in the multiple aspects of tumour cell biology, which is related to NHE1 activity (Orive *et al*, 2003). Overexpression of NHE1 has been shown to be a contributing factor in many transformed phenotypes of various cancer cells (McLean *et al*, 2000; Reshkin *et al*, 2000), and that cells with active NHE1 are more resistant to apoptotic stimuli (Putney et al*,* 2002). Furthermore, it has been established that NHE-mediated intracellular alkalinisation is directly responsible for serum and anchorage independent growth (Cardone *et al*, 2005a).

Intracellular pH (pH_i) is directly correlated to many aspects of tumour cell biology. Tumour cells contain an alkaline pH_i, ranging from 7.12 to 7.65 as compared to normal tissue at 6.9 to 7.2., and the extracellular environment is much more acidic, which has a range of 6.2 to 6.9, versus 7.3 to 7.4 for normal tissue (Cardone *et al*, 2005a). This resulting reversal of the pH gradient is an early step of neoplastic progression resulting from NHE1 activation (Siezkowski *et al*, 1994; Kaplan and Boron, 1994; Ober and Padree, 1989; Reshkin *et al*, 2000a) working in parallel with other membrane based ion exchangers including the Na^+-dependent HCO_3^-/Cl^- exchanger (NDBCE) and the H^+/lactate transporter (monocarboxylate transporter, MCT) (Cardone *et al*, 2005a). The alkalinisation of cell is completely reversible when NHE1 is inhibited with the pharmacological agent, 5-N-N-dimethylamiloride (DMA) (Reshkin *et al*, 2000a). Although this

cellular alkalinisation strongly correlates with neoplastic transformation (Harguindey *et al*, 1995), it has not been determined whether it is a key factor in the induction of cellular events that lead to cellular transformation, or a consequence of transformation (Harguindey *et al,* 1995).

It is possible that the long term stress loads of the tumour environment are responsible for elevated NHE1 activity, since it is known that factors such as increased glycolytic metabolism, acidosis, and hypoxia upregulate NHE1 (Haworth *et al*, 2003, Rios *et al*, 2005). With respect to the increase in aerobic glycolytic metabolism in tumour cells, Putney and Barber (Putney and Barber, 2004) demonstrated that stable expression of an ion exchange defective mutant of NHE1 in fibroblasts results in a decrease in expression of lactate dehydrogenase and several other glycolytic enzymes, suggesting that the activated state of NHE1 in tumours further drives the activation of aerobic glycolytic metabolism (Putney and Barber, 2004; Reshkin *et al,* 2000a). This increase in glycolytic metabolism protects hyper-proliferating cells against oxidative stress arising from reactive oxygen species produced during increased proliferation (Brand and Hermfisse, 1997).

Metastasis and invasion of tumour cells involves the alteration, coordination and regulation of a variety of cellular processes including neo-angiogenesis, cell adhesion, degradation and remodelling of the extracellular matrix, as well as motility and sustained exotopic proliferation in the invaded organ (Kerbel, 2000). The acidity of the intra-tumoural environment increases the potential for metastasis by promoting several factors such as neo-angiogenesis, anchorage independent growth, genetic instability and invasion (Orive *et al*, 2003; Gillies *et al*, 2002). Several mechanisms involved in the invasion of cancerous cells include a prominent role of NHE1 (Cardone *et al*, 2004a), such as the disruption of the extracellular matrix that arises from increased acid secretion, protease activity, and cell motility (Reshkin *et al*, 2000b; Paradiso *et al*, 2004; Lagana *et al*, 2000, Klein *et al*, 2000). Both degradation of the basement membrane and extracellular matrix by tumour cell acid secretion, as well as the release and activation of acid dependent proteases, are regulated by both

intracellular and extracellular pH of the tumour cell and its environment. Furthermore, once the region around the tumour cell has been modified, there is an increase in tumour cell locomotion in order to invade an organ or tissue (Cardone et al, 2005b). The resultant loosening of the extracellular matrix accompanies a change in tumour cell morphology, allowing the cell to undergo chemotaxis along growth factor gradients guided by the redistribution of NHE1 to the leading edge of the pseudopodia (Patel and Barber, 2005). Pharmacological inhibition of NHE1 has been shown to block both the formation of the pseudopodia as well as the invasive ability of the cell (Reshkin *et al*, 2000b; Bourguignon *et al*, 2004).

So far, little is known about the extrinsic promoting signals that activate NHE1 in cancer cells or the signal transduction system that is driven to regulate this process, both in regards to primary extracellular signals as well as subsequent signal-transduction that regulates NHE1 activity as a downstream function (Putney *et al*, 2002; Baumgartner *et al*, 2004; Orlowski and Grinstein, 2004). Several studies of various cancer cell lines have elucidated signalling regulation of NHE1-dependent motility and invasion. For instance, serum deprivation increases motility and invasion through NHE1-dependent remodelling of the actin cytoskeleton of the cell. Signalling pathways that regulate NHE1 are thought to be among the principle systems involved in driving cell metastasis and invasion (Mareel and Leroy, 2003).

Rationale and Objectives

The primary role of the ubiquitously expressed NHE1 is in the maintenance of both intracellular pH (pH_i) and the regulation of cell volume, although numerous secondary functions have also been elucidated to include roles in migration, proliferation, differentiation and apoptosis. NHE1 has also been demonstrated to play an important role as a scaffolding protein for the assembly of various signalling molecules (Brett *et al*, 2005; Orlowski and Grinstein 2007, 2004; Meima *et al*, 2007). Furthermore, NHE1 is the predominant isoform expressed within the myocardium where its activity is of crucial importance in maintaining intracellular myocardial pH and has been demonstrated to play an important role in several pathophysiological states of the heart. These include cardiac hypertrophy and heart failure as well as myocardial damage following ischemia/reperfusion (Fliegel, 2009; Karamzyn *et al*, 2005; Avkiran *et al*, 2008).

The focus of this thesis was to examine the mechanism of regulation of NHE1 by novel interacting proteins that are present within the myocardium, and to assess their significance during periods of metabolic stress. One such novel NHE1-interacting protein is calcineurin B homologous protein 3 (CHP3). This protein is a Ca^{2+}-binding protein that was originally termed Tescalcin (Mailander *et al*, 2002) due to its original isolation from developing mouse testis (Perrera *et al*, 2001). However, in adult human tissue, its expression is restricted predominantly to the myocardium (Gutierrez-Ford *et al*, 2003) and was reported to interact with the distal half the cytoplasmic tail of NHE1 and depress exchanger activity (Li *et al*, 2003). Interestingly, tescalcin shares both homology and an ability to regulate calcineurin phosphatase activity with a family of NHE1 interacting proteins that activate the exchanger, termed the calcineurin B homologous proteins (CHP), due to their homology to the regulatory B subunit of calcineurin (Lin and Barber, 1996; Pang *et al*, 2001, 2002, Gutierrez-Ford *et al*, 2003). Both CHP1 and 2 have been shown to bind the juxtamembrane region of the C-terminal tail of NHE1 and upregulate its activity (Pang *et al*, 2001, 2002). Where CHP1 is ubiquitously expressed and is important for maintenance of pH_i-

sensitivity of NHE1 as well as activation in response to various stimuli (Pang *et al,* 2001, 2004), CHP2 distribution is restricted to the intestinal epithelia (Inoue *et al*, 2004) and various malignant cell lines where it functions to constitutively activate the exchanger in the absence of external stimuli (Pang *et al*, 2002).

In chapter 1 of this thesis, we utilize mutational analysis and biochemical studies to map the precise location for the interaction of CHP3 on NHE1 as well as the molecular basis of its regulation of the exchanger. Our results demonstrate that, contrary to earlier findings, CHP3 activates the exchanger by promoting both maturation of NHE1 to the cell membrane as well as the cell surface stability of the exchanger. Chapter 2 further elucidates the roles of the N-myristoylation and Ca^{2+}-binding motifs of CHP3 with respect to the regulation of NHE1. Both motifs were found to be crucial for the stability of the exchanger at the plasma membrane, but not for binding to the transporter nor for its post-translational maturation .

Finally, chapter 3 examines the interaction between NHE1 and CHP3 during periods of severe metabolic stress. Previous studies in our lab demonstrated that NHE1 has a very distinct distribution within the heart, where it localizes to the intercalated discs and transverse tubules but not the lateral sarcolemmal membrane (Petrecca *et al*, 1999). Upon low flow ischemia/reperfusion or ATP depletion in rat hearts, NHE1 gradually translocates from the intercalated discs and transverse tubules to the lateral sarcolemmal membranes (Lamare et al., *unpublished data*). Furthermore, another study by our laboratory determined that ATP depletion partially inhibits NHE1 activity, which was partly attributed to the de-phosphorylation and reduction of plasmalemmal phosphatidylinositol-4,5-bisphosphate (PIP_2) which interacts with NHE1 at two sites on the juxtamembrane tail of the exchanger that flank the CHP binding site (Aharonovitz *et al,* 2000). Our results in CHO/AP-1 cells indicate that upon ATP depletion, there is indeed a loss of NHE1 activity and corresponding cell surface abundance, but that CHP3 remains bound to NHE1 and therefore cannot account for stress-induced alterations in NHE1 function and membrane localization.

Preamble to Chapter 1

NHE1 is a multifaceted protein that is involved in a wide variety of physiological processes in both normal and diseased states of an organism. The regulation of the exchanger is controlled, in part, through the interaction of the cytosolic regulatory domain with a wide variety of interacting proteins and molecules. One such interacting partner is the third member of a family of NHE1-binding proteins that share similarity with the regulatory B subunit of the serine/threonine protein phosphatase Calcineurin (CaN), hence the name Calcineurin B homologous proteins (CHPs). The third isoform, CHP3, also called Tescalcin, was demonstrated to interact with NHE1 both *in vitro* and *in vivo*. Interestingly, CHP3 was shown to interact with NHE1 at a region separate from the other isoforms, and unlike CHP1 and 2 which activate the exchanger, inhibits exchange activity. However, the exact interacting motif of NHE1, as well as the molecular mechanisms involved in Na^+/H^+ exchange regulation was not elucidated.

Chapter 1 of this thesis examines the underlying mechanisms involved in the regulation of NHE1 by CHP3. Utilizing various molecular, biochemical and cellular techniques we determined that CHP3 in fact does associate with NHE1 at the juxtamembrane region of the exchanger, involving the same critical residues that are responsible for the interaction with the other CHP isoforms. Furthermore, we demonstrate that CHP3 activates NHE1 by promoting the accumulation as well as stabilizing NHE1 at the plasma membrane.

Chapter 1:

Calcineurin B-Homologous Protein 3 Promotes the Biosynthetic Maturation and Cell Surface Stability of the Na^+/H^+ Exchanger NHE1 Isoform.

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ABSTRACT:

Calcineurin B-homologous protein (CHP) 1 and 2 are Ca^{2+}-binding proteins that modulate several cellular processes, including cytoplasmic pH by positively regulating plasma membrane-type Na^+/H^+ exchangers (NHEs). Recently another CHP-related protein termed tescalcin or CHP3, was also shown to interact with the ubiquitous NHE1 isoform, but seemingly suppressed its activity. However, the precise physical and functional nature of this association was not examined in detail. In this report, biochemical and cellular studies were undertaken to further delineate this relationship. Glutathione-S-transferase-NHE1 fusion protein pull-down assays revealed that full length CHP3 binds directly to the proximal juxtamembrane C-terminal regions (amino acids 505-571) of rat NHE1 in the same region that binds CHP1 and CHP2. The interaction was further validated by co-immunoprecipitation and co-immunolocalization experiments using full-length CHP3 and wild type NHE1 in transfected Chinese hamster ovary AP-1 cells. Combined mutation of several hydrophobic Phe and Leu residues within this region (^{530}FLDHLL^{535}) to Ala, Gln, or Arg (FL-A, FL-Q or FL-R) abrogated this interaction both *in vitro* and in intact cells. The NHE1 mutants were sorted properly to the cell surface, but showed markedly reduced (FL-A) or minimal (FL-R and FL-Q) activity. Interestingly, and contrary to an earlier finding, ectopic co-expression of CHP3 upregulated the cell surface activity of wild type NHE1. This stimulation was not observed with the CHP3 binding-defective mutants. Mechanistically, overexpression of CHP3 did not alter the H^+ sensitivity of the wild type transporter, but rather accelerated its rate of biosynthetic maturation and enhanced its half-life at the cell surface, thereby increasing the steady state abundance of the functional NHE1 protein.

INTRODUCTION:

Monovalent cations such as Li^+, Na^+, and K^+ are transported across biological membranes in exchange for H^+ by a family of alkali cation/proton countertransporters, commonly referred to as Na^+/H^+ exchangers (NHEs) or antiporters. Phylogenetic analysis and functional studies have revealed the existence of a least 11 mammalian NHE isoforms that display varied primary structure (~13-70% identity), tissue distribution, subcellular compartmentalization, cation selectivity, and function (Brett *et al,* 2005; Orlowski and Grinstein, 2007; Xiang *et al*, 2007) . Structurally, the NHEs are comprised of two major domains as follows: an N-terminus that contains 12 predicted membrane-spanning segments responsible for cation permeation and a C-terminus that faces the cytoplasm and serves to regulate transport activity, membrane targeting, anchorage to the underlying actin cytoskeleton, and as a scaffold for the assembly of other signalling complexes (Brett *et al,* 2005; Orlowski and Grinstein, 2007, 2004; Bobulescu *et al*, 2005; Donowitz and Li, 2007; Meima *et al*, 2007).

Of these isoforms, NHE1 has received considerable attention because it is widely expressed in plays a central role in several physiological processes, notably cytoplasmic pH homeostasis and maintenance of cell volume, but also events underlying cell shape, migration, proliferation, differentiation and apoptosis (Meima *et al*, 2007; Stock and Schwab, 2006; Malo and Fliegel, 2006; Wu *et al*, 2003, 2004; Sun *et al*, 2004; Konstantinidis *et al*, 2006). Accordingly, diverse signals (*e.g.*, hormones, mitogens and physical stimuli such as mechanical stress and hyperosmolarity) that regulate such phenomena also acutely stimulate NHE1 activity, mainly by enhancing the affinity of the transporter for intracellular H^+. Depending on the stimulus, this activation is often associated with direct phosphorylation of its C-terminus by assorted serine/threonine protein kinases, including extracellular signal-regulated protein kinase 1 and 2 (Malo and Fliegel, 2007), p38 mitogen-activated protein kinase (Khaleb *et al*, 2001), p90 ribosomal S6 kinase (Takahashi *et al*, 1999; Cuello *et al,* 2007), Rho-associated, coiled coil containing protein kinase 1 (Tominaga *et al*, 1998), and Nck-interacting kinase

(Yan *et al*, 2001). In turn, phosphorylation at certain sites has been shown to promote the binding of additional ancillary proteins such as carbonic anhydrase (Li *et al*, 2006) and the scaffolding protein 14-3-3 (Lehoux *et al*, 2001). Conversely, protein phosphatases such as PP1 and PP2A act as negative regulators of NHE1 phosphorylation and activity (Misik *et al*, 2005; Snabaitis *et al*, 2006). However, other interacting partners can bind independently of NHE1 phosphorylation, including Ca^{2+}-calmodulin (Bertrand *et al*, 1994), members of the calcineurin B-homologous protein (CHP) family (Lin and Barber, 1996; Pang *et al*, 2001, 2002; Inoue *et al*, 2003; Mailander *et al*, 2001), phosphatidylinositol 4,5-bisphosphate (PIP_2) (Aharonovitz *et al*, 2000), and the actin filament anchoring proteins ezrin, radixin, and moesin (ERM) (Denker *et al*, 2000). In general, these associations are thought to elicit a change in the conformation of its C-terminal regulatory domain, thereby altering H^+ affinity, but additional evidence supporting this conjecture is wanting.

Of the above mentioned regulatory molecules, the CHP family of proteins (CHP1-3) appear to be crucial partners for basal as well as regulated activity of NHE1 and other plasma membrane-resident NHE isoforms (Lin and Barber, 1996; Pang *et al*, 2001, 2002; Inoue *et al*, 2003; Mailander *et al*, 2001; Li *et al*, 2003). CHPs are N-myristoylated, EF-hand Ca^{2+}-binding proteins that exhibit ~29-61% identity to each other, and share ~40% identity with the regulatory B subunit of the protein phosphatase calcineurin. Indeed, the CHPs can alter calcineurin activity (Lin *et al*, 1999; Gutierrez-Ford *et al*, 2003). CHP1 is present in most tissues, binds to the proximal region of the cytoplasmic C-terminus of NHE1, and plays a significant role in setting the resting intracellular pH sensitivity of the transporter in the neutral range, but also its activation in response to various stimuli (Pang *et al*, 2001, 2004). By comparison, the expression of CHP2 is largely restricted to normal intestinal epithelia (Inoue *et al*, 2003), but is significantly induced in different malignant cell types (Pang *et al*, 2002). CHP2 interacts with the same region of NHE1 as CHP1, but binds with several-fold higher affinity (Pang *et al*, 2002). Intriguingly, heterologous expression of CHP2 in fibroblasts appears to constitutively activate the transporter

in absence of external stimuli, resulting in a marked elevation in steady-state intracellular pH relative to cells expressing only CHP1 (Pang *et al*, 2002). This activation also appears to correlate with a significant reduction in the incidence of cell death upon prolonged serum withdrawal, implicating a role for NHE1/CHP2 and intracellular pH in the progression of cancerous cells (Pang *et al*, 2002; Jin *et al*, 2007). By contrast, CHP3, originally isolated from the developing mouse testis and termed 'tescalcin' (Perera *et al*, 2001), is detected predominantly in adult mouse heart, brain, and stomach (Gutierrez-Ford *et al*, 2003), although in adult human tissue it appears to be restricted primarily to heart (Mailander *et al*, 2001). Interestingly, unlike CHP1 and -2, CHP3 was reported to bind a unique site within the distal half of the cytoplasmic C-terminus of NHE1 and to suppress the activity of the transporter in transfected cells (Li *et al*, 2003). However, the precise location and molecular basis for the negative regulation were not examined.

In this study, we further investigated the molecular interaction and mechanism of action of CHP3 on NHE1 function. Contrary to previous findings (Li *et al*, 2003), we found that CHP3 binds to the same juxtamembrane region of the cytoplasmic C-terminus of NHE1 as CHP1 and CHP2. An intact CHP3-binding motif was found to be crucial for optimal Na^+/H^+ exchange. Furthermore, rather than suppressing exchanger activity, CHP3 was found to increase NHE1 abundance at the cell surface by facilitating its maturation along the biosynthetic pathway and enhancing it half-life at the plasma membrane.

MATERIALS AND METHODS:

Materials:

Mouse monoclonal and rabbit polyclonal anti-hemagglutinin (HA) antibodies were purchased from Convance Inc. (Berkeley, CA); anti-Myc antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY); a rabbit polyclonal anti-green fluorescent protein (anti-GFP) antibody was obtained from Invitrogen; and antibodies specific to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Abcam Inc. (Cambridge, MA). Horseradish peroxidase-conjugated secondary IgG antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). All Alexa Fluor®-conjugated goat anti-mouse and anti-rabbit IgG antibodies were purchased from Molecular Probes. (Eugene, OR).

α-Minimum essential medium (α-MEM), fetal bovine serum, penicillin/streptomycin, geneticin (G418), and trypsin-EDTA and Lipofectamine™ 2000 transfection reagent were obtained from Invitrogen. Carrier-free ^{22}NaCl and [^{35}S]-methionine were obtained from PerkinElmer Life Sciences. Amiloride hydrochloride, nigericin, and ouabain were purchased from Sigma, and complete protease inhibitor mixture tablets were obtained from Roche Diagnostics. All other chemicals and reagents used in these experiments were obtained from Bio-Shop Canada (Burlington, Ontario, Canada), Sigma, or Fisher and were of the highest grade available.

cDNA Construction and Mutagenesis:

The construction of the mammalian expression vector containing the HA epitope-tagged form of the rat NHE1 cDNA ($NHE1_{HA}$) was described previously (Orlowski and Kandasamy, 1996). Previous experiments showed that when this modified NHE1 construct was expressed in AP-1 cells, no obvious effect was observed on basal activity or the functional properties of the exchanger (Aharonovitz *et al*, 2000). The full-length human CHP3/tescalcin cDNA was cloned using PCR methodology from a human heart Matchmaker™ cDNA library

(Clontech), and it was engineered to include a *myc* epitope (EQKLISEEDL) at its extreme C terminus for immunological detection. This construct, henceforth termed $CHP3_{myc}$, was inserted into the HindIII/XbaI sites of the mammalian expression vector pcDNA3 (Invitrogen), as well as the mammalian expression vector pCMV (Orlowski, 1993).

Mutations of the hydrophobic amino acids in $NHE1_{HA}$ crucial for interaction with $CHP3_{myc}$ were accomplished using a commercially available QuickChange® site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) according to the manufacturer's protocol. These amino acids (^{530}FLDHLL^{535}) were substituted for either Ala (^{530}AADHAA^{535}, FL-A), Gln (^{530}QQDHQQ^{535}, FL-Q), or Arg (^{530}RRDHRR^{535}, FL-R). Deletion of segments at the N-terminal juxtamembrane region of the cytoplasmic tail of $NHE1_{HA}$ (Δ505–540 and Δ505–566) was accomplished by PCR mutagenesis. All constructs were sequenced using the Sanger method (Sanger *et al*, 1977) to validate the fidelity of the sequences.

Cell Culture and DNA Transfection:

Chinese hamster ovary cells devoid of plasma membrane Na^+/H^+ exchange activity (AP-1 cells) (Rotin and Grinstein, 1989) were maintained in α-MEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 mm $NaHCO_3$ (pH 7.4) and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. To generate AP-1 cells stably expressing $NHE1_{HA}$ and its derived mutants, plasmid DNA (0.5–1.0 μg/ml) was transfected into subconfluent cells in a 6-well plate using Lipofectamine™-2000 according to the manufacturer's recommended procedure. Twenty four hours post-transfection, cells were split (1:10 to 1:50, depending on cell density) into 10-cm dishes and selected for stably expressing clones over a 2-week period using repeated acid selection as described previously (Orlowski, 1993). $NHE1_{HA}$ and $CHP3_{myc}$ double expressing cells were obtained by transfecting a single clone expressing $NHE1_{HA}$ with $CHP3_{myc}$ using Lipofectamine™-2000, as described

above, and selecting in α-MEM culture medium supplemented with geneticin (G418) (600 μg/ml) over a 2–4-week period.

Construction of Glutathione-S-Transferase (GST)-Fusion Protein and in-vitro Binding Assay:

GST-fusion proteins of segments of the C-terminal regulatory region of NHE1 were produced by PCR amplification using primers containing *BamHI* at the 5'-terminus and *EcoRI* at the 3'-terminus. These PCR products were subcloned in-frame into the bacterial expression vector pGEX-2T (Amersham Bioscience, Piscataway, NJ). Inserts were sequenced to confirm their fidelity and then plasmid constructs were transformed into the *Epicurian Coli® BL-21-CodonPlus™* strain (Stratagene, Cedar Creek, TX) strain.

Individual clones were cultured overnight, then diluted 1:25 in 50 ml bacterial growth medium, and incubated further at 37º with vigorous shaking to attain a sufficient population density. Protein expression was then induced with the addition of 0.4mM isopropyl-1-thio-β-galactopyranoside (IPTG) and cultures were incubated a further 2.5 hours at 30ºC. The bacterial cultures were centrifuged and the resulting pellets were resuspended in 500 μl GST-lysis buffer (1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitors in standard phosphate buffered saline (PBS). Bacteria were subsequently lysed by sonication (Model 100 Sonic Dismembrator, Fisher Scientific) on ice and cleared by centrifugation at 4ºC for 20 min. Proteins were then purified by incubating cell lysates with a reduced form of glutathione-Sepharose™ beads (Amersham Bioscience) for several hours at 4ºC. Then purified GST-fusion proteins bound to glutathione-Sepharose beads were washed six times with GST-lysis buffer, and then incubated with either 2.5 μl in vitro translated full-length ^{35}S-labelled CHP3 or lysates from Chinese hamster ovary cells (CHO) transfected with CHP_{myc} for several hours at 4ºC.

In vitro translated CHP3 was accomplished by subcloning the CHP3 cDNA into a vector containing a T7 promoter to enable *in vitro* transcription-translation coupling reaction using rabbit reticulocyte lysates (Promega, Madison,

WI) in the presence of ^{35}S-methionine. $CHP3_{myc}$ cDNA containing cell lysates were obtained by transfecting CHO cells with $CHP3_{myc}$ cDNA using Lipofectamine™-2000 following the manufacturers recommended procedure. Twenty-four hours post transfection, cells were lysed on ice by washing two times with ice cold PBS, followed by the addition of 1 ml ice-cold RIPA buffer (150 mM NaCl, 0.25% deoxycholic acid, 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, and protease inhibitors), scrapping, and then incubation of the cells on ice for 1 h. Cell lysates were then centrifuged for 10 min at 4ºC to remove cellular debris.

NHE1-fusion protein complexes were washed six times with GST-lysis buffer, then eluted in sodium dodecyl sulphate (SDS)-sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 50 mM DTT, 10% glycerol, 1% bromophenol blue), and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels containing *in vitro* translated ^{35}S-labelled CHP3 were dried and resulting bound [^{35}S]CHP3 was resolved using PhosphorImager™ (Molecular Dynamics Inc., Sunnyvale, CA), whereas gels containing CHP_{myc} from CHO cell lysates were subject to electrophoretic protein transfer onto polyvinylidene fluoride (PVDF) membranes (Millipore, Nepean, ON) for Western blotting. Membranes were blocked with 5% nonfat powdered milk and exposed to 1:1000 dilution of mouse monoclonal antibody against the *myc* epitope, and a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) at a dilution of 1:5000. Immunoreactive bands were then visualized using ECL™ Western Blotting Detection reagents (Amersham Bioscience).

Coimmunoprecipitation:

Immunoprecipitation of wild-type and mutant forms (FL-A, FL-Q, FL-R, Δ505–540, and Δ505–566) of $NHE1_{HA}$ were performed in 10-cm plates of CHO cells cotransfected with 5 μg of both $NHE1_{HA}$ and $CHP3_{myc}$ cDNA constructs. Transfections were performed using Lipofectamine™-2000 according to the manufacturer's recommended procedure. Twenty four to 36 h post-transfection, cells lysates were obtained by washing cells twice on ice with ice-cold PBS,

followed by the addition of 1 ml of RIPA buffer. The cells were removed from the dish by scraping and then incubating for an additional 20 min at 4 °C. The lysates were then centrifuged for 20 min at 4 °C to pellet the cellular debris. The supernatants were pre-cleared with 100 µl of 50% protein G-Sepharose slurry (Amersham Biosciences) in RIPA buffer for 2 h at 4 °C. The protein G-Sepharose slurry was removed by brief centrifugation, and a fraction of each lysate was removed for Western blotting. Five µg of primary mouse monoclonal antibody against the HA epitope or 10 µg of polyclonal rabbit antibody against the *myc* epitope was added to the remaining lysates and incubated with gentle rocking overnight at 4 °C. One hundred µl of 50% protein G-Sepharose slurry was added to each tube and allowed to incubate for several hours at 4 °C with gentle rocking, followed by six washes in RIPA buffer. Protein conjugates were eluted by SDS-sample buffer and incubated for 30 min at room temperature without boiling to minimize aggregation of the NHE1 proteins. Samples were then subjected to SDS-PAGE and Western blotting as described previously. Blots from monoclonal anti-HA immunoprecipitates were detected with rabbit polyclonal antibodies against the HA and *myc* epitopes followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Polyclonal anti-Myc immunoprecipitates were detected using mouse monoclonal antibodies to the respective epitopes followed by goat anti-mouse secondary antibody conjugated to horseradish peroxidase. All blots were visualized with ECL™ Western blotting detection reagents.

Immunocytochemistry:

For colocalization studies of NHE1 and CHP3, AP-1 cells overexpressing wild-type and mutant $NHE1_{HA}$ were grown on glass coverslips in 6-well plates and transiently transfected with 1 µg of $CHP3_{myc}$. Thirty six hours post-transfection, cells were fixed with 2% paraformaldehyde for 20 min at room temperature, rinsed several times with PBS (pH 7.4), and permeabilized and blocked in 0.2% saponin, 2% BSA in PBS for 30 min at room temperature. Cells were subsequently rinsed in PBS, and then incubated with a mouse monoclonal

antibody against the HA epitope at a dilution of 1:1000, and a rabbit polyclonal antibody against the *myc* epitope at a dilution of 1:500 in 2% BSA in PBS for 2 h. Cells were then washed three times in 0.01% saponin in PBS, followed by incubation with anti-mouse Alexa Fluor™ 488 and anti-rabbit Alexa Fluor™ 568 secondary antibodies at a dilution of 1:2000 in 2% BSA in PBS for 1 h. Coverslips were subsequently washed several times with 0.01% saponin in PBS and mounted onto glass slides with Immuno-Fluor™ mounting medium (ICN Biomedicals, Aurora, OH). Transfected cells were analyzed by laser scanning confocal microscopy using a Zeiss LSM 510, and images were analyzed using LSM software and Corel® CorelDraw™ version 12.

Measurement of Na^+/H^+ Exchanger Activity:

Cells were grown to confluence in 24-well plates. NHE activity was determined by preloading the cells with H^+ using the NH_4Cl technique (Boron and DeWeer, 1976) and then measuring the initial rates of $^{22}Na^+$ influx essentially as described (Orlowski, 1993). Briefly, the cell culture medium was aspirated and replaced by isotonic NH_4Cl medium (50 mM NH_4Cl, 70 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4). Cells were incubated in this media for 30 min at 37ºC in a nominally CO_2-free atmosphere. After acid loading, the monolayers were rapidly washed twice with isotonic choline chloride solution (125 mM choline chloride, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4). $^{22}Na^+$ influx assays were initiated by incubating the cells in isotonic choline chloride solution containing 1 mM ouabain and 1 μCi of $^{22}NaCl$ (carrier-free)/ml (~120 nM NaCl) for 5 minutes. The assay medium was K^+-free and included ouabain to prevent transport of $^{22}Na^+$ by the Na^+-K^+-Cl^- cotransporter and the Na^+, K^+-ATPase. Influx of $^{22}Na^+$ was terminated by rapidly washing the cell monolayers three times with 4 volumes of ice-cold isotonic saline solution (130 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM HEPES-NaOH, pH 7.4). The washed cell monolayers were solubilized in 0.25 ml of 0.5 N NaOH, and the wells were washed with 0.25 ml of 0.5 N HCl. Both the solubilized cell extract and the wash solutions were added to

vials, and radioactivity was assayed using a liquid scintillation counter. Under the conditions of H^+-loading used in this study, uptake of $^{22}Na^+$ was linear with time 8-10 min (at low Na^+ concentrations, 22 ºC). Therefore, $^{22}Na^+$ uptakes were measured after 5 min. Measurements of $^{22}Na^+$ influx specific to the Na^+/H^+ exchanger were determined as the difference between the initial rates of H^+-activated $^{22}Na^+$ influx in absence and presence of 1 mM amiloride, an NHE antagonist. Protein content was determined using the Bio-Rad *DC* protein assay procedure.

To examine NHE activity as a function of intracellular H^+ concentration, the pH_i was clamped at different concentration over a range of 5.4-7.4 by suspending the cells in media of varying K^+ concentration containing the K^+/H^+ exchange ionophore, nigericin as described previously (Aharonovitz *et al*, 2000).

Measurement of NHE1 Half-life:

To determine the half-life of $NHE1_{HA}$ in the absence and presence of $CHP3_{myc}$, AP-1 cells stably expressing $NHE1_{HA}$ alone or in conjunction with $CHP3_{myc}$ were grown on 10 cm dishes to near confluence. Plates were treated with cycloheximide (100 μg/ml) in α-MEM supplemented with 10% FBS and penicillin/streptomycin for up to 48 h. At appropriate time points, cell lysates were obtained, and equal volumes were subjected to SDS-PAGE and immunoblotting. Spot densitometry of visualized bands obtained by immunoblotting was performed on an FC1000 gel imaging system and software (Alpha Innotech Corp., San Leandro, CA). Multiple exposures of the same blot were used to obtain relative intensities to account for under or oversaturation of individual bands on each film.

Cell Surface Biotinylation and Pulse-Chase Assay:

A cell surface biotinylation assay (LeBivic *et al*, 1989) was used to measure the relative abundance of plasma membrane $NHE1_{HA}$ in the absence and presence of $CHP3_{myc}$. AP-1 cells grown to subconfluence on 10-cm dishes were co-transfected with 8 μg of plasmid DNA containing $NHE1_{HA}$ and an increasing

ratio of $CHP3_{myc}$ (0-2 μg) to empty vector (pCMV). Cells were also cotransfected with an expression plasmid (1μg) that constitutively expresses green fluorescent protein (pGFP) as a control for transfection efficiency. Thirty six hour post-transfection, cells were washed quickly on ice with ice-cold PBS containing 1mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS-CM), and incubated for 20 min on ice with 0.5 mg/ml sulfo-NHS-SS biotin (Pierce), a water soluble, membrane-impermeable, thiol-cleavable, amine-reactive biotinylation reagent. Cells were quickly washed and incubated twice in quenching buffer (20 mM glycine in PBS-CM) for 7 min each on ice to remove excess biotin. After two more washes in ice-cold PBS-CM, cell lysates were harvested in RIPA buffer. A small fraction of the cell lysates was removed for immunoblotting, and the remainder was incubated with 200 μl 50% NeutrAvidin-Sepharose slurry (Pierce) in RIPA buffer overnight at 4ºC. The bound biotinylated protein complexes were washed six times in RIPA buffer and then eluted with SDS-sample buffer for 20 min at room temperature with gentle rocking. All samples were then subject to SDS-PAGE and immunoblotting analysis.

The half-life of $NHE1_{HA}$ in AP-1 cells in the absence and presence of $CHP3_{myc}$ was determined by growing AP-1 cells stably expressing either $NHE1_{HA}$ or both $NHE1_{HA}$ and $CHP3_{myc}$ to ~90% confluence. Cells were biotinylated and quenched as described above, and after extensive washing to remove excess biotin, cells were returned to growth media at 37ºC, and then cell lysates were prepared at varying times over a 48-h period, with fresh media being added every 12 h to maintain cell viability. At each time point, a small fraction of the cell lysates was removed for immunoblotting, and the remainder was incubated with 200 μl of 50% NeutrAvidin-Sepharose beads to extract the biotinylated proteins. Cell lysates and extracted biotinylated proteins were subject to SDS-PAGE and immunoblot analysis. Relative band intensities of Western blots were obtained through multiple exposures of the same blot to ensure that the signal was within the linear range of the X-ray film. Densitometry measurements were obtained using the FC1000 gel imaging system and software.

RESULTS:

Delineation of the CHP3-binding Domain of NHE1:

An earlier study (Li *et al*, 2003) indicated that CHP3/tescalcin bound to a histidine-tagged fragment of NHE1 encompassing the distal half (amino acids 633 and 815) of its cytoplasmic C-terminus using an *in vitro* affinity blotting assay. However, the precise binding site within this fragment was not delineated nor was the analysis extended to other regions of the cytoplasmic C-terminus where its close paralogs, CHP1 and CHP2, were shown to bind (*i.e.* amino acids 516-540 of human NHE1) (Pang *et al*, 2001, 2002, 2004).

To provide a more comprehensive survey of the potential CHP3-binding site(s) within the cytoplasmic C-terminus of rat NHE1, we performed *in vitro* protein-binding pulldown assays. Purified GST fusion proteins containing the entire cytoplasmic C-terminus of rat NHE1 (amino acids 505-820) as well as smaller segments spanning that region were incubated with ^{35}S-labeled CHP3 protein synthesized *in vitro* using rabbit reticulocyte lysates. Complexes of GST-NHE1 and ^{35}S-labeled CHP3 were isolated, subjected to SDS-PAGE, and analyzed using a PhosphorImager. Contrary to the study by Li *et al* (Li *et al*, 2003), CHP3 associated only with the juxtamembrane region of the C-terminus between residues 505 and 571 (***Fig. 1.1***), similar to the binding region of CHP1 and CHP2.

To further define the binding motif, four hydrophobic amino acids within this region of NHE1 (^{530}FLDHLL^{535}) that were shown previously to be crucial for direct binding of CHP1 and CHP2 (Pang *et al*, 2001, 2002) were simultaneously mutated in the GST-NHE1(505-820) construct to either Ala, Gln, or Arg (FL-A, FL-Q, or FL-R). Mutation of these amino acids completely abolish the interaction of *in vitro* synthesized ^{35}S-labeled CHP3 with GST-NHE1 (505-820) (***Fig. 1.2A***). Because the ^{35}S-labeled CHP3 protein synthesized *in vitro* using rabbit reticulocyte lysates may lack potential post-translational modifications important for binding to other potential sites in the NHE1 C-terminus, we repeated the pulldown assay using whole cell lysates of transfected CHO cells expressing a

Fig. 1.1.

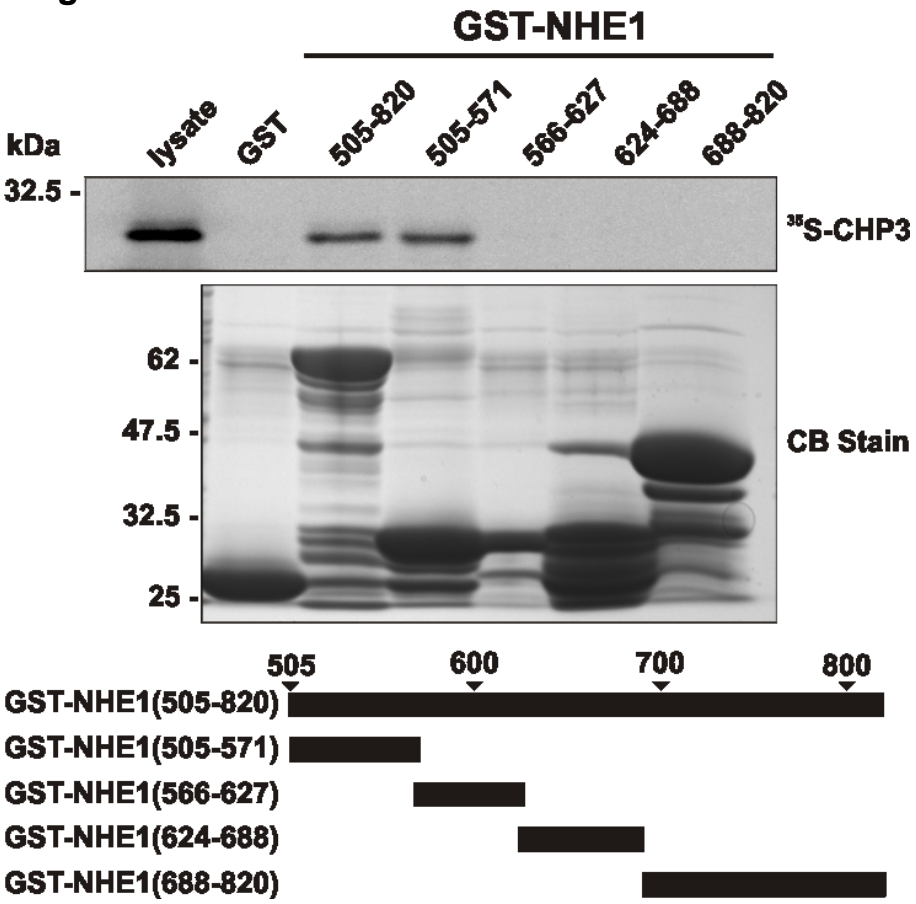


Figure 1.1. **Mapping the CHP3-binding region of NHE1:** Protein binding pulldown assays were used to delineate the site of interaction of CHP3 within the cytoplasmic C terminus of NHE1. GST fusion proteins containing full-length (amino acids 505–820) or partial segments spanning the length of the C terminus of NHE1 were generated in *E. coli*. Purified GST fusion proteins were incubated with ^{35}S-labeled CHP3 protein synthesized *in vitro* in rabbit reticulocyte lysates using a transcription-translation coupling reaction in the presence of [^{35}S]methionine. Complexes of GST-NHE1 and ^{35}S-labeled CHP3 were isolated from the lysates using glutathione-Sepharose™ beads and subjected to SDS-PAGE, as described under "Experimental Procedures." The radioactivity was analyzed using a PhosphorImager (*upper panel*). To verify equivalent gel loading of the GST fusion proteins, a parallel gel was stained with Coomassie Blue (*CB*) dye (*lower panel*). Data shown are representative of at least three independent experiments.

myc-tagged form of CHP3 ($CHP3_{myc}$) and immunoblotting. As shown in ***Fig 1.2B***, $CHP3_{myc}$ bound to wild type GST-NHE1 (505-820) but not to the FL-A, -Q, or –R mutants.

To verify the interaction between NHE1 and CHP3 in intact cells, a mutagenized CHO cell line that lacks endogenous NHE1 (*i.e.* AP-1 cells) was transiently transfected with either full length wild type or mutant forms (FL-A, -Q, or –R) of an HA epitope-tagged construct of NHE1 ($NHE1_{HA}$) and $CHP3_{myc}$. Following a 36-h incubation period, cell lysates were prepared an incubated with an anti-Myc antibody to precipitate $CHP3_{myc}$, and aliquots of the initial lysates and resultant immunoprecipitates were resolved by SDS-PAGE and Western blotting. As shown in ***Fig 1.3A***, each of the cell lysates expresses similar amounts of $CHP3_{myc}$, which migrated as a single band of ~24 kDa, whereas the wild type and mutant $NHE1_{HA}$ proteins migrated as two bands as described previously (Shrode *et al*, 1998), *i.e.* a slower migrating, fully glycosylated form with an apparent molecular mass of ~100 kDa that is present in the plasma membrane and a faster migrating, core-glycosylated form of ~75 kDa that resides intracellularly, likely within the endoplasmic reticulum. Interestingly, each of the NHE1 mutants showed a noticeable reduction in expression of the fully glycosylated form relative to the core glycosylated form, suggesting the processing of these mutants may be partially impaired. Probing of the blots containing the anti-Myc immunoprecipitates with an anti-HA antibody revealed strong immunoreactive bands corresponding to both core- and fully glycosylated forms of wild-type $NHE1_{HA}$, but negligible signals from lysates of cells expressing the $NHE1_{HA}$/FL-A, -Q, or –R mutants. The presence of both immature and mature forms of wild-type $NHE1_{HA}$ in the anti-Myc immunoprecipitates indicates that $CHP3_{myc}$ associates with $NHE1_{HA}$ at an early stage of the transporter's biosynthesis. The reciprocal experiment, whereby wild-type and mutant NHE1 proteins were immunoprecipitated with an anti-HA antibody, also demonstrated that $CHP3_{myc}$ associates only with wild-type $NHE1_{HA}$ (data not shown). In addition, internal deletions of segments of the C-terminus of NHE1 encompassing the CHP3-

Fig. 1.2.

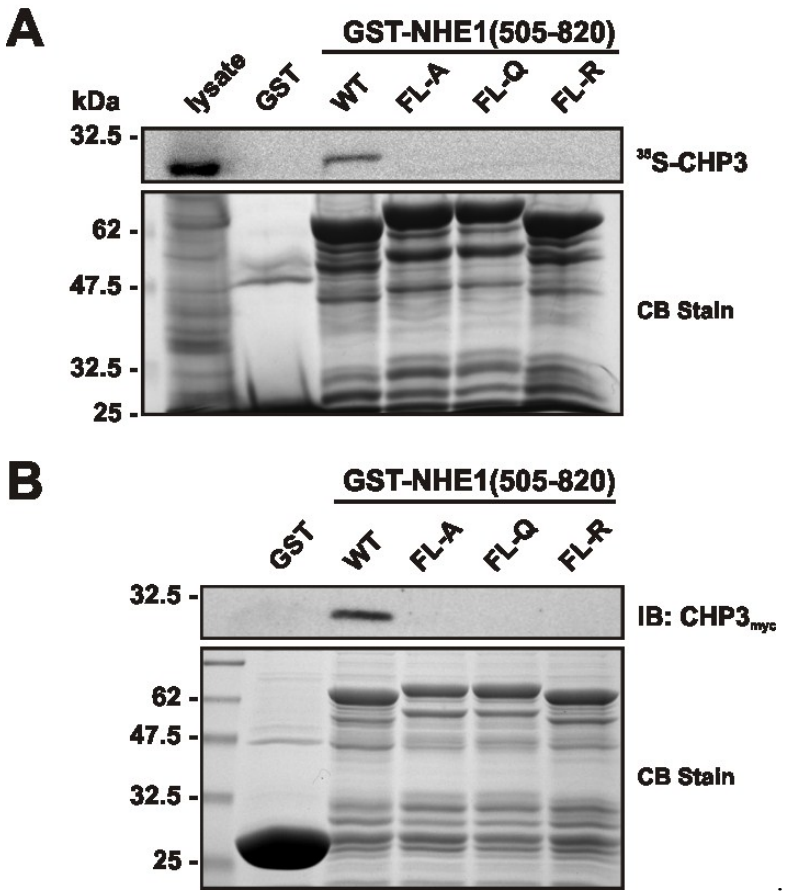


Figure 1.2. **Mutational analysis of the CHP3-binding site of NHE1.** Four hydrophobic amino acids within the juxtamembrane region of NHE1 (^{530}FLDHLL^{535}) that were shown previously to be crucial for direct binding of CHP1 and CHP2 were simultaneously mutated in the GST-NHE1(505–820) construct to either Ala, Gln, or Arg (FL-A, FL-Q, or FL-R). Purified wild-type (*WT*) and mutant GST-NHE1 fusion proteins were incubated with either rabbit reticulocyte lysates containing *in vitro* synthesized ^{35}S-labeled CHP3 protein (***A***) or lysates of CHO cells transiently expressing exogenous CHP3$_{myc}$ (***B***). Complexes of GST-NHE1 and ^{35}S-labeled CHP3 or CHP3$_{myc}$ were isolated using glutathione-Sepharose™ beads and subjected to SDS-PAGE. The levels of ^{35}S-labeled CHP3 were analyzed using a PhosphorImager (***A****, upper panel*), whereas the amounts of CHP3$_{myc}$ were detected by immunoblotting (*IB*) using a primary mouse monoclonal anti-Myc antibody and a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (***B****, upper panel*). To verify equivalent gel loading of the GST fusion proteins, parallel gels were stained with Coomassie blue (*CB*) dye (***A*** and ***B***, *lower panel*). Data shown are representative of at least three independent experiments.

binding domain, either Δ505-540 or Δ505-566, also resulted in the loss of interaction with $CHP3_{myc}$ when evaluated by coimmunoprecipitation (***Fig.* 1.3B**).

To further characterized the association of $NHE1_{HA}$ with $CHP3_{myc}$, AP-1 cells stably expressing either wild-type or mutant forms (FL-A, -Q, or –R) of $NHE1_{HA}$ were transiently transfected with $CHP3_{myc}$ and their subcellular distribution was compared by immunofluorescence confocal microscopy. As shown in ***Fig. 1.4***, wild-type $NHE1_{HA}$ and $CHP3_{myc}$ showed low diffuse staining throughout the cell (non-nuclear), but strongly co-localized at the plasma membrane. By comparison, although CHP3 binding-deficient mutants of $NHE1_{HA}$ also trafficked to the cell surface, $CHP3_{myc}$ was distributed largely throughout the cytoplasm and nucleus. CHP3 also displayed a diffuse distribution in transfected AP-1 cells that lack NHE1 expression (data not shown). Collectively, these analyses confirm that the juxtamembrane region of the C-terminus of NHE1 serves as the principle binding site for CHP3. In addition they indicate that the binding of CHP3 is not essential for targeting of NHE1 to the plasma membrane but may facilitate the processing of the exchanger to its fully glycosylated state (as hinted by data in ***Fig. 1.3***).

Having ascertained that the mutants were appropriately targeted to the cell surface, we proceeded to evaluate the effect of the mutations on the basal rate of transport. The expression level of the exchangers varied among the transfected cell lines, and meaningful comparison of their rates of transport required normalization with respect to the number of plasmalemmal exchangers. This was estimated from the relative intensities of the fully glycosylated NHE1 band in immunoblots of the stably expressed cell lines, as assayed by densitometry. Using this procedure, we compared the rates of Na^+/H^+ exchange in acid-loaded wild-type and mutant cells by measuring rates of H^+-activated $^{22}Na^+$ influx, expressed as picomoles/min/mg or total cellular protein *per* unit density of plasmalemmal NHE1. A comparison of near maximal rates of transport is shown in ***Fig. 1.5A***. Compared with wild type, the efficiency of transport of the FL-A, -Q, and –R mutants was markedly reduced to ~26, 2, and 4%, respectively. Further examination of the rates of transport as a function of H^+ concentration

Fig. 1.3.

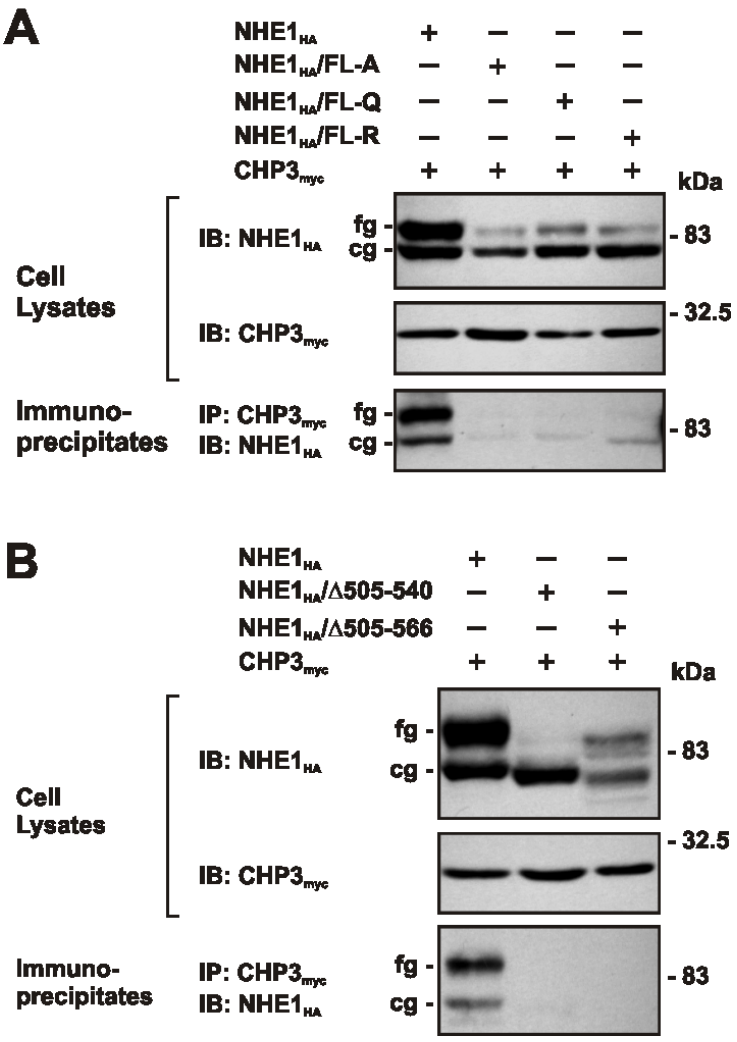


Figure 1.3. **NHE1 forms a complex with CHP3 in transfected cells.** Chinese hamster ovary AP-1 cells were transiently cotransfected with expression plasmids containing full-length $CHP3_{myc}$ and wild-type or mutant $NHE1_{HA}$ containing site-specific substitutions (FL-A, FL-Q, or FL-R) (***A***) or internal deletions (Δ505–540 and Δ505–566) of the CHP3-binding region (***B***), as indicated. After transfection (~36 h), the cells were lysed, and a major fraction was used for immunoprecipitation (*IP*) analyses using a rabbit polyclonal anti-Myc antibody. Immunoblot (*IB*) analyses of the cell lysates and immunoprecipitates were performed as described under "Experimental Procedures." Immunoreactive bands corresponding to the fully glycosylated (*fg*) and core-glycosylated (*cg*) forms of NHE1 are indicated beside the gels. Data shown are representative of three independent experiments.

Fig. 1.4.

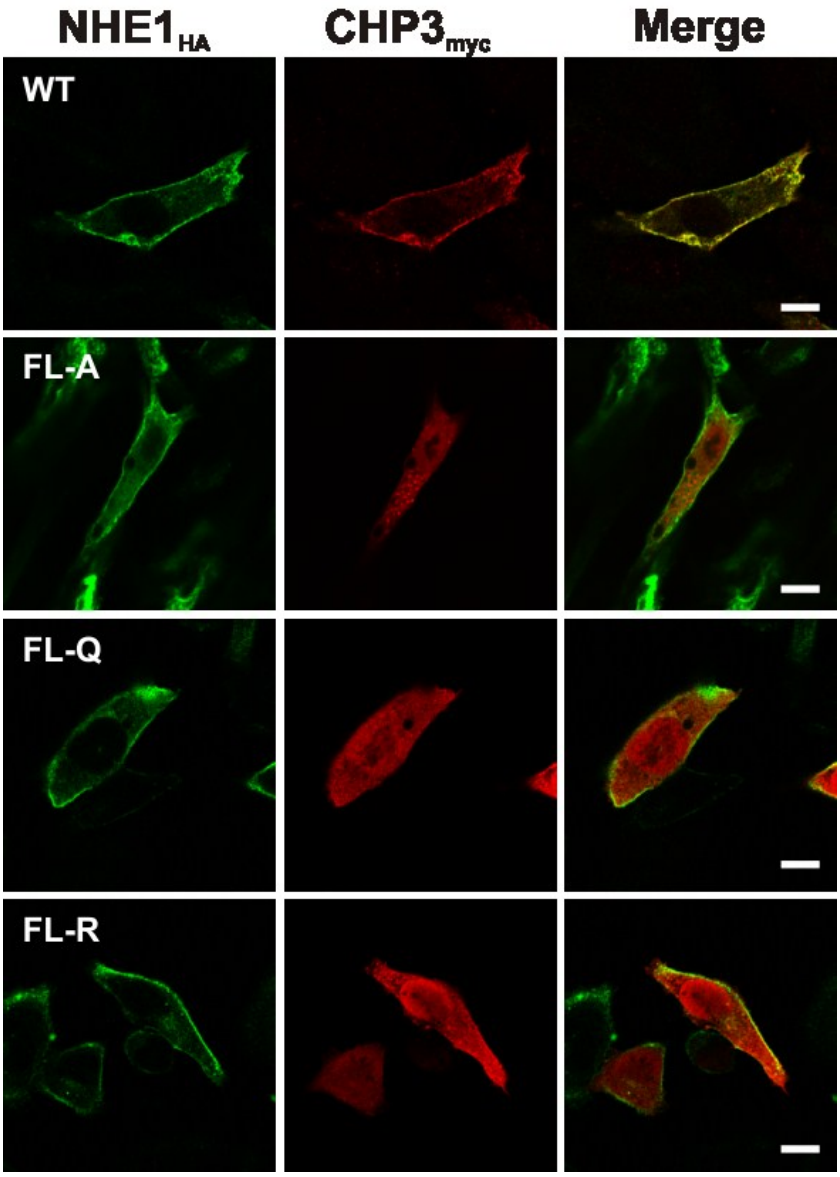


Figure 1.4. **Colocalization of NHE1 and CHP3 in intact cells.** AP-1 cells were stably transfected with either wild-type (*WT*) or mutant forms (FL-A, -Q, or -R) of $NHE1_{HA}$, followed by transient coexpression of $CHP3_{myc}$, and their subcellular distribution was compared by immunofluorescence confocal microscopy. $NHE1_{HA}$ was detected with a primary mouse monoclonal anti-HA antibody and a secondary goat anti-mouse antibody conjugated to AlexaFluor™ 488. $CHP3_{myc}$ was detected with a rabbit polyclonal anti-Myc antibody and a secondary goat anti-rabbit antibody conjugated to Alexa Fluor™ 568. The *scale bar* in the panels on the *right* represents 10 μm.

(normalized to 100% at pH 5.4) showed that substitution of the critical Phe and Leu amino acids with polar residues (*i.e.* Gln or Arg) within the CHP3-binding region caused a profound acidic shift in the H^+ sensitivity of the transporter, whereas substitution with the non-polar Ala residue had no appreciable effect (***Fig. 5B***). Although the basis for the differential effects of the amino acid substitution on H^+ sensitivity is unclear, it is possible that Gln and Arg substitutions, unlike Ala, may cause a more pronounced conformational change in the C-terminus of NHE1 that significantly compromises H^+ sensitivity independent of their effects on the maximal transport velocity (V_{max}) of the transporter. Notwithstanding, the results indicate that the motif capable of binding CHP3 (as well as other CHP isoforms) is essential for optimal Na^+/H^+ exchange, but the nature of the mutations that disrupt CHP binding can also autonomously affect the kinetic properties of the transporters (*i.e.* V_{max} alone or V_{max} and H^+ affinity).

CHP3 Promotes the Maturation and Cell Surface Activity of NHE1:

As mentioned earlier, the data presented in ***Fig. 1.3*** suggests that the binding of CHP3 may also influence the maturation of NHE1. To further examine this possibility, AP-1 cells stably expressing either wild-type $NHE1_{HA}$ or the mildly active $NHE1_{HA}$/FL-A mutant were transiently transfected with increasing amounts of $CHP3_{myc}$-containing expressing plasmid (***Fig. 1.6A***). Increasing levels of $CHP3_{myc}$ resulted in a corresponding increase in the abundance of both the core- and fully glycosylated forms of wild-type $NHE1_{HA}$, whereas the abundance of both forms of $NHE1_{HA}$/FL-A were largely unchanged. The increasing abundance of wild-type $NHE1_{HA}$ as a function of $CHP3_{myc}$ expression also closely correlated with an elevation in its transport activity (***Fig. 1.6B***). By contrast, the effect of $CHP3_{myc}$ on $NHE1_{HA}$/FL-A activity was marginal.

Previous studies have reported that the interaction of CHP2, but not CHP1, with wild-type NHE1 increases the exchanger's affinity for intracellular H^+ in the absence of hormonal or mitogenic stimulation (Pang et al, 2002). To determine whether activation of NHE1 in the presence of CHP3 could also reflect a change

Fig. 1.5.

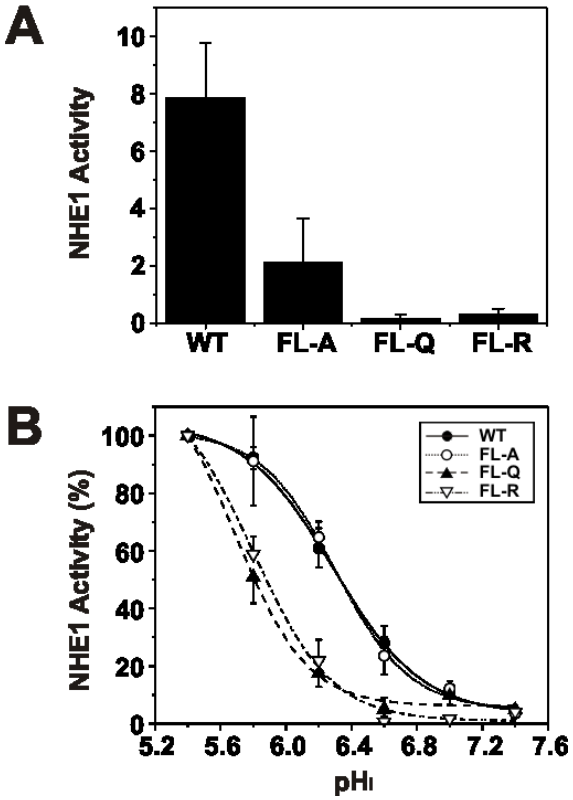


Figure 1.5. **Comparative analysis of rates of H^+-activated $^{22}Na^+$ influx of AP-1 cells transfected with wild-type or mutant forms of NHE1.** AP-1 cells stably transfected with wild-type (*WT*) or mutant forms (FL-A, -Q, or -R) of $NHE1_{HA}$ were grown to confluence in 24-well plates. Initial rates of amiloride-inhibitable H^+-activated $^{22}Na^+$ influx were measured at various intracellular H^+ concentrations over the range of pH_i 5.4 to 7.4. The pH_i was adjusted by the K^+-nigericin method, as described under "Experimental Procedures." To facilitate comparison of the effects of mutating the CHP-binding site, the rates of $^{22}Na^+$ influx (pmol/min/mg total cellular protein) of wild-type and mutant forms of $NHE1_{HA}$ were normalized to their respective plasmalemmal (fully glycosylated) protein levels, as determined by densitometry. ***A***, comparison of the near-maximal velocities of the various NHE1 constructs. ***B***, percentage of the transport velocities of WT and mutant NHE1 as a function of the intracellular H^+ concentration, each normalized to their respective maximal rates of uptake. Values represent the mean ± S.E. of three experiments, each performed in triplicate. *Error bars* smaller than the symbol are absent.

Fig. 1.6.

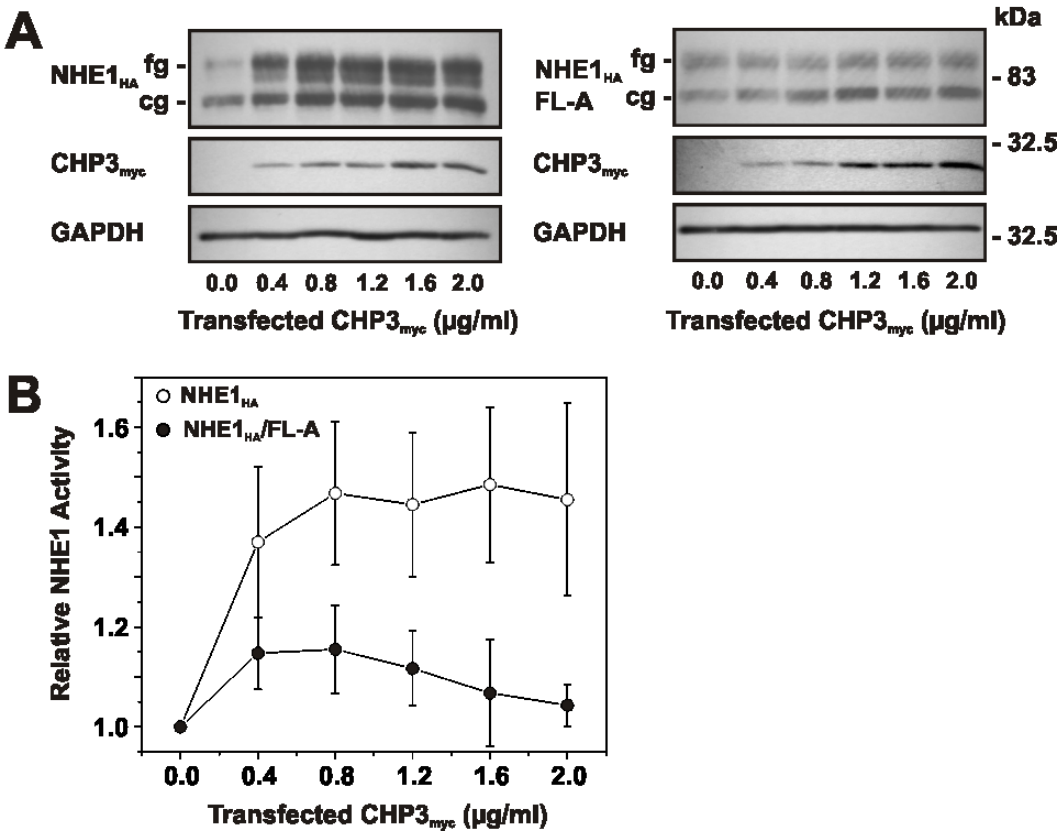

A
NHE1HA fg
cg
CHP3myc
GAPDH
0.0 0.4 0.8 1.2 1.6 2.0
Transfected CHP3myc (μg/ml)
NHE1HA fg
FL-A cg
CHP3myc
GAPDH
kDa
83
32.5
32.5
0.0 0.4 0.8 1.2 1.6 2.0
Transfected CHP3myc (μg/ml)
B
NHE1HA
NHE1HA/FL-A
Relative NHE1 Activity
1.6
1.4
1.2
1.0
0.0 0.4 0.8 1.2 1.6 2.0
Transfected CHP3myc (μg/ml)

Figure 1.6. **Effect of overexpression of CHP3 on NHE1 abundance and activity.** AP-1 cells stably expressing either wild-type $NHE1_{HA}$ or CHP3-binding defective mutant $NHE1_{HA}$/FL-A were cultured to subconfluence in 6-well plates (10-cm^2/well) for immunoblot analyses (***A***) or 24-well plates (2-cm^2/well) for measurement of NHE1 activity (***B***), and then transiently transfected with empty vector or increasing amounts of the $CHP3_{myc}$-containing expression plasmid relative to empty vector. The total concentration of the transfected plasmid DNA remained constant at 2 μg of DNA/ml serum-free culture medium (2.5 ml per 10-cm^2 dish or 0.5 ml per 2-cm^2 dish). ***A***, following transfection (24 h), cell lysates were prepared and analyzed for $NHE1_{HA}$ and $CHP3_{myc}$ expression by SDS-PAGE and immunoblotting. Immunoreactive bands corresponding to the fully glycosylated and core-glycosylated forms of $NHE1_{HA}$ and CHP_{myc} were detected using a primary mouse monoclonal anti-HA and anti-Myc antibody, respectively, and a secondary goat anti-mouse antibody conjugated to horseradish peroxidase. As a control for protein loading, the blots were stripped and reprobed for expression of endogenous GAPDH using a primary mouse monoclonal anti-GAPDH antibody and a secondary goat anti-mouse antibody conjugated to horseradish peroxidase. ***B***, Na^+/H^+ exchange activity of cells expressing wild-type $NHE1_{HA}$ and mutant $NHE1_{HA}$/FL-A were measured as a function of CHP3 abundance. NHE activity was determined as the initial rates of amiloride-inhibitable $^{22}Na^+$ influx following an acute intracellular acid load induced by prepulsing with NH^+_4, as described under "Experimental Procedures." To facilitate comparison, the data were normalized to cells that do not express CHP3. Values represent the mean ± S.E. of three experiments, each performed in triplicate.

in H^+ affinity, $CHP3_{myc}$ was stably coexpressed in the established AP-1/$NHE1_{HA}$ cell line. As shown in ***Fig. 1.7***, the pH profile and calculated half-maximal activity or H^+ affinity (K_H) of $NHE1_{HA}$ in the presence of $CHP3_{myc}$ (K_H = 6.36 ± 0.04) was not markedly different from $NHE1_{HA}$ alone (K_H = 6.29 ± 0.04). Collectively, the above data support the notion that CHP3 promotes the maturation and accumulation of wild-type NHE1 at the cell surface.

To further explore a potential role for CHP3 in the maturation of NHE1, we examined the processing of newly synthesized NHE1 in the absence or presence of CHP3 as a function of time. To this end, $NHE1_{HA}$ alone or in combination with $CHP3_{myc}$ was transiently transfected into AP-1 cells, and their expression was analyzed at various time points over a 72-h period. As shown in ***Fig. 1.8*****,** cells expressing $NHE1_{HA}$ alone showed a gradual increase in the formation of both the core- and fully glycosylated forms of the exchanger, with the production of the latter lagging expectedly behind the former, but both peaking at 36 h. By comparison, cells coexpressing $NHE1_{HA}$ and $CHP3_{myc}$ showed a similar time course for production of core- and fully glycosylated forms, but the abundance of the fully glycosylated form was markedly increased relative to core-glycosylated form and correlated closely with the relative abundance of $CHP3_{myc}$.

To verify that the CHP3-mediated increase in production of fully glycosylated NHE1 protein and transport activity also paralleled its accumulation at the cell surface, we performed an analogous experiment to that described in ***Fig. 1.6***. AP-1 cells were transiently transfected with fixed amounts of $NHE1_{HA}$ and an increasing ration of $CHP3_{myc}$ to empty vector (pCMV). The cells were also cotransfected with an expressing plasmid (pGFP) that constitutively expresses green fluorescent protein as a control for transfection efficiency. Thirty six hours post-transfection, a cell surface biotinylation method (LeBivic et al, 1989) was used to selectively extract the plasma membrane proteins for analysis of NHE1 abundance by immunoblotting. As illustrated in ***Fig. 1.9***, the fully glycosylated form of $NHE1_{HA}$ was the predominant species detected at the cell surface and increased as a function of the level of $CHP3_{myc}$.

Fig. 1.7.

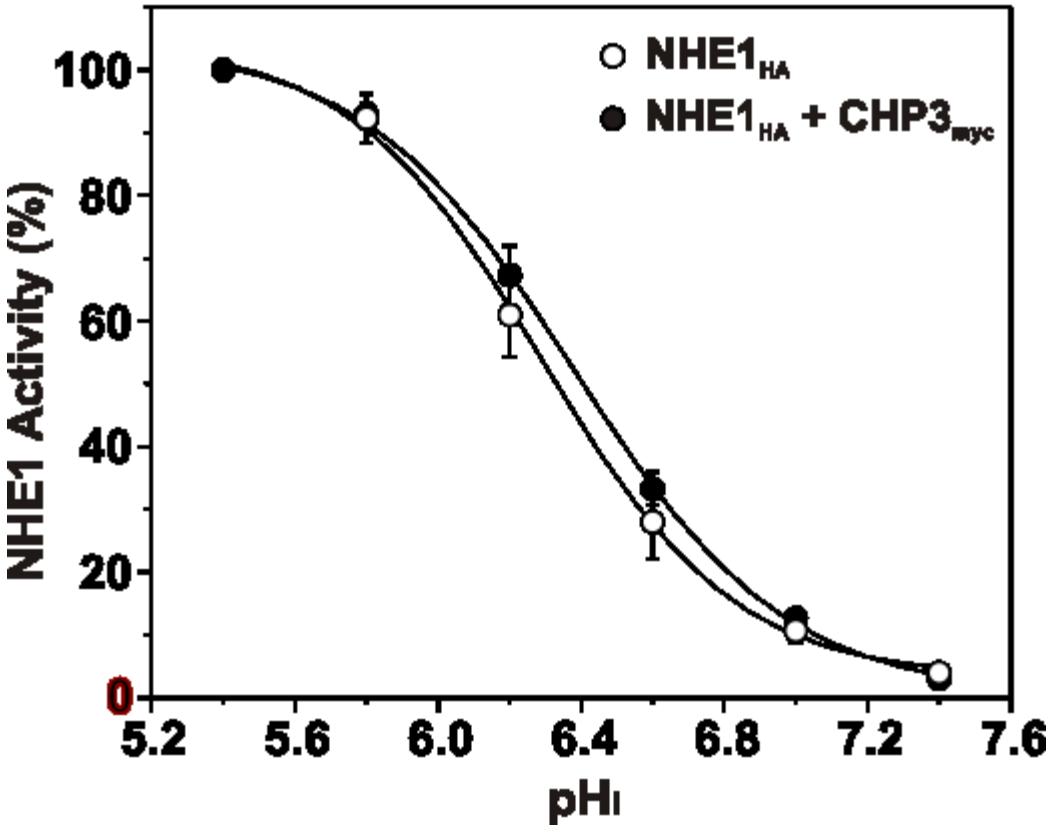


Figure 1.7. **Effect of CHP3 on affinity of NHE1 for intracellular protons.** AP-1 cells stably expressing wild-type $NHE1_{HA}$ alone or stably coexpressing $NHE1_{HA}$ and $CHP3_{myc}$ were cultured to confluence in 24-well plates. Initial rates of amiloride-inhibitable H^+-activated $^{22}Na^+$ influx were measured at various intracellular H^+ concentrations over the range of pH_i 5.4 to 7.4. The pH_i was adjusted by the K^+-nigericin method, as described under "Experimental Procedures." To facilitate comparison of the effects of CHP3, the data were normalized to their respective maximal rates of uptake. Values represent the mean ± S.E. of three experiments, each performed in triplicate. *Error bars* smaller than the symbol are absent.

Fig. 1.8.

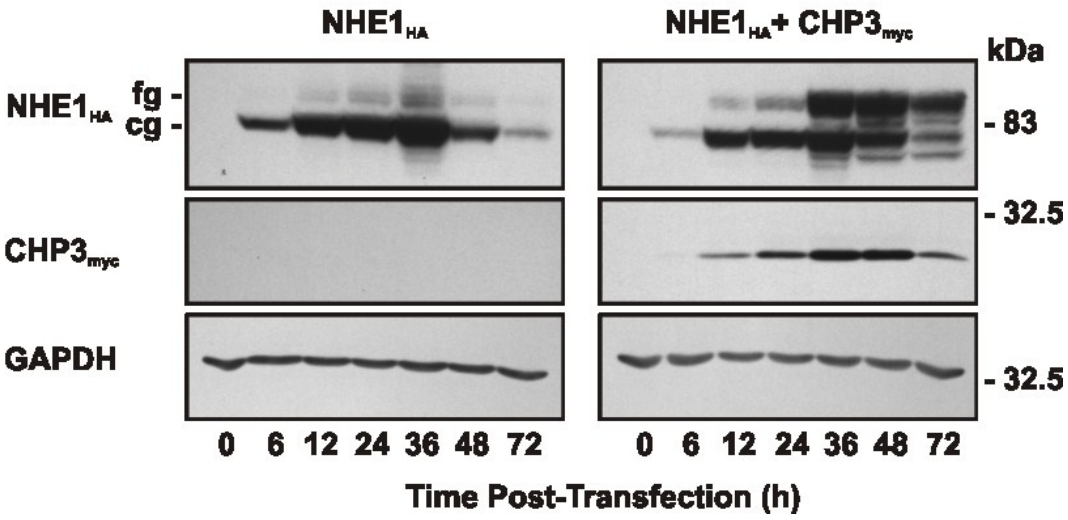


Figure 1.8. **Effect of CHP3 on biosynthetic maturation of NHE1.** AP-1 cells were transiently cotransfected with $NHE1_{HA}$ and empty vector or $CHP3_{myc}$. Cell lysates were prepared at the indicated time points following transfection and subjected to SDS-PAGE and immunoblotting to detect expression of fully glycosylated (*fg*) and core-glycosylated (*cg*) forms of $NHE1_{HA}$ and $CHP3_{myc}$ as described in the legend to *Fig. 6.* Blots were stripped and reprobed for expression of endogenous GAPDH as a control for protein loading. Data shown are representative of three independent experiments.

Fig. 1.9.

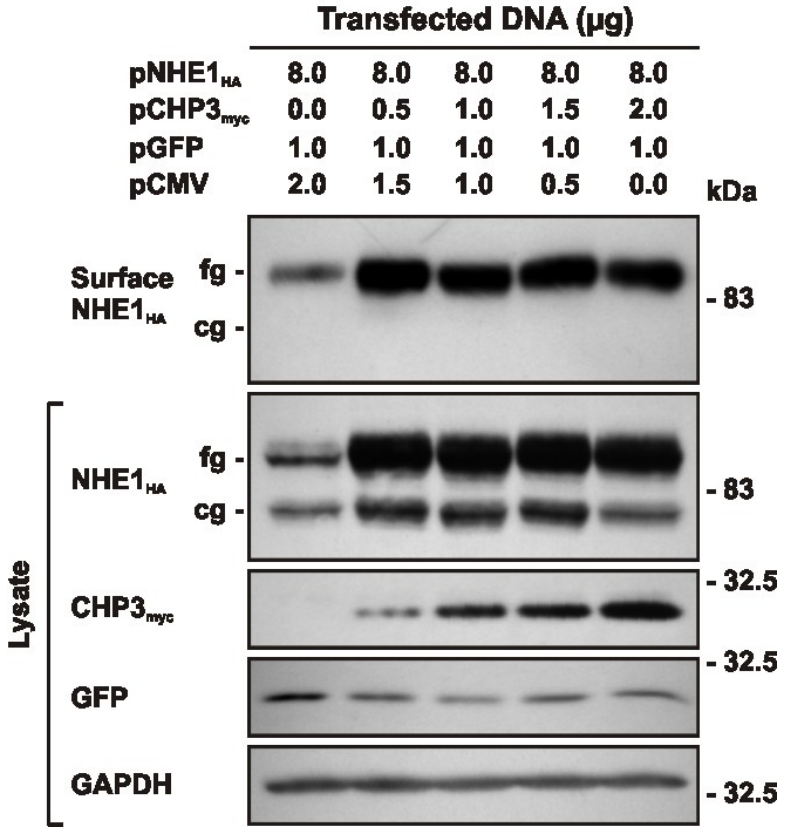


Figure 1.9. **Effect of CHP3 on abundance of the cell surface fully glycosylated form of NHE1.** AP-1 cells were transfected with a fixed amount of expression plasmid DNA containing $NHE1_{HA}$ and an increasing ratio of $CHP3_{myc}$ to empty expression vector (pCMV). Cells were also cotransfected with a plasmid that constitutively expresses green fluorescent protein (pGFP) as a control for transfection efficiency. At 36 h post-transfection, cells were subjected to surface biotinylation as described under "Experimental Procedures," and whole cell lysates were prepared. A major fraction of the lysates was subsequently incubated with NeutrAvidin-Sepharose beads to isolate the biotinylated cell surface proteins. Aliquots of the whole cell lysates and biotinylated cell surface proteins were subjected to SDS-PAGE and immunoblotting. Expression of fully glycosylated (*fg*) and core-glycosylated (*cg*) forms of $NHE1_{HA}$ and $CHP3_{myc}$ were detected as described in *Fig. 6.* GFP was detected using a primary rabbit polyclonal anti-GFP antibody and a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase. Immunoblots of the lysates were stripped and reprobed for endogenous GAPDH as a control for protein loading. Data shown are representative of three independent experiments.

Role for CHP3 in Cell Surface Half-life of NHE1:

Mechanistically, the CHP3-mediated increase in fully glycosylated NHE1 levels could arise from accelerated processing of newly translated NHE1 along the biosynthetic pathway, as suggested by the data in ***Fig. 1.8***, but could also reflect increased stability of the mature protein at the cell surface, albeit these processes are not necessarily mutually exclusive. To test the latter hypothesis, the half-life of fully glycosylated $NHE1_{HA}$ at the cell surface was measured in the absence and presence of $CHP3_{myc}$ using two different approaches as follows: (i) monitoring the disappearance of fully glycosylated NHE1 over time following cycloheximide blockage of de novo protein synthesis, and (ii) measuring the longevity of fully glycosylated $NHE1_{HA}$ by tagging the cell surface transporter with biotin (to facilitate its isolation) and trafficking its existence as a function of time. For these assays, we generated AP-1 cells that stably express either $NHE1_{HA}$ alone or both NHE_{HA} and $CHP3_{myc}$.

With respect to the first approach (presented in ***Fig. 1.10A***, it is noteworthy that in untreated AP1/$NHE1_{HA}$ cells, relative fractions of core- and fully-glycosylated $NHE1_{HA}$ are similar, whereas in AP-1/$NHE1_{HA}$ + $CHP3_{myc}$ cells, the bulk of $NHE1_{HA}$ exists in its fully glycosylated state. These relative shifts in the proportions of the two glycosylated states of $NHE1_{HA}$ closely parallel the patterns observed using transiently transfected cells. Upon treatment with cycloheximide (100 μg/ml), the core-glycosylated form of $NHE1_{HA}$ in AP-1/$NHE1_{HA}$ cells gradually decreases with time, presumably reflecting maturation to the fully glycosylated form and/or protein degradation. Similarly, the fully glycosylated pool of $NHE1_{HA}$ was also markedly reduced by ~58% over the 48-h treatment period, as quantified by densitometry (***Fig. 1.10B***). In AP-1 cells stably coexpressing both $NHE1_{HA}$ and $CHP3_{myc}$, the minor levels of core-glycosylated $NHE1_{HA}$ also diminished as a function of time in the presence of cycloheximide.

To provide a more direct measure of the half-life of fully glycosylated $NHE1_{HA}$ present at the plasma membrane, we also performed a pulse-chase assay. Cell surface proteins were labelled with biotin using the membrane-impermeant reagent, sulfo-NHS-SS-biotin, for 30 min on ice. The excess reagent was then

Fig. 1.10.

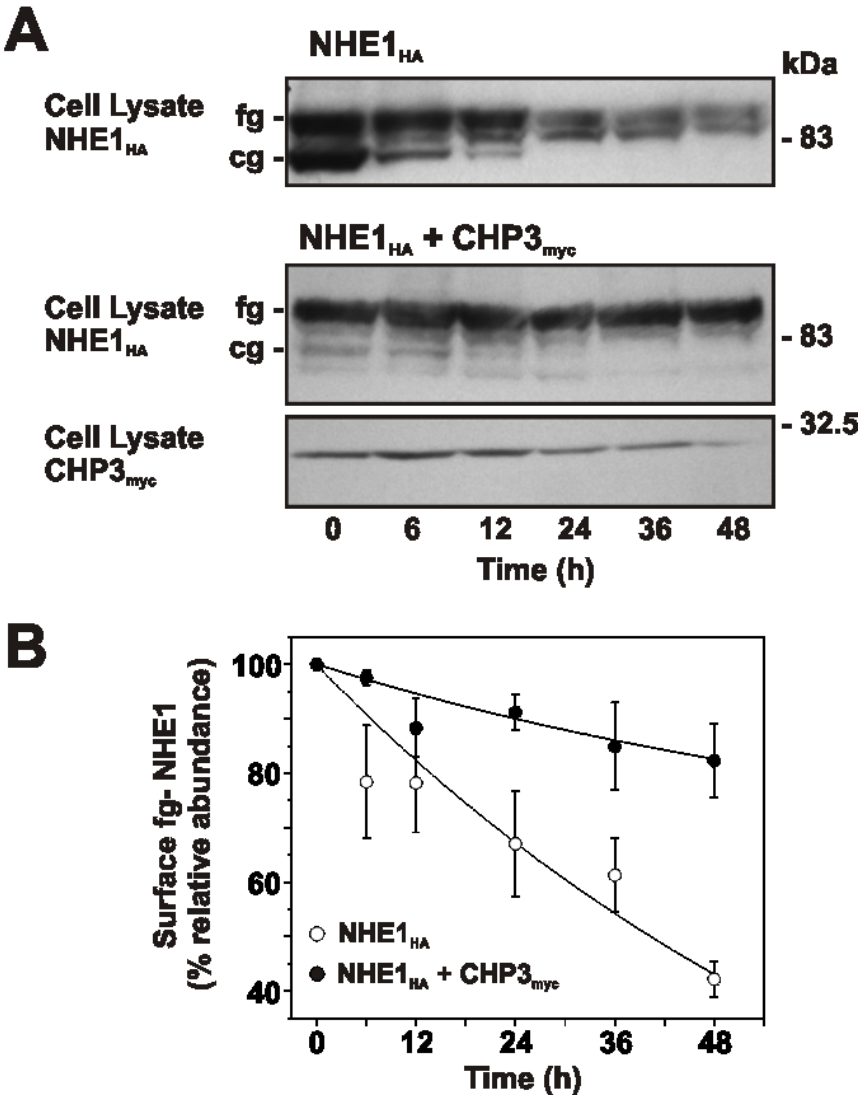


Figure 1.10. Effect of CHP3 expression on half-life of NHE1 upon arrest of cellular protein synthesis. ***A***, AP-1 cells stably expressing either $NHE1_{HA}$ alone or coexpressing both $NHE1_{HA}$ and $CHP3_{myc}$ were grown to subconfluence and then subjected to treatment with cycloheximide (100 μg/ml) to block *de novo* protein synthesis. At the indicated time points, cell lysates were prepared and fractionated by SDS-PAGE, followed by immunoblotting to visualize fully glycosylated (*fg*) and core-glycosylated (*cg*) $NHE1_{HA}$ and $CHP3_{myc}$. ***B****,* fully glycosylated bands of $NHE1_{HA}$ presented in *A* were quantified by densitometry and displayed as a percentage of their maximal levels as a function of time following cycloheximide treatment. Values represent the mean ± S.E. of four experiments.

removed by quenching with glycine-enriched buffer and extensive washing, followed by incubation in regular culture media over a 48-h period. At each time point, the biotinylated proteins were extracted from the various cell lysates using NeutrAvidin™-Sepharose beads, fractionated by SDS-PAGE, and analyzed by immunoblotting. As shown in ***Fig. 1.11***, A and B, the half time of biotinylated, fully glycosylated $NHE1_{HA}$ was 3-fold higher in the presence of $CHP3_{myc}$ then in its absence (12.3 ± 1.5 h versus 4.1 ± 0.3 h, respectively). For each time point, the total cellular steady-state levels of $NHE1_{HA}$, or $NHE1_{HA}$ and $CHP3_{myc}$ were constant. These results corroborate the finding using cycloheximide and validate the hypothesis that CHP3 enhances the stability of NHE1 at the plasma membrane.

Fig. 1.11.

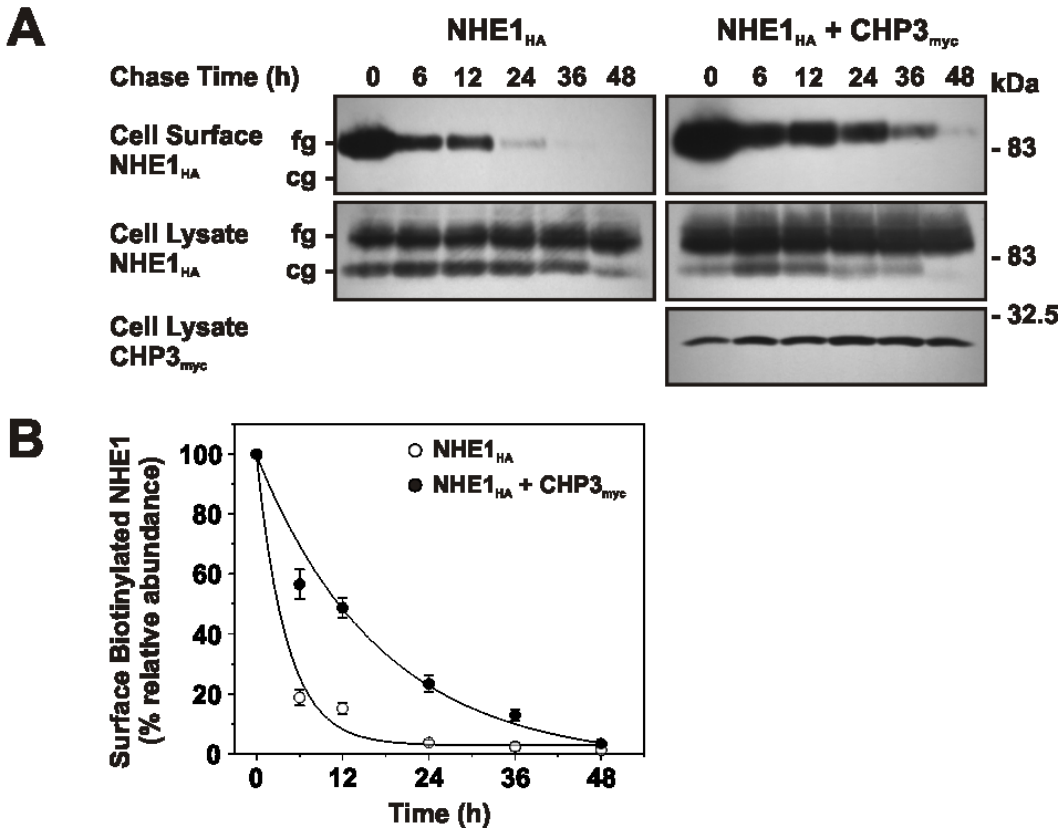


Figure 1.11. Effect of CHP3 on half-life of cell surface NHE1 tagged by biotinylation. ***A,*** AP-1 cells stably expressing $NHE1_{HA}$ or stably coexpressing $NHE1_{HA}$ and $CHP3_{myc}$ were subject to cell surface biotinylation, as described under "Experimental Procedures." The cells were returned to growth media at 37 °C, and then cell lysates were prepared at varying times over a 48-h period. At each time point, a small fraction of the cell lysates was removed for immunoblotting, and the remainder was incubated with 200 μl of NeutrAvidin-Sepharose beads to extract the biotinylated proteins. Total cellular levels of fully glycosylated (*fg*) and core-glycosylated (*cg*) $NHE1_{HA}$ and $CHP3_{myc}$ as well as levels of surface biotinylated, fully glycosylated $NHE1_{HA}$ were monitored as a function of time by SDS-PAGE and immunoblotting, as described in the legend to *Fig. 6.* ***B,*** data represent densitometric analysis of the cell surface biotinylated $NHE1_{HA}$ presented in ***A***, normalized as a percentage of its maximal abundance at time 0 h. Values represent the mean ± S.E. of four experiments. *Error bars* smaller than the symbol are absent.

DISCUSSION:

This study delineates the site of interaction and functional relevance of the association between the ubiquitously expressed NHE1 isoform and the third member of the CHP family of EF-hand Ca^{2+}-binding proteins, CHP3/tescalcin. Using a combination of biochemical, immunological, and cellular techniques, we demonstrate that CHP3 binds to the same juxtamembrane segment of the cytoplasmic C-terminus of NHE1 (amino acids 516-540 of human or 520-544 of rat NHE1, which are identical between these two species as well as other mammals) as CHP1 and CHP2. Recent high resolution structural analyses have revealed that this segment is an amphipathic α-helix whose apolar face is embrace by an extended hydrophobic cavity present in both CHP1 (Naoe *et al*, 2005; Mashima *et al*, 2007) and CHP2 (Ammar *et al*, 2006). Because the same amino acids of NHE1 that confer binding to CHP1 and CHP2 are also important for the interaction of CHP3, it is likely that a similar NHE1-CHP3 complex is formed.

Functional analyses have revealed that overexpression of CHP3 enhances wild-type NHE1 activity at the cell surface chiefly by enhancing its biosynthetic maturation and stability at the plasma membrane, while having little detectable impact on its intracellular H^+ sensitivity. Mutations of NHE1 (FL-A, -R, and –Q) that disrupt the binding of CHP3 (as well as other CHPs) also appeared to impede (see ***Fig. 3A***), but not prevent, the trafficking of fully glycosylated NHE1 to the cell surface. Interestingly, these mutations also caused a pronounced reduction in intrinsic NHE1 activity. In the case of the FL-A mutant, loss of CHP3 binding markedly reduced its maximal rate of transport (V_{max}) but did not appreciably alter its affinity for intracellular H^+. However, for the FL-Q and FL-R mutants, drastic reductions were observed not only in V_{max}, but also in H^+ sensitivity. These more radical substitutions, aside from disrupting CHP3 binding and NHE1 stability, may cause a more dramatic change in conformation of the C-terminus that is sufficient to broadly perturb cation translocation. Despite these differences, collectively the results indicate that the binding of CHP3 (as well as other CHP

isoforms) is essential for optimal Na^+/H^+ exchange, both in terms of protein stability and catalysis.

Our finding are in contrast to an earlier study (Li *et al*, 2003) showing that CHP3/tescalcin bound to the distal half of the C-terminal NHE1 and suppressed NHE1 activity in transfected CHO cells. The apparent discrepancies between the two studies are difficult to reconcile but may reflect differences in experimental approaches. For instance, in the former report (Li *et al*, 2003), the binding of CHP3 was tested only on a single fragment derived from the extreme C-terminus of human NHE1 (between amino acids 633-815; equivalent to amino acids 638-820 of rat NHE1) using an *in vitro* affinity blotting assay. Under the conditions employed, this technique may have revealed a low affinity binding site for CHP3 that was not readily detected using the more stringent approaches applied in this study. Alternatively, amino acid differences in this region (18 of 183 amino acids) between human and rat NHE1 could also be a factor. However, these sites are not highly conserved among mammalian species, suggesting they are unlikely to play a major role in the binding for critical regulatory proteins such as CHP. Because the precise location of this distal C-terminal site in human NHE1 was not delineated further nor manipulated experimentally, its functional relevance remains obscure. A more difficult issue to resolve between the two studies is the opposing effects of CHP3 on NHE1 activity. Although different assay methods were used to assess NHE1 activity, *i.e.* fluorimetric measurements of Na^+-dependent H^+ efflux using the pH sensitive dye 2'7'-bis(carboxyethyl)-5,6-carboxyfluorescene (Li *et al*, 2003) *versus* radioisotopic measurements of H^+-activated $^{22}Na^+$ influx used herein, they are complementary techniques and in principle should provide equivalent outcomes. Further investigation will be required to resolve these disparities.

In comparison to CHP3, earlier studies indicated that CHP1 and CHP2 were primarily involved in regulating the H^+ sensitivity of NHE1 as well as other plasma membrane-type NHEs (Pang *et al*, 2001, 2002, 2004), albeit in subtly different ways. CHP1 appeared critical for setting the resting intracellular H^+ sensitivity of the exchanger in the neutral pH range and modulating its

responsiveness to various stimuli (Pang *et al*, 2001, 2004; DiSole *et al*, 2004). By contrast, CHP2 bound NHE1 with several fold higher affinity than CHP1 and appeared to constitutively activate the transporter in the absence of external stimuli, resulting in a marked alkalinisation of steady-state intracellular pH relative to cells expressing only CHP1 (Pang *et al*, 2002). However, more recent evidence indicates that CHP1 may also influence NHE1 protein stability (Matsushida *et al*, 2007). Analyses of chicken B lymphoma DT40 cells containing null mutations of CHP1 showed a significant reduction in total cellular abundance of NHE1 protein without affecting its mRNA expression, an effect consistent with either a decreased rate of translation and/or reduced post-translational processing and stability of NHE1 (Matsushida *et al*, 2007). Whether CHP2 also regulates NHE1 protein stability is unknown. Taken together, these data suggest that members of the CHP family act as positive regulators of NHE1 (and possibly other plasmalemmal NHEs) by enhancing its post-translational maturation and stability, maximal velocity, and/or intrinsic sensitivity to intracellular H^+ in a manner that is dependent on the CHP isoform. In cells expressing multiple CHP isoforms, it is conceivable that these ancillary proteins could act in a temporal and spatial manner to control different facets of NHE1 biosynthesis and function.

Other studies have also highlighted the critical importance of the proximal cytoplasmic C-terminus for the proper functioning of the exchanger. In addition to binding CHP, this region contains two positively charged amino acid clusters that immediately flank the CHP-binding site and interact directly with the inositol phospholipid PIP_2 located in the inner leaflet of the plasma membrane (Aharonovitz *et al*, 2000). Mutations of NHE1 that disrupt the binding of PIP_2 or biochemical manipulations that sequester or deplete PIP_2 in intact cells greatly reduce the transport capability of NHE1. In addition, the distal positively charged amino acid cluster has also been shown to bind the actin binding proteins ezrin, radixin, and moesin, an association that appears vital for proper organization of focal adhesions, actin stress fibres, and cell shape (Denker *et al*, 2000). Thus, although the precise structural basis for these functional effects is uncertain, the binding of CHP1, -2, -3, PIP_2, and ezrin/radixin/moesin, operating separately or in

conjunction, may act to orient the juxtamembrane cytoplasmic C-terminus of NHE1 in tight apposition with the inner membrane surface. Such a configuration may support enhanced protein maturation, stability, and optimal transport at the cell surface.

In addition to their effects on the NHEs, the CHPs also regulate other cellular processes. CHP1 (also referred to a p22) was found to play a significant role in constitutive transcytotic targeting of apical membrane proteins in epithelial cells (Barroso *et al*, 1996). Subsequent analysis revealed that CHP1/p22 interacts with several proteins linked to the movement of vesicles along the microtubules, including the kinesin-related motor protein KIF1Bβ2 (Nakamura *et al*, 2002) and the multifunctional enzyme glyceraldehydes-3-phosphate dehydrogenase (Andrade *et al*, 2004). Moreover, CHP1/p22 expression was shown to modulate the assembly and dynamics of the microtubule cytoskeleton and endoplasmic reticulum network (Andrade *et al*, 2004). Thus, CHP1 appears to play a broad role in the movement of membrane proteins along the biosynthetic pathway. With regard to CHP3/tescalcin, up-regulation of this isoform was found to be essential for the expression of members of the Ets family of transcription factors (*i.e.* Fli-1 and Ets-2) that promote differentiation of hematopoietic progenitor cells along the megakaryocyte lineage (Levay and Slepak, 2007). Whether this phenomenon is linked to changes in cellular pH or through an independent pathway is unknown.

In summary, these data provide new insight into the importance of CHP3 for the optimal functioning of the Na^+/H^+ exchanger NHE1 isoform. CHP3 positively regulates NHE1 activity by promoting its rate of biosynthetic maturation and half-life at the cell surface as well as its maximal rate of transport.

Preamble to Chapter 2:

Having ascertained the role of CHP3 in the maturation and stability of NHE1 at the plasma membrane, we next examined the possible roles of N-myristoylation and the binding of Ca^{2+} to CHP3 and the effects these post-translational modifications may have on the NHE1/CHP3 interaction. Numerous studies of various EF-hand containing proteins have demonstrated the important role Ca^{2+} binding has on the function of these proteins, including calmodulin (CaM) and CHP1/p22. Furthermore, the Ca^{2+}-myristoyl switch proteins are a class of proteins by which the binding of Ca^{2+} results in a conformational change that exposes the N-myristoylation motif, thereby allowing the protein to fulfill physiological functions when activated by elevated concentration of intracellular Ca^{2+}. Thus far, Ca^{2+} stimulation of NHE1 activity has been largely attributed to the binding of activated Ca^{2+}/CaM to the transporter. This binding is thought to induce a conformation change that enhances the H^+ affinity of NHE1. However, it is conceivable that part of the Ca^{2+}-mediated upregulation of NHE1 may be conferred by the binding of the Ca^{2+}/CHP3 complex. To this end, the N-myristoylation and Ca^{2+}-binding sites of CHP3 were modified by site-directed mutagenesis and the functional consequences on NHE1 activity were evaluated by biochemical, immunological and cellular techniques. Our results demonstrate that these two modifications are not crucial for CHP3 binding to NHE1 or for post-translational maturation and trafficking of the exchanger to the cell membrane, but they are critical for cell surface stability and optimal Na^+/H^+ exchange activity.

Chapter 2:

N-myristoylation and Ca^{2+}-Binding of the Calcineurin B-Homologous Protein 3 (CHP3) is Required for Cell Surface Stability, but not Biosynthetic Maturation of the Na^{+}/H^{+} Exchanger Isoform 1.

Hans-Christian Zaun, Alvin Shrier, John Orlowski.

The contents of this chapter are in preparation for submission.

ABSTRACT:

The plasma membrane sodium/proton exchanger isoform 1 (NHE1) plays a critical role in regulating intracellular pH in order to provide a suitable ionic environment for optimal cell metabolism, growth proliferation and survival. Various stimuli, including hormones, growth factors and second messengers such as Ca^{2+}, regulate NHE1 activity by either phosphorylation-dependent or independent mechanisms; the latter involving binding of ancillary proteins and phospholipids to the cytoplasmic C-terminus of the transporter. One such class of interacting proteins is a family of N-myristoylated EF-hand Ca^{2+}-binding proteins called the calcineurin B homologous proteins (CHPs). Previous work has shown that the heart-enriched isoform, CHP3, functions to increase the NHE1 activity by promoting its biosynthetic maturation and cell surface stability (Zaun *et al*, 2008). Previous studies have shown that the CHP proteins bind Ca^{2+} at micromolar affinities and are N-myristoylated. However, the functional relevance of these domains with respect to CHP3 regulation of NHE1 has not been determined. Mutation of either the N-myristoylation site (G2A) or the calcium-binding domain (D123A) demonstrated that neither is crucial for the interaction of CHP3 with NHE1 nor for the post-translational oligosaccharide maturation and membrane trafficking of the exchanger. However, both sites were required to enhance the half-life and activity of NHE1 at the cell surface. These results suggest that both domains in CHP3 are interdependent and selectively stabilize the NHE1/CHP3 complex at the cell surface.

INTRODUCTION

The maintenance of intracellular pH (pH_i) and cell volume is of crucial importance for the survival of all living cells. Almost all cellular processes including metabolism, cell growth and proliferation, as well as contraction of cardiac, skeletal, and smooth muscles cells, are dependent on the maintenance of intracellular pH homeostasis within an optimal range. The Na^+/H^+ exchangers (NHEs) are a family of at least eleven (glyco)phosphoproteins that are responsible for the electroneutral countertransport of alkali cations such as Na^+, but in some cases also Li^+ or K^+, for H^+ across the plasma membrane as well as organellar membranes (Wakabayashi *et al*, 1997; Counillon and Pouysségur, 2000; Orlowski and Grinstein 2004, 2007;Brett *et al*, 2005).

NHE1 has been the most extensively studied isoform, mainly due to its ubiquitous expression and role in numerous cellular processes. This isoform has also been found to play an major role in various pathophysiological states including hypertension (Bobulescu *et al*, 2005), cancer (Harguinday *et al,* 2005), cellular damage as a result of ischemia/reperfusion, as well as cardiac hypertrophy and heart failure (Karmazyn *et al,* 2005; Orlowski and Grinstein 2004). Although NHE1 is considered to be the housekeeping isoform responsible for pH homeostasis and cell volume regulation, (Orlowski and Grinstein, 2004), it is also involved in a variety of other processes, including cell migration (Denker and Barber, 2002), differentiation (Li *et al*, 2009; Dyke and Fliegel, 1995), proliferation and apoptosis (Putney *et al*, 2002; Wu *et al,* 2003). Structurally, the NHE isoforms are comprised of two domains; a large conserved N-terminal transmembrane domain consisting of 12 transmembrane spanning α-helices that is responsible for ion translocation, and a more divergent cytoplasmic C-terminal tail that regulates exchanger activity through the interactions with a wide variety of signalling molecules (Orlowski and Grinstein, 2004, Wakabayashi *et al*, 1997). Interacting molecules that bind NHE1 include numerous serine/threonine protein kinases that phosphorylate the exchanger at sites in the C-terminal tail, including extracellular signal-protein kinase 1 and 2 (ERK1/2) (Haworth *et al*, 2003, Malo and Fleigel, 2007), p38 mitogen-activated protein kinase (Khaled *et al,* 2001), p90

ribosomal S6 kinase ($p90^{RSK}$) (Takahashi *et al,* 1999; Cuello *et al*, 2007), Rho-associated coil-coil containing protein kinase (ROCK) (Tomininga *et al,* 1998) and Nck-interacting kinase (Nck) (Yan *et al*, 1999). Phosphorylation has been shown to have either a direct affect by enhancing the exchanger's affinity for intracellular protons (H^+_i), or by promoting the binding of additional proteins such as carbonic anhydrase (CA) (Li *et al*, 2006) and the scaffolding protein 14-3-3 (Lehoux *et al*, 2001). Other interacting proteins that regulate exchange activity independent of phosphorylation include Ca^{2+}-calmodulin (Bertrand *et al*, 1999), members of the calcineurin B homologous protein (CHP) family (Lin and Barber, 1996; Pang *et al*, 2001, 2002; Mailander *et al*, 2001), and the phospholipids, phosphatidylinositol-4,5-bisphosphate (PIP2) (Aharonovitz *et al*, 2000). Furthermore, the exchanger has also been shown to bind the ezrin-radixin-moesin (ERM) family of actin-binding proteins independent of ion translocation and modulate cell shape (Denker *et al*, 2000).

The Calcineurin B homologous proteins are a family of three N-myristoylated, EF-hand Ca^{2+}-binding domain containing proteins (CHP1-CHP3) that share homology with the calcineurin B subunit of the phosphatase, calcineurin (26 - 43% identity, 46 - 70% similarity), and indeed have the ability to regulate the phosphatase activity of the calcineurin A catalytic subunit (Lin *et al*, 1999; Li *et al,* 2003; Gutierrez-Ford *et al*, 2003). All members of the CHP family have been shown to bind the juxtamembrane region of NHE1 and differentially influence basal and regulated activity of the exchanger (Lin and Barber, 1996, Pang *et al*, 2001, 2002; Mailander *et al*, 2001, Zaun *et al*, 2008). CHP1 and CHP2 are both essential cofactors of several NHE isoforms, where CHP2 constitutively enhances the intracellular pH sensitivity of NHE1 in an absence of serum (Pang *et al*, 2002) and CHP1 sets the intracellular pH sensitivity of the exchanger in the neutral range, but also its activation in response to various stimuli (Pang *et al*, 2001, 2004). CHP1 also plays an essential role in stabilization of NHE1 at an early stage of biogenesis (Matsushita *et al,* 2007). CHP3 has been shown to promote the late stage maturation and stability of NHE1 at the cell surface, resulting in a dramatic upregulation of exchanger activity (Zaun *et al,* 2008).

CHP1 (also known as p22), which is widely expressed, and in addition to binding integral membrane proteins such as NHE1, also interacts with an assortment of soluble proteins including the death-associated protein kinase-related apoptosis-inducing protein kinase 2 (DRAK2) (Matsumoto *et al,* 2001), kinesin family member 1Bb2 (KIF1Bβ2) (Nakamura *et al*, 2002) and GAPDH (Andrade *et al*, 2004; Barroso *et al*, 1996; Timm *et al*, 1999). CHP2 expression is restricted to the intestinal epithelia (Inoue *et al*, 2003), but is induced in several malignant cell types where the CHP2-dependent activity of NHE1 results in a more alkaline intracellular pH (pH_i) that promotes the survival of malignant cells (Pang *et al*, 2002).

The third isoform of the CHP family, CHP3 or Tescalcin, was originally discovered as an autosomal gene that is differentially expressed in mouse developing testis (Perrera *et al,* 2001). Unlike the ubiquitously expressed CHP1, CHP3 displays a very limited expression pattern. In adult mouse, it is restricted to heart, brain, and stomach, while in humans, it is predominantly expressed in heart and hematopoietic progenitor cells (Levay and Slepak, 2007; Gutierrez-ford *et al*, 2004). CHP3 contains both an N-myristoylation motif and a single functioning EF-hand Ca^{2+}-binding motif that binds calcium with micromolar affinity (Gutierrez-Ford *et al*, 2003). It has been previously shown that a majority of EF-hand Ca^{2+}-binding proteins undergo Ca^{2+}-dependent conformational change (Strynadka and James, 1989) that modulate their function or influences the activity of their effectors. N-myristoylation involves the irreversible attachment of myristate, a 14-carbon saturated fatty acid, to the N-terminal glycine residue of a wide variety of eukaryotic and viral proteins following removal of the initiator methionine by cellular methionylaminopeptidases. This covalent modification promotes the reversible tethering of proteins to the inner-leaflet of membrane bilayers and is thought to regulate protein targeting and function (Maurer-Stroh *et al,* 2002; O'Callaghan and Burgoyne, 2003).

In this study, we examined the biological contributions of these domains in CHP3 to the regulation of NHE1. We demonstrate that although N-myristoylation and Ca^{2+}-binding of CHP3 is not required for the interaction of the

two proteins, or the biosynthetic maturation of NHE1, they are both crucial for the stability of NHE1 at the plasma membrane.

MATERIALS AND METHODS

Materials and Solutions:

Monoclonal antibodies to a decapeptide derived from influenza virus hemagglutinin (HA) were purchased from Convance Inc. (Berkeley, CA) and to the peptide of the c-myc proto-oncogene (myc) from Millipore (Temecula, CA). Polyclonal antibodies to the HA- and myc-epitopes were purchased from Abcam Inc. (Cambridge, MA) and Upstate (Lake Placid, NY) respectively, and antibodies specific to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Abcam. All AlexaFluor®-conjugated goat anti-mouse or anti-rabbit IgG antibodies were purchased from Molecular Probes (Eugene, OR).

Vent polymerase, DNA ligase, restriction endonucleases, as well as protein and DNA makers were purchased from New England Biolabs (Ipswich, MA). α-Minimum Essential Medium (α-MEM), fetal bovine serum (FBS), penicillin-streptomycin, Geneticin (G418), trypsin-EDTA and Lipofectamine-2000™ transfection reagent were all purchased from Invitrogen Corporation (Grand Island, NY). Carrier-free ^{22}NaCl (range of specific activity, 900–950 mCi/mg; concentration, ~10 mCi/ml) was obtained from PerkinElmer Life Sciences (Woodbridge, ON). Amiloride hydrochloride, nigericin and ouabain were all purchased from SigmaAldrich Co. (St. Louis, MO) and complete protease inhibitor cocktail tablets were obtained from Roche Diagnostics (Indianapolis, IN). All other chemicals and reagents were purchased from Bioshop Canada (Burlington, ON), Sigma-Aldrich or Fisher Scientific, and were of highest grade available.

cDNA construction and Mutagenesis:

A mammalian expression vector under the control of the enhancer/promoter region from the immediate early gene of human cytomegalovirus (pCMV) (Orlowski, 1993) and expressing either the cDNA of NHE1 containing a C-terminal hemagglutinin (HA) epitope tag ($NHE1_{HA}$) or a C-terminally myc epitope tagged CHP3/tescalcin cDNA ($CHP3_{myc}$) were

constructed as described previously (Orlowski and Kandasamy, 1996; Zaun *et al*, 2008). It has previously been shown that when the modified NHE1 construct is expressed in AP-1 cells, no obvious effect was observed on either the basal or the regulated functional properties of the exchanger (Aharonovitz *et al*, 2000).

Mutations of the myristoylation site was accomplished by way of a PCR of the full length CHP_{myc} utilizing a N-terminal primer containing a HindIII restriction site followed by a Kozak consensus sequence immediately followed by the start methionine and a substitution of the glycine at the second residue for alanine (**Fig 2.1, ↓**). The PCR product was purified using the UltraClean™ DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA) and subcloned into the pCMV expression vector ($CHP3_{myc}$ G2A). This mutation has been shown previously to abolish the ability of the CHP3 to become N-myristoylated (Pang *et al*, 2004; Gutierrez-Ford *et al*, 2003; Otto-Bruce *et al*, 1997). A mutation to the EF-hand Ca^{2+}-binding motif of CHP3 previously demonstrated to be devoid of calcium binding ability ($CHP3_{myc}$D123A) (Gutierrez-Ford *et al*, 2003) was performed using a 2-step mutually priming PCR-based strategy. Briefly, two complementary oligonucleotides containing a point mutation converting the aspartic acid at residue 123 to an alanine (**Fig 2.1, ↓**) and the flanking six amino acids on either side were used to obtain two PCR products consisting of the mutation as well as either the N-terminal or C-terminal sides of CHP3 construct. A small portion of these two products were then used as mutually-priming template to allow both sides of the CHP3 construct to PCR together. The PCR reactions proceeded for five cycles to extend the polymerization of either end of the CHP3 sequence before the external 3' and 5' primers were added to amplify the complete CHP3 product. The final PCR product was purified using UltraClean™ DNA purification kit and subcloned into the pCMV expression vector with the incorporated HindIII and XbaI restriction sites. The $CHP3_{myc}$ mutant containing both the mutations of the myristoylation motif and EF-hand Ca^{2+}-binding motif ($CHP3_{myc}$G2/D123A) was obtain by the PCR procedure identical to that used for the G2A mutant, but using the D123A mutant as a template and subcloning into the pCMV expression vector. All constructs were

sequence in order to confirm the presence of the desired mutation and to ensure that other random mutations were not introduced.

Cell Culture and Transfection:

A cell line devoid of endogenous Na^+/H^+ exchanger activity derived from Chinese hamster ovary fibroblasts (CHO), termed AP-1 (Rotin and Grinstein, 1989) were maintained in α-MEM medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml / 100 µg/ml) and 25 mM sodium bicarbonate. Cells were incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37ºC. To obtain AP-1 cells expressing either $NHE1_{HA}$ or co-expressing $NHE1_{HA}$ along with either wild-type or mutated $CHP3_{myc}$, a total of 2 µg of DNA was transfected in a 6-well plate using Lipofectamine™-2000 reagent according to manufacturer recommended procedure. Twenty four hour post-transfect, the cells were split into 10 cm dishes at a dilution of 1:10 and 1:50 and selected for cell stably expressing $NHE1_{HA}$ by repeated acid-selection over a two-week as described previously (Orlowski, 1993), and for cells expressing $CHP3_{myc}$ by selecting in α-MEM culture medium supplemented with geneticin (G418) (600 µl/ml) over a two to four week period.

Co-Immunoprecipitation and Western blotting:

Co-immunoprecipitation of $NHE1_{HA}$ and wild-type or mutant forms of $CHP3_{myc}$ (G2A, D123A, and G2/D123A) was performed in 10 cm dishes by transfecting AP-1 cells stably expressing $NHE1_{HA}$ with 10 µg of the desired $CHP3_{myc}$ constructs using Lipofectamine™-2000 according to the manufacturers recommended procedure. Twenty four hours post-transfection, cell lysates were obtained by washing cell in ice-cold PBS and adding 1 ml Radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 2.5% deoxycholate, 0.5 % NP-40, and protease inhibitors). For Ca^{2+}-dependence of $NHE1_{HA}/CHP3_{myc}$ co-immunoprecipitation, RIPA buffer was either supplemented with 1mM Mg^{2+} and 2mM EDTA, or 0.1 mM Ca^{2+} without EDTA. Cell were scraped from the dish and incubated for 20 min. at 4ºC,

followed by centrifugation for 20 min. at 4ºC to pellet cellular debris. Supernatants were then pre-cleared with 100 µl of a 50% Protein G-sepharose (GE Healthcare BioScience) slurry for 2 hours at 4ºC. After brief centrifugation to remove the Protein G-sepharose and removing a small fraction for western blotting, the remaining supernatants were incubated with either 5 µl of primary rabbit polyclonal antibody against either the HA- or myc-epitopes, overnight at 4ºC with gentle rocking. 100 µl of Protein G-Sepharose was then added and lysates were incubated for 6 hours at 4ºC with gentle rocking, followed by multiple washes with RIPA buffer to remove non-bound proteins. Protein conjugates were then eluted by SDS-sample buffer (2% SDS, 50 mM Tris pH 6.8, 100 mM dithiothreitol) by incubating samples for 30 min. at room temperature without boiling in order to prevent aggregation of NHE1 proteins. Samples were then subjected to SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) and subject to western blotting. Membranes were blocked with 5% nonfat powdered milk and incubated with either 1:10,000 dilution of primary mouse monoclonal antibody recognizing the HA epitope or a 1:1000 dilution of a mouse primary antibody recognizing the myc-epitope. After several washes with PBS containing 0.1% Tween-20, blots were incubated with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) at a dilution of 1:10,000. Immunoreactive bands were then visualized using the Western Lightning™ Plus-ECL western blotting detection reagents (Perkin Elmer Inc., Waltham, MA).

Immunocytochemistry:

AP-1 cells and AP-1 cells stably expressing $NHE1_{HA}$ were cultured on glass coverslips treated with fibronectin (1.5 µg/ml) (Sigma-Aldrich) to subconfluence, and transfected with 2 µg of either $CHP3_{myc}$ wild-type and mutant constructs using Lipofectamine™-2000 according to manufacturer's recommendations. 24 hours post-transfection, cells were washed with PBS and fixed in 2% paraformaldehyde/PBS for 30 minutes. Cells were then permeabilized in PBS containing 0.1% saponin for 30 min, followed by repeated washing with

10 mM glycine in PBS / 0.01% saponin. After blocking for 1 hour in PBS/10% goat serum/0.01% saponin, cells were incubated with a combination of a 1:1000 dilution of mouse monoclonal antibody recognizing the HA epitope and 1:500 dilution of rabbit polyclonal antibody recognizing the myc epitope overnight at 4ºC. The reverse of a 1:500 dilution of anti-HA polyclonal rabbit antibody along with 1:250 dilution of a mouse monoclonal antibody to the myc epitope was also used. All antibodies were diluted in PBS containing 10% goat serum and 0.01% saponin. After several washes with PBS/0.01% saponin cell were incubated with secondary goat anti-mouse and anti-rabbit antibodies conjugated to AlexaFluor™-488 and AlexaFluor™-569 respectively at a dilution of 1:2000 in PBS/10% goat serum/0.01% saponin for 2 hours at room temperature. Coverslips were subsequently washed several times in PBS/0.01% saponin and mounted onto glass slides with ImmunoFluore™ mounting medium (ICN Biomedicals, Aurora, OH). Transfected cells were analyzed by laser scanning confocal microscopy using a Zeiss LSM 510 inverted microscope, and images were analyzed using LSM software and Corel® CorelDraw™ version 12.

Measurement of Na^+/H^+ Exchanger activity by ^{22}Na uptake.

$NHE1_{HA}$ activity in cells co-expressing the exchanger and $CHP3_{myc}$ constructs (wild-type, G2A, D123A, and G2/D123A) was assessed using a radioisotope influx assay as described previously (Orlowski, 1993) on 24-well plates cultured to confluency. To measure absolute activity of $NHE1_{HA}$ cells were washed two times and then pulsed with NH_4Cl solution (50 mM NH_4Cl, 70 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2 \bullet 2H_2O$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) for 30 min. at 37ºC and 0% CO_2. This was followed by two rapid washes with choline chloride solution (125 mM choline chloride, 1 mM $MgCl_2$, 2 mM $CaCL_2 \bullet 2H_2O$, 5 mM glucose, 20 mM HEPES-Tris, pH7.4) followed by incubation for 5 min. with 0.25 ml choline chloride solution containing 1 mM ouabain and carrier free $^{22}Na^+$ (1 μCi/ml) in absence and presence of the NHE1 inhibitor amiloride (1 mM).

To measure the activity of the exchanger as a function of the expression of the different $CHP3_{myc}$ constructs, 10 cm dishes of AP-1 cells stably expressing $NHE1_{HA}$ were grown to subconfluence and transfected with an increasing ratio of $CHP3_{myc}$-containing expression vector relative to empty vector (0 μg to 10 μg) using Lipofectamine™-2000 according to manufacturers recommended procedure. 24 hours post-transfection, cells were split into 24 well plates (6 wells per transfection) as well as 6-well plates (1 well per transfection) for western blotting. At 48 hours post-transfection, cells in the 6-wells were harvested and lysates were obtained for Western blotting as mentioned previously. The 24-well plates were assayed for $^{22}Na^+$-incorporation after NH_4Cl acid load as mentioned above.

$NHE1_{HA}$ activity was also measured as a function of intracellular H^+ concentration (pH_i) by clamping the intracellular pH (pH_i) at a range of 5.4 – 7.4, using the KCl/nigericin pH_i-clamp method as described previously (Thomas *et al*, 1979; Aharonovitz *et al*, 2000). Cells were grown to confluence in 24-well plates, washed twice in choline-chloride solution followed incubated for 4 min at room temperature in NMG-balanced salt solutions specific for each pH_i (pH-clamp solution). Clamp solutions contained 2 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5, with varying concentrations K^+ as well as 10 μM nigericin and 1 mM ouabain. $^{22}Na^+$-influx measurements were performed in the same pH-clamp solutions containing $^{22}Na^+$ (1 μCi/ml) with and without amiloride for 10 min at room temperature.

All influx assays were rapidly terminated after appropriate time by washing the cells three times with four volumes of ice-cold NaCl stop solution (130 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM HEPES – NaOH, pH 7.4). Incorporated $^{22}Na^+$ was extracted by solubilizing cell monolayers with 0.25 ml of NaOH (0.5 N), followed by washing each well with 0.25 ml HCl (0.5 N). Both solutions were transferred into scintillation vials containing 5 ml of scintillation fluid, and radiolabel was assayed by liquid scintillation spectroscopy. $^{22}Na^+$-measurements specific to NHE1 were determined as the difference between the initial rates of H^+-activated $^{22}Na^+$-influx in absence and presence of 1mM

amiloride and expressed as amiloride-inhibitable H^+-activated $^{22}Na^+$-influx. When required, protein content was determined using the Bio-Rad DC protein assay kit according to the manufacturers protocol. Measurements are represented by three independent experiments performed in triplicate and expressed as the mean ± S.E.

Transient Expression Assay of $NHE1_{HA}$ and $CHP3_{myc}$ (wild-type and mutants forms).

AP-1 cells were grown to subconfluence on 10 cm dishes and transfected with 5 µg of $NHE1_{HA}$ containing expression vector along with 5 µg of empty vector or expression vectors of $NHE1_{HA}$ as well as $CHP3_{myc}$ constructs (wild-type, G2A, D123A,) using Lipofectamine™-2000 according to manufacturer's recommendations. At each time point, cell lysates were obtained as described previously and assayed for protein-content using the BioRad DC protein assay kit. Equal amounts of total protein were subjected to SDS-PAGE and immunoblotting as previously described.

Cell Surface biotinylation and pulse-chase assay:

In order to determine the relative amount of cell surface $NHE1_{HA}$ relative to $CHP3_{myc}$ (wild type, G2A, D123A, G2/D123A), AP-1 cells were grown to subconfluence on 10 cm dishes and transfected with 8 µg of $NHE1_{HA}$ cDNA along with an increasing ratio of the different $CHP3_{myc}$ cDNA constructs (0 to 2 µg) to empty expression vector using Lipofectamine™-2000. 1 µg of cDNA containing green fluorescent protein (GFP) was also transfected as a control for transfection efficiency. 36 hours post-transfection, cells were placed on ice and washed rapidly with ice-cold PBS-CM (PBS supplemented with 0.1 mM $CaCl_2$, 1 mM $MgCl_2$). Cells were incubated for 30 minutes on ice with PBS-CM containing 0.5 mg/ml of sulfo-NHS-SS-biotin (Thermo Scientific, Rockford, IL), a water soluble membrane impermeable, thiol-cleavable, amine-reactive biotinylation reagent. Cells were quickly washed and incubated twice in quenching buffer (20 mM glycine in PBS) for seven min each on ice to remove excess biotin. Following several rapid washes in PBS-CM, cell lysates were obtained in RIPA

buffer by scrapping cells and incubating for 20 minutes on ice, followed by centrifugation for 20 min to remove cellular debris. A small fraction supernatant was removed for western blotting, and the remaining supernatant was incubated with 200 µl of a 50% NeutrAvidin® agarose slurry (Thermo Scientific) in RIPA buffer overnight at 4ºC. The bound biotinylated protein complexes were washed six times in RIPA buffer and eluted with SDS-sample buffer for 30 minutes at room temperature with rocking. After vortexing and centrifugation for 5 min, samples were subjected to SDS-PAGE and immunoblot analysis with monoclonal antibodies specific to the HA- and myc- epitopes of $NHE1_{HA}$ and $CHP3_{myc}$ respectively.

The cell surface stability of $NHE1_{HA}$ in relation to expression of $CHP3_{myc}$ wild-type or mutants was determined through a pulse chase of biotinylated NHE_{HA} as described previously (Zaun *et al*; 2008). Briefly, 6-well plates containing AP-1 cells expressing either NHE_{HA} alone or co-expressing NHE_{HA} along with $CHP3_{myc}$ wild type or mutants forms (G2A, D123A, G2/D123A) were grown to roughly 90% subconfluency and cell surface proteins were biotinylated and quenched as described above. After extensive rapid washing to remove excessive biotin, cells were returned to growth media supplemented with 10% FBS and cultured at 37ºC in 5% CO_2/95% air for various time point with fresh media added every 12 hours to maintain cell viability. At the indicated time points, cells lysates and biotinylated proteins were obtained as described above, and subject to SDS-PAGE and immunoblotting.

The relative band intensity for each time point on the western blots were obtained through multiple exposures of the same blot to ensure the signal was within the linear range of the X-ray film. Densitometry measurements were obtained using ImageJ™ image processing software and plotted in relation to time. All measurements were obtained from a minimum of three independent experiments and expressed as a mean ± S.E.

Measurement of Total NHE1 half-life:

Half-life of $NHE1_{HA}$ in the absence and presence of $CHP3_{myc}$ wild type and mutants (G2A/ D132A) was performed by plating AP-1 cells expressing $NHE1_{HA}$ alone or co-expressing $NHE1_{HA}$ along with $CHP3_{myc}$ (wild-type, G2A or D123A) on 10 cm dishes cultured to near confluency. Plates were treated with cycloheximide (100 μg/ml) in α-MEM growth medium supplemented with 10% FBS and 1% penicillin/streptomycin for up to 36 hours. Fresh media was added every 12 hours to maintain maximal cell viability. At appropriate time points, cell lysates were obtained through lysis in RIPA buffer and centrifugation, and equal volumes were subject to SDS-PAGE and immunoblotting with antibodies specific to the epitopes of $NHE1_{HA}$ and $CHP3_{myc}$ as described previously. Spot densitometry measurements of visualized bands from western blotting were obtained by ImageJ™ image analysis software. All measurements are representative of three independent experiments and expressed as the means ± S.E.

RESULTS:

Role of N-myristoylation and Ca^{2+}-binding in the interaction between NHE1 and CHP3:

In order to characterize the biological significance of N-myristoylation and Ca^{2+}-binding of CHP3 in relation to its regulation of NHE1, the critical glycine residue of the N-myristoylation motif (G2A) (***Fig. 2.1,*** ↓) and the crucial aspartic acid at position 123 in the EF-hand Ca^{2+}-binding motif were mutated separately to alanine (D123A) (***Fig. 2.1,*** ↓). Although the CHP proteins contain four potential EF-hand domains, only the third domain that contains D123 was shown to bind Ca^{2+} in CHP3 (Gutierrez-Ford *et al*, 2003).

Previously, our lab demonstrated conclusively that NHE1 interacts with CHP3 when co-expressed in intact cells (Zaun *et al*, 2008). To determine whether the interaction between NHE1 and CHP3 is dependent on N-myristoylation or Ca^{2+}-binding of CHP3, co-immunoprecipitation studies of NHE1 with either wild type (WT), N-myristoylation-defective (G2A), or Ca^{2+}-binding deficient (D123A) CHP3 were performed. To this end, a Chinese hamster ovary cell line (CHO) that is devoid of endogenous plasmalemmal NHE activity, termed AP-1 (Rotin and Grinstein, 1989), and stably expressing an HA-epitope tagged form of NHE1 ($NHE1_{HA}$) and transiently cotransfected with each of the CHP3 constructs (WT, G2A, and D123A) tagged at their C-terminus with a myc-epitope ($CHP3_{myc}$). At 24-hours post-transfection, cell lysates were obtained and incubated with a mouse polyclonal antibody that recognizes the HA-epitope on the $NHE1_{HA}$ protein in order to precipitate the exchanger along with any bound proteins. After extensive washing, immunoprecipitated complexes as well as aliquots from the cell lysates were subjected to SDS-PAGE and immunoblotting in order to visualize $NHE1_{HA}$ and $CHP3_{myc}$ proteins. Immunoprecipitates of $NHE1_{HA}$ revealed that all three forms of the $CHP3_{myc}$ protein (WT, G2A, and D123A) formed complexes with NHE1 (***Fig. 2.2***). As a control, $NHE1_{HA}$ expressing cells were transfected with wild-type $CHP3_{myc}$ and cell lysates were incubated with a non-specific rabbit IgG

Fig. 2.1.

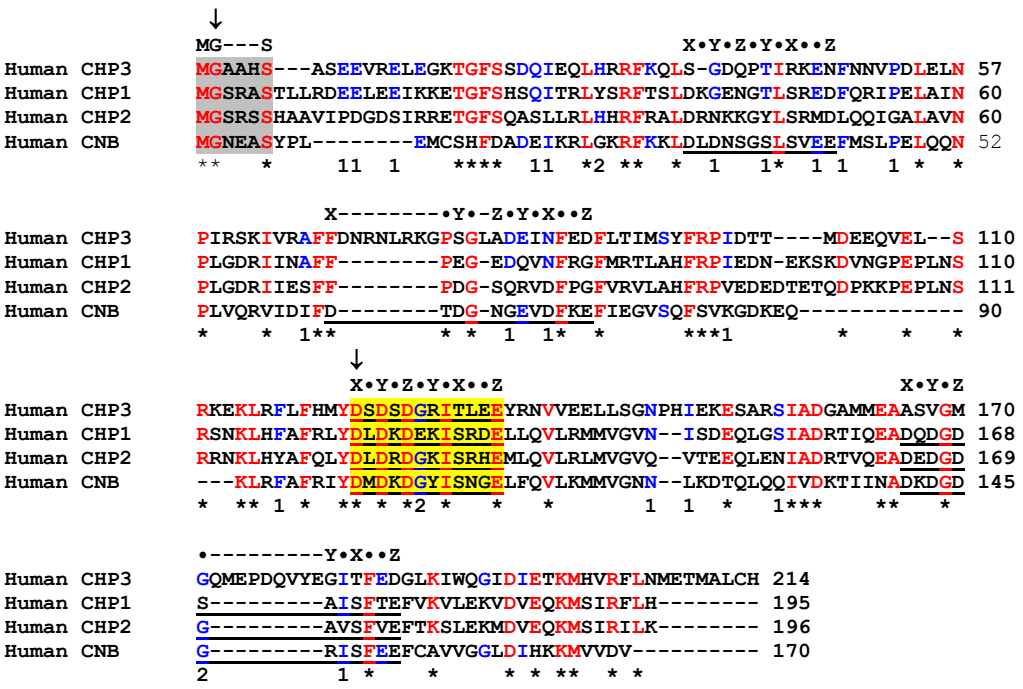


Figure 2.1. **Amino acid alignment the CHP3 with CHP1 and 2 and calcineurin B (CNB).** Amino acid alignment of CHP3 showing its homology to the other two CHP isoforms (CHP1 and CHP2) as well as the related regulatory B subunit of calcineurin (CNB). The N-myristoylation site is indicated by light grey box (**MGxxxS/T**) and the critical residues the make up the coordinating loop of the EF-hand calcium-binding domains are highlighted by the underscore. EF-hand calcium-binding motifs consist of a helix-loop-helix structure of about 29 amino acids in length, with the Ca^{2+} ion coordinated by a characteristic 12 amino acids within the loop, consisting of the X•Y•Z•-Y•-X••-Z (dots represent intervening residues) (Gutierrez-Ford et al, 2003; Lewit-Bentley and Réty, 2000) as shown. Whereas Calcineurin B (CNB) contains four functional EF-hand domains, and CHP1 has been shown to contain two functional EF-hand domains, and two non-functional ancestral helix-loop-helix domains (Pang et al, 2004) and CHP3 contains only one functional EF-hand domain (Gutierrez-Ford et al, 2003), corresponding to EF-3 of CNB and CHP1 (indicated by yellow shading). Residues that are identical between the three CHP1 proteins are indicated by an asterix (*) and numbers 1 and 2 correspond to sequence identity of CHP3 and corresponding isoform. Mutation sites for the Gly (G) of the N-myristoylation site (G2A) and the Asp (D) of the EF-hand domain (D123A) are indicated by the downward arrow (↓).

Fig. 2.2.

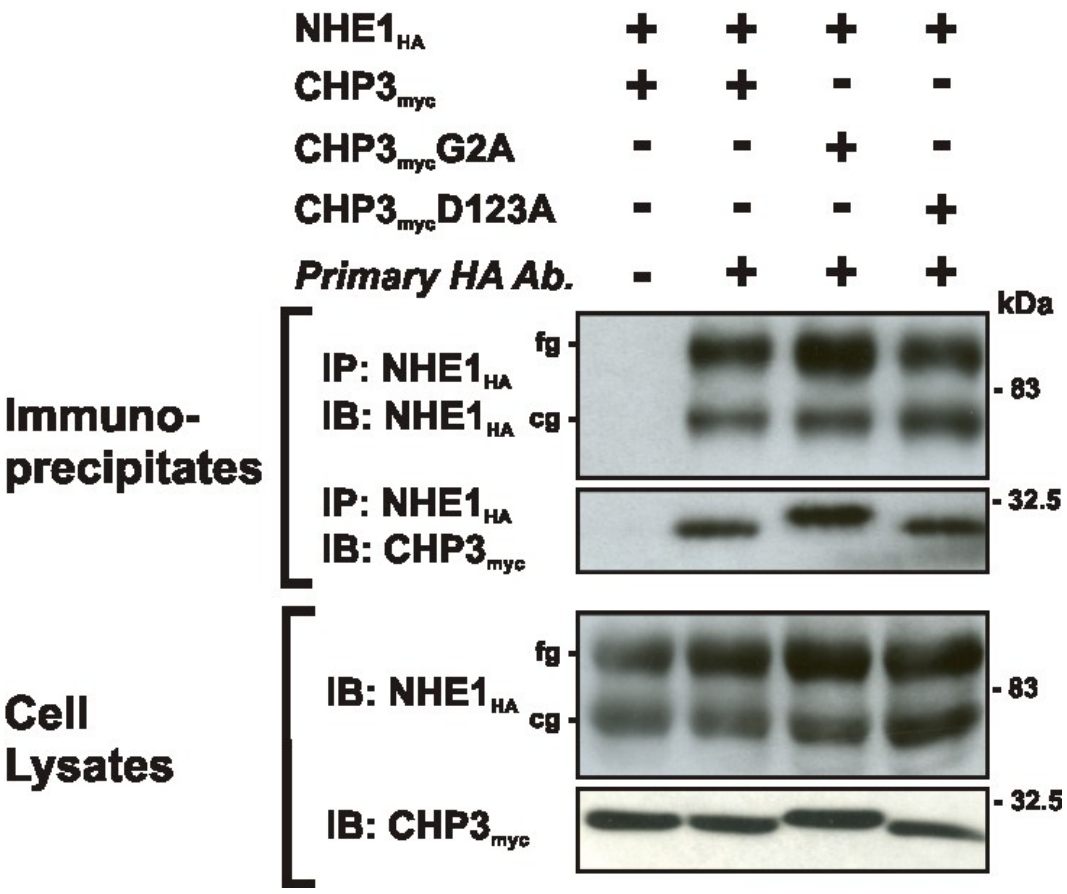


Figure 2.2. **N-Myristoylation and Ca^{2+}-binding defective mutants of CHP3 form a complex with NHE1 in transfected cells.** Transfection of Chinese hamster ovary AP-1 cells stably expressing $NHE1_{HA}$ with wild-type and mutant constructs of $CHP3_{myc}$ that are devoid of the ability to be either N-myristoylated (G2A) or bind calcium (D123A), result in co-immunoprecipitation of $NHE1_{HA}$/$CHP3_{myc}$ protein complexes. Twenty-four hours post-transfection cell lysates were obtained and $NHE1_{HA}$ was immunoprecipitated with a rabbit polyclonal antibody specific to the HA-epitope (**IP**) and bound $CHP3_{myc}$ was analysed by Western blotting using a mouse monoclonal antibody specific to the myc-epitope (**IB**). The two immunoreactive bands visualized in the $NHE1_{HA}$ blots represent both the immature core-glycosylated (***cg***) and mature fully-glycosylated (***fg***) forms of the exchanger. Data shown is representative of three separate experiments.

antibody (Southern Biotech, Birmingham, AL) in order to verify that antibodies did not bind non-specifically. The interaction between $NHE1_{HA}$ and the $CHP3_{myc}$ proteins was verified by the reciprocal experiments of immunoprecipitating $CHP3_{myc}$ and immunoblotting for $NHE1_{HA}$ (*Data not shown*).

To further demonstrate the association between $NHE1_{HA}$ and N-myristoylation- and Ca^{2+}-binding defective $CHP3_{myc}$, their respective subcellular distributions were compared using dual immunolabeling and fluorescence confocal microscopy. Previous studies showed that when co-expressed in AP-1 cells, $NHE1_{HA}$ and $CHP3_{myc}$ co-localize at the cell surface. However, when expressed in AP-1 cells devoid of NHE1, or co-expressed with mutant forms of NHE1 that do not interact with CHP3, CHP3 did not concentrate at the cell surface but instead was diffusely distributed throughout the cytoplasm (Zaun *et al*, 2008). These results indicate that CHP3 requires NHE1 for its targeting to the cell surface, and is not responsible for NHE1 trafficking (Zaun *et al*, 2008). In agreement with this previous study, $NHE1_{HA}$ and $CHP3_{myc}$ strongly co-localized at the plasma membrane as did the N-myristoylation-defective (G2A) and Ca^{2+}-binding deficient (D123A) mutants of CHP3 (***Fig 2.3A***). However when the $CHP3_{myc}$ constructs were expressed in AP-1 cells that were devoid of NHE1, all three forms of CHP3 were distributed throughout the cytoplasm and to a lesser extent, in the nucleus (***Fig. 2.3B***). These results demonstrate that N-myristoylation and Ca^{2+}-binding of CHP3 are not a requirement for the interaction with NHE1.

Previous studies by Pang and colleagues suggested that Ca^{2+}-binding greatly influences the interactions between CHP1 and NHE1, and when the two functional EF-hand domains of CHP1 were mutated, the interaction between CHP1 with NHE1 was greatly impaired (Pang *et al*, 2004). Furthermore, Gutierrez-Ford *et el*, suggested that CHP3 likely binds Mg^{2+} in its inactive state and upon cellular stimulation that increases intracellular Ca^{2+}, Mg^{2+} is replaced with Ca^{2+} allowing CHP3 to undergo a conformational change to an "active" state (Gutierrez-Ford *et al,* 2003). In our current study, co-immunoprecipitation and

Fig. 2.3.

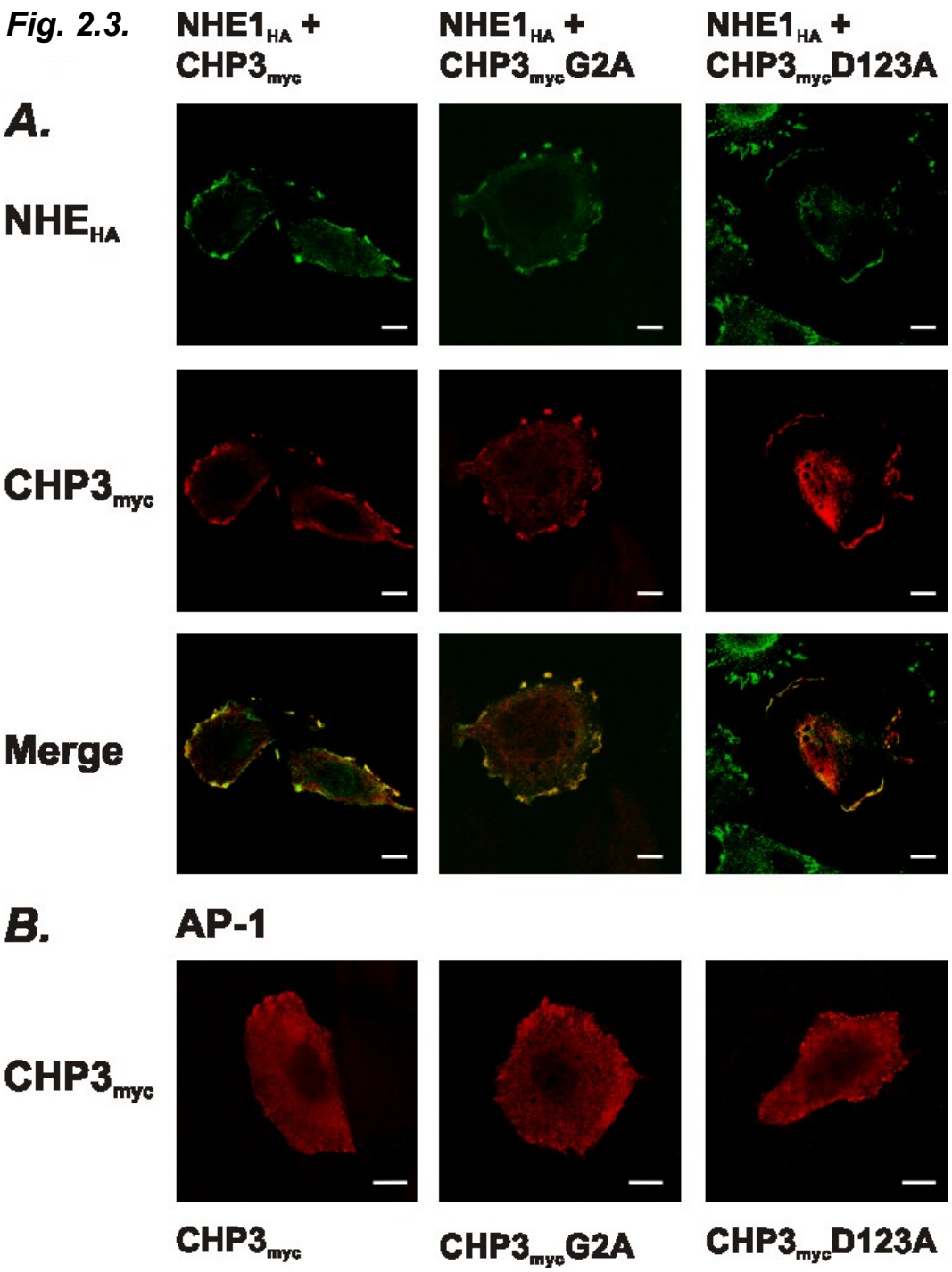

NHE1HA + CHP3myc
NHE1HA + CHP3mycG2A
NHE1HA + CHP3mycD123A
A.
NHEHA
CHP3myc
Merge
B.
AP-1
CHP3myc
CHP3myc
CHP3mycG2A
CHP3mycD123A

Figure 2.3. **Subcellular co-localization and distribution of NHE1 and CHP3 in transfected cells.** Immunofluorescence confocal microscopy of AP-1 cells stably expressing $NHE1_{HA}$ and transiently transfected with $CHP3_{myc}$ wild-type and mutant forms (G2A, D123A). Cells grown on glass coverslips treated with 1.5 µg/ml of fibronectin were fixed permeabilized, and labelled. Subcellular distribution of $NHE1_{HA}$ (Green) was visualized using mouse monoclonal antibodies specific to the HA-epitope followed by labelling with a goat anti-mouse secondary antibody conjugated to AlexaFluor™-488. $CHP3_{myc}$ (Red) distribution was identified through a primary rabbit polyclonal antibody specific to the myc-epitope followed by a secondary goat anti-rabbit antibody conjugated to AlexFluor™-568. Overlapping signals in the merged images are shown in yellow. Data is representative of between two and four independent experiments. Scale bars at the bottom right of each panel represent 10 µm.

co-localization studies did not show any great diminishment in the ability of the Ca^{2+}-binding deficient mutant (D123A) of CHP3 to interact with $NHE1_{HA}$. However, during some preliminary co-immunoprecipitation studies, the amount of $CHP3_{myc}$D123A complexed with NHE1 appeared to be lower when compared to wild-type or G2A versions of the protein, although there seemed to be a corresponding decrease in the total amount of the D123A protein (*Data not shown*). Furthermore, in co-localization studies of $NHE1_{HA}$ and $CHP3_{myc}$, the D123A mutant protein had a slightly stronger intracellular appearance when co-expressed with $NHE1_{HA}$ then did the wild-type and G2A constructs (***Fig. 2.3A)***.

In order to investigate whether the binding of Ca^{2+} to CHP3 influences its interaction with $NHE1_{HA}$, we performed an immunoprecipitation assay to test the influence of Ca^{2+}. AP-1 cells co-expressing $NHE1_{HA}$ with either $CHP3_{myc}$ or $CHP3_{myc}$D123A (as a control) were used in order to be certain of equal NHE1 and CHP3 expression. Each cell line was seeded equally on three separate 10-cm dishes and cultured to confluency, after which each plate was harvested in RIPA buffer either supplemented with 1 mM $MgCl_2$ and 1 mM EDTA or with 0.1 mM $CaCl_2$. After pre-clearing cell lysates with Protein-G-sepharose, $CHP3_{myc}$ was immunoprecipitated with a rabbit polyclonal antibody recognizing the myc-epitope. Immunoprecipitates and whole cell lysates were subject to SDS-PAGE and immunoblotting to visualize the resulting protein complexes. Immunoblotting the $CHP3_{myc}$ immunoprecipitates with a monoclonal mouse antibody recognizing the HA-epitope of $NHE1_{HA}$ indicates that in the presence of Ca^{2+}, the amount of $NHE1_{HA}$ that forms a complex with the $CHP3_{myc}$ (***Fig. 2.4A***) is increased by roughly 35 ±8% (***Fig 2.4B***) . In the case of the $CHP3_{myc}$D123A Ca^{2+}-binding deficient form of CHP3, there was no detectable difference between the interaction in the absence or presence of Ca^{2+} (***Fig. 2.4A, 2.4B***). Although this result indicates that Ca^{2+}-binding to CHP3 influences its interaction with NHE1, whether this increase in the band intensity is representative of an increase in the affinity between the two proteins, or an increased stabilization of the $NHE1_{HA}/CHP_{myc}$ complex remains to be resolved.

Fig. 2.4.

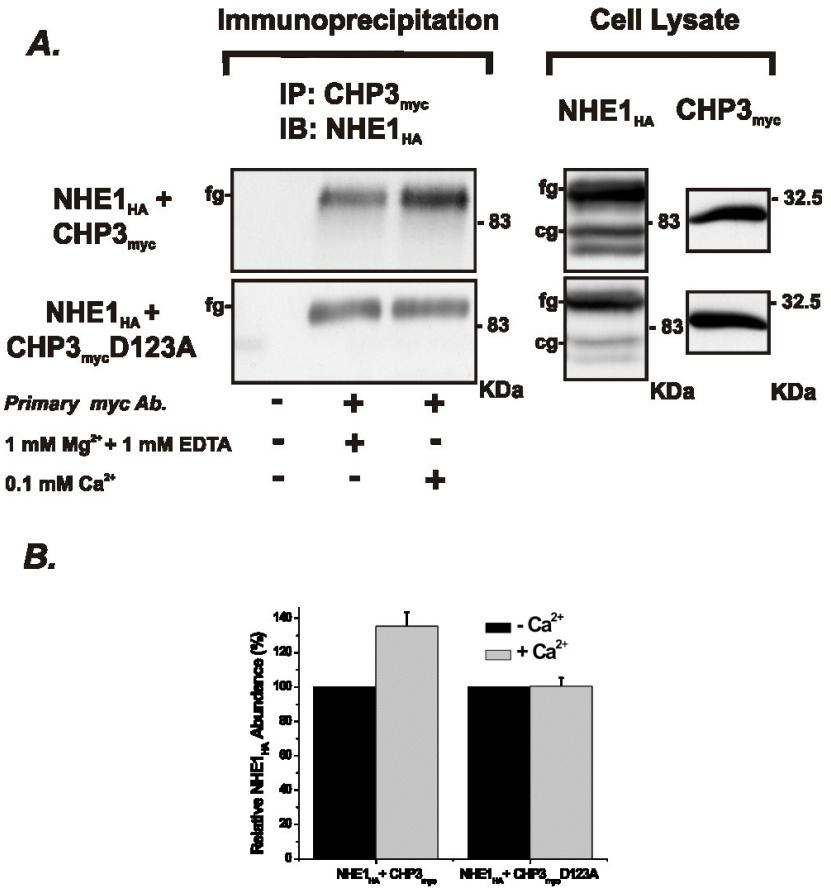


Figure. 2.4. **Interaction between NHE1 and CHP3 in transfected cells is influenced by presence of Ca^{2+}.** AP-1 cells co-expressing $NHE1_{HA}$ and $CHP3_{myc}$ were used in order to be sure of equal protein quantity. Cell were cultured to confluence and lysates were extracted in RIPA buffer either devoid of EDTA and supplemented with 0.1 mM Ca_2Cl or containing 1 mM $MgCl_2$ and 1 mM EDTA. A Fraction of the cell lysates were removed for western blotting and all the lysates were subject to immunoprecipitation a rabbit polyclonal antibody specific to the myc-epitope in their respective RIPA buffer. $NHE1_{HA}$ bound to $CHP3_{myc}$ was visualized with a monoclonal mouse antibody specific to the HA-epitope and resulting Western blot band intensities were compared (**A**) As a control, AP-1 cells doubly expressing $NHE1_{HA}$ and the Ca^{2+}-binding deficient mutant $CHP3_{myc}D123A$ was used (**A**). Band intensity was measured of two separate experiments and densitometry measurements were obtained using ImageJ®, normalized to Mg^{2+} and EDTA lane and graphed as a representation of the increased band intensity in the presence of Ca^{2+} ± S.E. (**B**)

N-myristoylation and Ca^{2+}-binding of CHP3 are required for optimal NHE1 activity through accumulation of the exchanger at the cell surface:

Previously we demonstrated that expression of CHP3 increases NHE1 activity by enhancing its biosynthetic maturation and cell surface stability, resulting in higher steady-state levels of NHE1 at the plasma membrane (Zaun *et al*, 2008). To determine whether N-myristoylation or Ca^{2+}-binding of CHP3 influences the cell surface accumulation or activity of NHE1, we transiently transfected a series of 10-cm dishes of AP-1 cells stably expressing $NHE1_{HA}$ with an increasing ratio of an expression vector coding for either wild-type or mutant forms of CHP3 (G2A and D123A) to empty vector. At 24 hours post-transfection, these dishes were split into 24-wells plates for activity assessment, as well as a 6-well plate to obtain corresponding cell lysates for immunoblotting. At 48 h post-transfection, the activity of the exchanger was assessed using the $^{22}Na^{+}$-influx assay as described in "Materials and Methods", and the cell lysates were obtained from the 6 well plates. The activity of $NHE1_{HA}$ in relation to increasing $CHP3_{myc}$ expression was determined as amiloride-inhibitable activity and normalized to non-CHP3 transfected $NHE1_{HA}$ activity (***Fig. 2.5A***). The corresponding cell lysates were subjected to SDS-PAGE and immunoblotting with mouse monoclonal antibodies specific for the HA- and myc-epitopes in order to visualize the expression of $NHE1_{HA}$ and $CHP3_{myc}$ respectively (***Fig. 2.5B***). Membranes were then stripped and re-probed with an antibody specific to endogenous GAPDH to verify equal protein loading (***Fig. 2.5B***). In agreement with our previous study, an increase in transfected $CHP3_{myc}$ results in both an increase in the activity of the exchanger and a corresponding increase in the expression of both the immature core-glycosylated (*cg*) and mature fully-glycosylated (*fg*) forms of $NHE1_{HA}$. Both the N-myristoylated deficient and Ca^{2+}-binding defected mutants of $CHP3_{myc}$ also increased the activity of $NHE1_{HA}$ and its corresponding expression, albeit to a much lesser extent, thereby confirming that an interaction between the two proteins is maintained when $CHP3_{myc}$ is unmyristoylated or unable to bind Ca^{2+} (***Fig. 2.5A, B***). This suggests that N-myristoylation and Ca^{2+}-binding are not critical requirements for CHP3 to bind and influence the

Fig. 2.5.

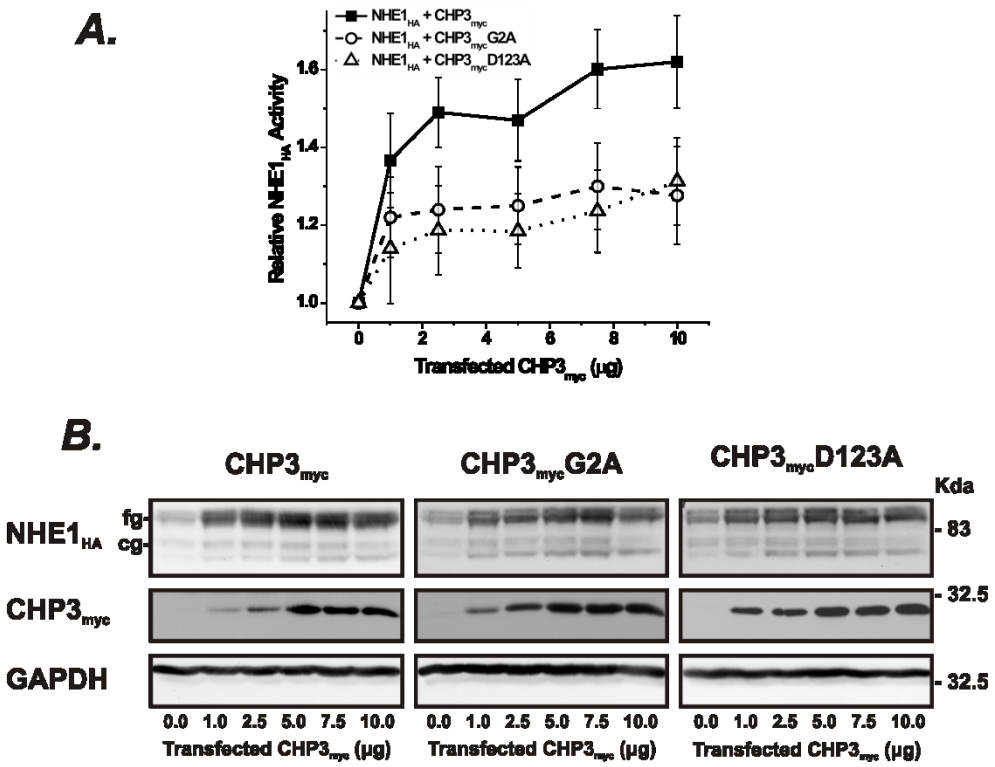


Figure 2.5. **Over-expression of either N-myristoylation-defective (G2A) or Ca^{2+}-binding deficient (D123A) of CHP3 effects both NHE1 activity and cellular abundance.** AP-1 cells stably expressing $NHE1_{HA}$ were cultured in a series of 10-cm dishes and transfected with an increasing ratio of CHP3 (WT) and mutants (G2A, or D123A) containing expression plasmids to empty vector (0 to 10 µg per dish) in order to maintain total amount of DNA transfected at 10 µg per dish. At 24 h post-transfection, each plate was split into 6-wells of a 24-well plate and one well of a 6-well plate to assess activity and protein expression respectively. At 48 h post-transfection, cells in the 24-well pate we subject to NH_4-acid load and assayed for amiloride inhibitable $^{22}Na^+$-influx assay as described in "Materials and Methods", and normalized to rate of influx to cells devoid of CHP3 expression (**A**). Values represent the mean ± S.E. of a minimum of three experiments done in triplicate. Cell lysates were obtained from the 6-well plates and subjected to SDS-PAGE and immunoblotting with primary mouse monoclonal antibodies specific to the HA-epitope and myc-epitope of $NHE1_{HA}$ and CHP_{myc} respectively, in order to determine relative abundance of $NHE1_{HA}$ in response to $CHP3_{myc}$ expression (**B**). Blots were stripped and probed for the expression of endogenous GAPDH as a control for equal loading. Blots are representative of a minimum of three separate experiments.

maturation of NHE1 to the cell surface. However, it does not exclude that these alterations in CHP3 are not involved to a lesser degree, particularly since the binding of Ca^{2+} to CHP3 seems to be involved in its interaction with NHE1 (***Fig. 2.4)***.

In order to assay the activity, half-life and cell surface stability of $NHE1_{HA}$ in the absence and presence of $CHP3_{myc}$, wild type and mutant forms, we required cells in which each protein was expressed at a consistent, reproducible level. An AP-1 cell line stably co-expressing both $NHE1_{HA}$ and either wild type $CHP3_{myc,}$ the N-myristoylation- (G2A), or Ca^{2+}-binding deficient (D123A) forms were obtained by co-transfecting the corresponding expression plasmids and selecting for $NHE1_{HA}$ and $CHP3_{myc}$ expression over a two to four week period with repeated acid challenging ($NHE1_{HA}$) and G418 selection ($CHP3_{myc}$). The corresponding pools of doubly-expressing cells were then subjected to both a $^{22}Na^{+}$-influx, to assess activity of the exchanger, and Western blotting to assess protein expression. A control cell line was also obtained expressing only $NHE1_{HA}$ without $CHP3_{myc}$ by transfecting the NHE1 expression plasmid along with an empty vector. Equal quantities of protein were subjected to SDS-PAGE and immunoblotting to determine relative expression of both $NHE1_{HA}$ and $CHP3_{myc}$ through the use of antibodies recognizing the respective epitopes in each protein. An antibody that recognizes endogenous GAPDH was also used to verify equal protein loading. (***Fig. 2.6A***).

Further analysis of the NHE1 activity as a function of intracellular H^{+} concentration (pH_i) was obtained by subjecting the above cell lines to pH-clamp $^{22}Na^{+}$-influx measurements using the K^{+}/nigericin technique as described in "Materials and Methods". Results normalized to 100% at the exchangers maximal activity at pH_i 5.4, revealed that mutations in the CHP3 proteins did not affect the H^{+} affinity of the exchanger (***Fig 2.6C***), supporting the notion that in interaction of NHE1 and CHP3, either wild-type of mutant forms, optimizes the activity of the exchanger solely through its increased expression at the cell surface.

Fig. 2.6.

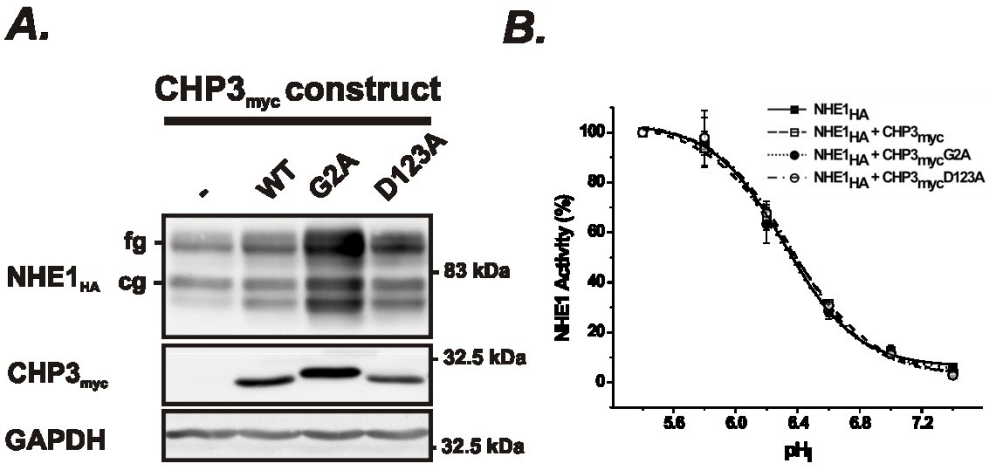


Figure 2.6. **Comparison of NHE1 exchange as a factor of CHP3 expression:** AP-1cells stable expressing $NHE1_{HA}$ or $NHE1_{HA}/CHP3_{myc}$ (WT or mutants) were obtained by transfecting $NHE1_{HA}$ with either with empty vector (-) or $CHP3_{myc}$ constructs (WT, G2A, D123A) followed by selection with repeated acid-challenge and G418. Expression of $NHE1_{HA}$ and the $CHP3_{myc}$ wild-type and mutants (G2A, D123A) was assessed by SDS-PAGE and immunoblotting as described in "Materials and Methods" (**A).** Na^+/H^+ transport activity of $NHE1_{HA}$ in cells expressing either $NHE1_{HA}$ alone or co-expressing $NHE_{HA}/CHP3_{myc}$ (WT, G2A, D123A) as a function of intracellular H^+ concentration (pH_i 5.4 to 7.4) was performed by clamping pH_i at different concentration using the K^+-nigericin method as described in "Materials and Methods". Data was normalized as a percentage of maximal rate of amiloride-inhibitable $^{22}Na^+$ influx at pH_i 5.4 (**C**). All values are an average of a minimum of three independent experiments, performed in triplicate represented as the mean ± S.E.. *Error bars* smaller than the symbol are absent.

N-myristoylation and Ca^{2+}-binding of CHP3 does not influence the cell surface maturation of NHE1.

Previously we determined that co-expression of CHP3 with NHE1 promotes the activity of NHE1 by affecting the exchangers maturation and cell surface stability (Zaun *et al*, 2008). Our current activity and expression studies (***Fig. 2.5, 2.6***) suggest that N-myristoylation and Ca^{2+}-binding of CHP3 are involved in the regulation of NHE1, since overexpression of wild type CHP3 upregulates NHE1 to a greater extent than the mutant forms (***Fig. 2.5***). In order to further investigate the effects of the CHP3 mutants (G2A and D123A) on the biosynthetic maturation of NHE1 over time, we analyzed the expression of transfected NHE1 expression either alone, or co-expressed with either CHP3 wild-type or mutants (G2A, D123A). AP-1 cells were cultured in multiple 10 cm dishes at equal cell density and transfected with either $NHE1_{HA}$ and empty vector or in conjunction with the different constructs of CHP3 (WT, G2A, or D123A). Cell lysates were obtained at time points up to 72 h, and analyzed by SDS-PAGE and immunoblotting to determine the expression of $NHE1_{HA}$ and $CHP3_{myc}$ (***Fig. 2.7***). $NHE1_{HA}$ alone showed an increase in both core- (**cg**) and fully glycosylated (**fg**) forms up to a maximum at roughly the 24 h time point with a majority of the expression as core glycosylated. $NHE1_{HA}$ co-transfected with wild-type $CHP3_{myc}$ also showed an increase in expression of both core- and fully glycosylated $NHE1_{HA}$, reaching its maximal expression between 24 to 36 h, but unlike the solely $NHE1_{HA}$ expressing cells, there was a much greater expression of the fully glycosylated form of $NHE1_{HA}$, which is in agreement with previous studies (Zaun *et al*, 2008). Co-transfection of $NHE1_{HA}$ with the N-myristoylated-defective or Ca^{2+}-binding deficient mutants of $CHP3_{myc}$, closely resembled the expression pattern of the $NHA_{HA}/CHP3_{myc}$ expression pattern (***Fig. 2.7***). A large fraction of fully glycosylated NHE1 that peaks between the 24 and 36 h time points that lags behind the core-glycosylated form as the immature $NHE1_{HA}$ is processed into the cell surface fully-glycosylated form. Furthermore in all cases the maximal expression of $NHE1_{HA}$ correlates closely with the expression level $CHP3_{myc}$.

Fig. 2.7.

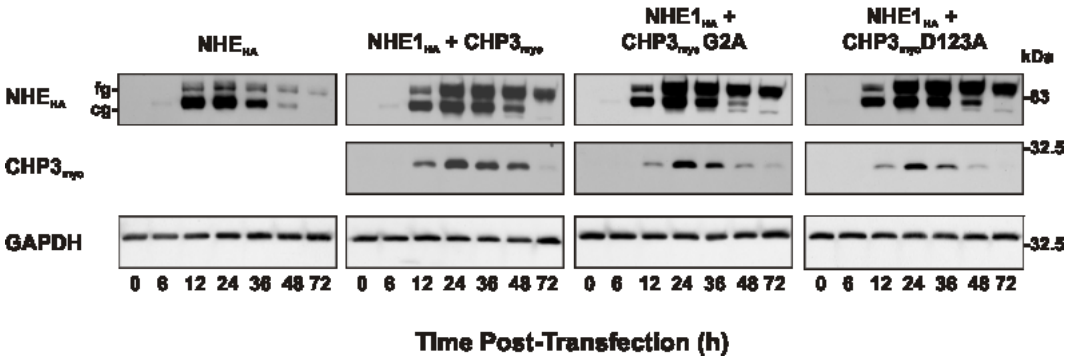


Figure 2.7. **N-myristoylation-defective and Ca^{2+}-binding deficient mutants of CHP3 and their effect on the biosynthetic maturation of NHE1.** AP-1 cells were transiently co-transfected with equal quantities of $NHE1_{HA}$ expression plasmid and plasmids containing either wild-type $CHP3_{myc}$ (WT) or mutants constructs (G2A and D123A). At indicated time points post-transfection, cell lysates were obtained, and equal amounts of protein were subject to SDS-PAGE and immunoblotting with a mouse monoclonal antibody specific to the HA-epitope to detect expression of core- (cg) and fully- (fg) glycosylated forms of $NHE1_{HA}$ in relation to time. $CHP3_{myc}$ expression was visualized by incubating blots with mouse monoclonal antibody specific to the myc-epitope. Blots were stripped and reprobed with a mouse monoclonal antibody recognizing endogenous GAPDH to verify equal loading.

These results suggest the non-myristoylatable and Ca^{2+}-binding defective CHP3 does not influence the late stage maturation of $NHE1_{HA}$.

In order to verify the above observations, we performed a cell surface biotinylation assay that closely mirrors the experimental protocol followed in *Fig 2.5*. Multiple 10-cm dishes of equally seeded and cultured AP-1 cells were transiently co-transfected with a fixed quantity of $NHE1_{HA}$ (8 μg/dish) and an increasing ratio of $CHP3_{myc}$ wild-type or mutant cDNA (0 – 2 μg/dish) to empty vector (pCMV). One μg of an expression vector containing the green fluorescent protein (pGFP) cDNA was also transfected as a control for equal transfection efficiency. At 24 hours post-transfection, cell surface proteins were biotinylated, in order to extract cell surface NHE1, and expression was analysis by SDS-PAGE and immunoblotting (***Fig. 2.8***) as described in "Materials and Methods". Purification of cell surface proteins with NeutrAvidin™-agarose followed by SDS-PAGE and immunoblotting with antibodies recognizing the HA-epitope of $NHE1_{HA}$ showed that the fully glycosylated form of NHE1 (***fg***) is the predominant species that resides at the cell surface. Furthermore, expression of wild-type as well as mutated forms of $CHP3_{myc}$ with $NHE1_{HA}$ increases the level of cell surface NHE1 as a function of $CHP3_{myc}$ expression, reinforcing the conclusion that N-myristoylation and Ca^{2+}-binding of CHP3 do not seem to influence the cell surface maturation of NHE1.

N-myristoylation and Ca^{2+}-binding of CHP3 are required for cell surface stability of NHE1.

Although we have demonstrated that N-myristoylation and Ca^{2+}-binding of CHP3 does not seem to influence the cell surface maturation of the exchanger (*Fig. 7 and 8*), the activity of $NHE1_{HA}$ in AP-1 cells when transfected with non-myristoylated or Ca^{2+}-binding deficient mutants of $CHP3_{myc}$ are roughly half the value of those transfected with wild-type CHP3. This suggests that another mechanism is involved by which CHP3 exerts its influence on the exchanger. Apart from enhancing the maturation of $NHE1_{HA}$ to the cell surface, CHP3 is also known to stabilize NHE1 at the plasma membrane. Therefore, to test whether the

Fig. 2.8.

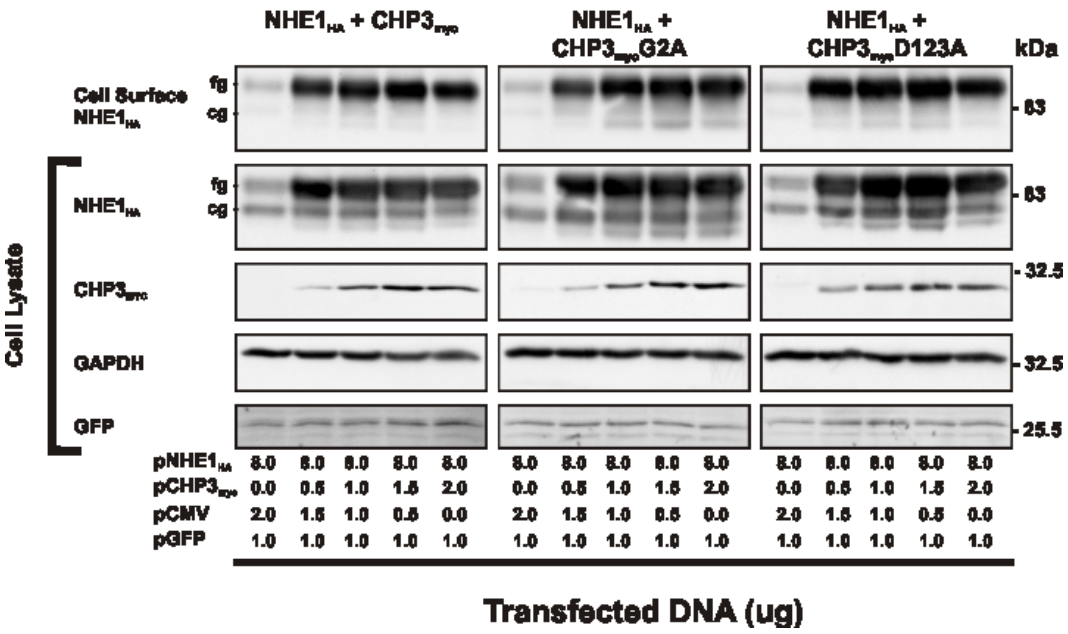


Figure 2.8. **Effect of CHP3 N-myristoylation-deficient and Ca^{2+}-binding defective mutants on cell surface abundance of NHE1.** AP-1 cells cultured equally on multiple 10-cm dishes and grown to subconfluence were transiently co-transfected with 8 µg of $NHE1_{HA}$ expression plasmid and an increasing ratio of $CHP3_{myc}$ (WT, G2A, or D123A) expression plasmids (0 to 2 µg per dish) to empty vector. Dishes were also simultaneously transfected with 1 µg of a green fluorescent protein expression vector (pGFP) in order to verify equal transfection efficiency. Twenty-four hours post-transfection, cells were subjected to surface biotinylation as described in "Materials and Methods". Surface biotinylated proteins and whole cell extracts were subject to SDS-PAGE and immunoblotting and cell surface fully glycosylated $NHE1_{HA}$, whole cell $NHE1_{HA}$, was analyzed as a function of increasing $CHP3_{myc}$ expression. Cell lysate immunoblots were stripped and reprobed for specific to endogenous GAPDH as a control for equal protein loading and GFP to verify equal transfection efficiency. Data shown are representative of three independent experiments.

CHP3 mutations affect the stability of NHE1 at the plasma membrane, AP-1 cells stably co-expressing $NHE1_{HA}$ alone or in conjunction with the three different forms of $CHP3_{myc}$ (WT, G2A and D123A) were assayed for the stability of the fully-glycosylated form of NHE1 using cycloheximide to block new protein synthesis in order to determine the half-life of existing NHE1, or a biotinylation pulse-chase assay to determine more directly the stability of cell surface NHE1.

Cells were cultured on multiple 10-cm dishes and treated with 100 µg/ml cycloheximide to block protein synthesis, and cell lysates were obtained at time points up to 36 h. After subjecting cellular proteins to SDS-PAGE and immunoblotting for expression of $NHE1_{HA}$ (***Fig. 2.9A***), the amount of fully glycosylated $NHE1_{HA}$ was measured by densitometry and analyzed as a decrease in the expression of fully glycosylated (fg) NHE1 over time and normalized to maximum expression at time point 0 h (***Fig. 2.9B***). As we expected, the level of core glycosylated NHE1 in each case disappears rather quickly, most likely due to either maturation to the fully glycosylated form, degradation, or both. In the case of cells expressing $NHE1_{HA}$ alone, there is rapid decrease in the level of the fully glycosylated NHE1, retaining 52.7 ± 5.4 % of the protein at the 36 h time period. In agreement with previous findings (Zaun *et al*, 2008), in the $NHE1_{HA}/CHP3_{myc}$ wild-type expressing cells, fully glycosylated $NHE1_{HA}$ appeared much more stable with a retention of 74.2 ± 8.5% at 36 h. Interestingly, when the stability of fully glycosylated $NHE1_{HA}$ was analyzed in cells co-expressing either of the two mutant forms of $CHP3_{myc}$ (G2A and D123A), the exchanger retains stability with values roughly equal to the $NHE1_{HA}/CHP3_{myc}$ cell line up to 24 h, before rapidly degrading to levels equivalent to cells expressing $NHE1_{HA}$ exclusively. Interestingly, when the levels of fully glycosylated $NHE1_{HA}$ are compared to the levels of $CHP3_{myc}$ expression, there appears to be a correlation between the two. In the stable $NHE1_{HA}$ levels in cells co-expressing wild type $CHP3_{myc}$, CHP3 expression is also much more stable, with expression up to the 36 h time point. However, in cells where $NHE1_{HA}$ is co-expressed with either $CHP3_{myc}$G2A or $CHP3_{myc}$ D123A mutants, the expression of the CHP3 proteins are reduced rather rapidly at the 24 and 12 h time points, respectively. This suggests that the

Fig. 2.9.

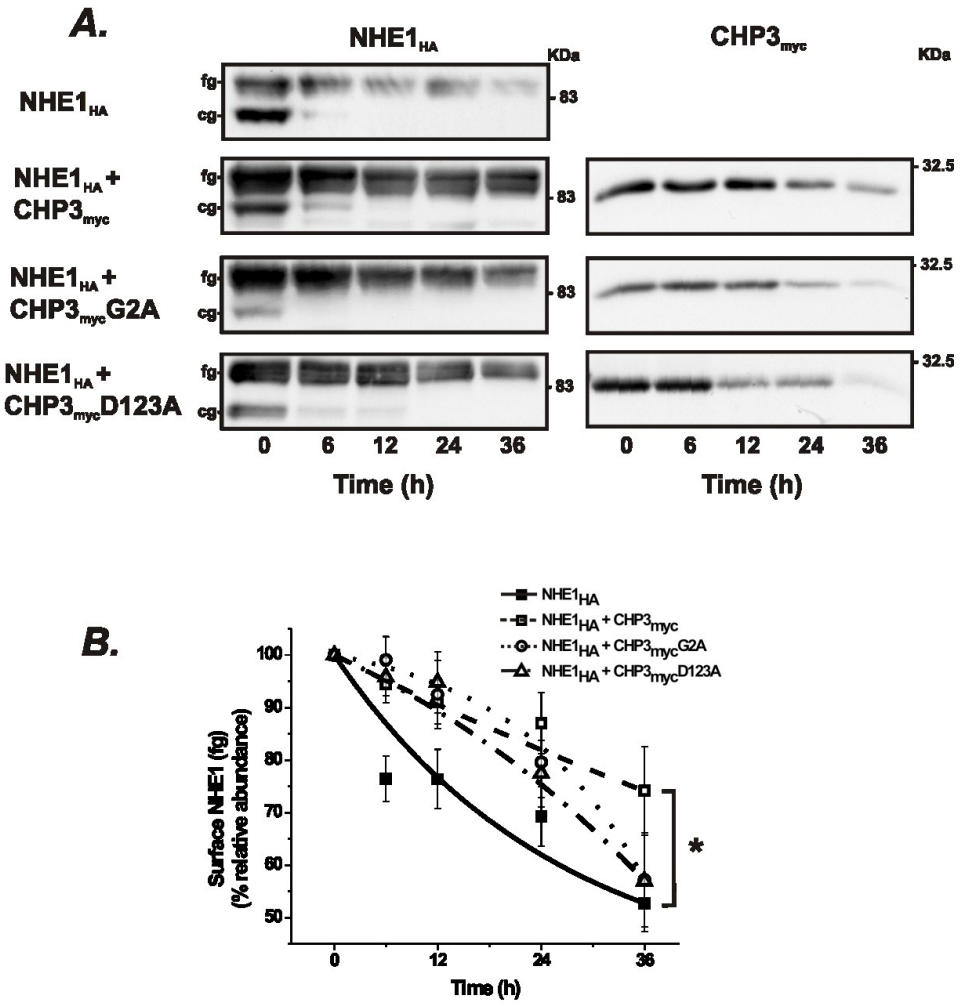


Fig. 2.9: **Effect of CHP3 on half-life of NHE1 during cessation of protein synthesis.** The half-life of $NHE1_{HA}$ in the absence and presence of the different $CHP3_{myc}$ constructs (WT, G2A and D123A) was measured in AP-1 cells stably expressing $NHE1_{HA}$ alone or in concert with $CHP3_{myc}$ forms. Cells were cultured in 6-well plates to subconfluence and treated with 100 µg/ml of cycloheximide in growth media to arrest protein synthesis for up to 36 h. Cell lysates were acquired at time points indicated and lysates were subjected to SDS-PAGE and immunoblotted with primary mouse antibodies specific to the HA- and myc-epitopes to visualize decreasing levels of both $NHE1_{HA}$ and $CHP3_{myc}$ respectively (**A**). Densitometry measurements of multiple exposures were collected in order to account for under or over-saturation using ImageJ® software and plotted as a function of time (**B**). Values represent a mean of a minimum of three experiments ± S.E. and significant difference (*) measure of student t-test with a null hypothesis of 0.05.

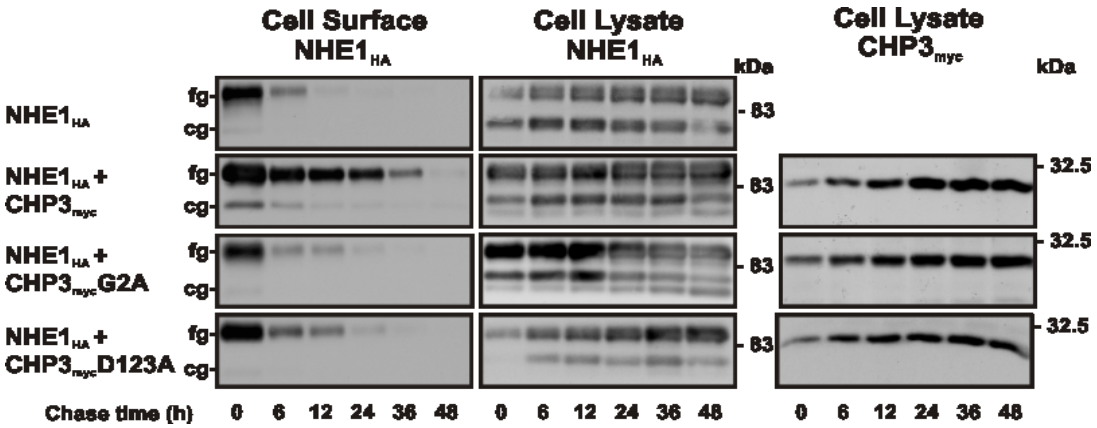
stability of NHE1 is directly related to the stability of the CHP3 proteins, and that the more stable NHE1/CHP3 complex formed with wild-type CHP3 retains the exchanger at the cell surface.

The second assay that was performed to determine the stability of NHE1 at the cell surface when co-expressed with the different CHP3 proteins was a surface labelling biotinylation-chase assay. AP-1 cells stably expressing either $NHE1_{HA}$ alone or in combination with the different $CHP3_{myc}$ proteins were cultured in 6 well plates to subconfluency, and biotinylated for 30 min on ice to label cell surface $NHE1_{HA}$. After repeated washes and quenching in buffer supplemented with 20 mM glycine to remove excess biotin, cells were incubated in growth media supplemented with 10% FBS for up to 48 h in 37ºC, 5% CO_2. At each time point, cell lysates were obtained and biotinylated proteins were extracted with NeutrAvidin™-Agarose beads and subjected to SDS-PAGE and immunoblotting in order to visualize the originally labelled cell surface $NHE1_{HA}$ (***Fig. 2.10A***). Expression of fully glycosylated cell surface $NHE1_{HA}$ at each time point was measured by densitometry, normalized to maximum NHE_{HA} expression at time point 0 h, and plotted as a decrease in band intensity over time (***Fig. 2.10B***).

As expected, the half-life of surface NHE1 in cells dually expressing NHE_{HA} and wild-type $CHP3_{myc}$ showed a much greater stability than cells expressing solely $NHE1_{HA}$ (14.5 ± 3.6 h vs. 3.8 ± 0.6h). However, the half-life of $NHE1_{HA}$ in cells co-expressed with the N-myristoylation-defective (G2A) and the Ca^{2+}-binding deficient (D123A) mutants of CHP3 closely paralleled that of cells expressing $NHE1_{HA}$ alone (6.1 ± 1.1 h and 4.8 ± 0.8 h respectively). These results indicate that although N-myristoylation and Ca^{2+}-binding of CHP3 are not critical for the interaction of CHP3 with NHE1, nor influence the late stage maturation of the exchanger, they are crucial for maintaining the stability of NHE1 at the cell membrane, and regulating Na^+/H^+ exchange accordingly.

Fig. 2.10.

A.


Cell Surface
NHE1HA
Cell Lysate
NHE1HA
kDa
Cell Lysate
CHP3myc
kDa
NHE1HA
fg
cg
NHE1HA +
CHP3myc
NHE1HA +
CHP3mycG2A
NHE1HA +
CHP3mycD123A
83
32.5
Chase time (h)
0 6 12 24 36 48

B.

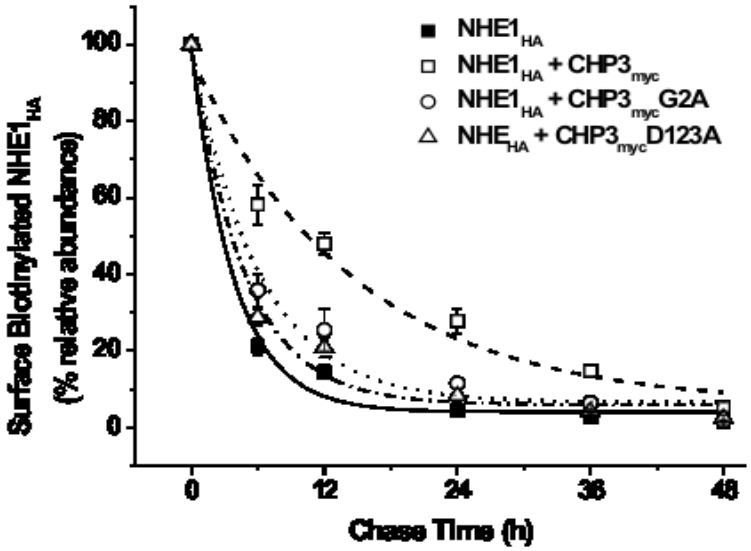

NHE1HA
NHE1HA + CHP3myc
NHE1HA + CHP3mycG2A
NHEHA + CHP3mycD123A
Surface Biotinylated NHE1HA
(% relative abundance)
100
80
60
40
20
0
0
12
24
36
48
Chase Time (h)

Figure 2.10. **Effect of CHP3 on cell surface stability of biotinylated NHE1.** AP-1 cells stably expressing $NHE1_{HA}$ separately or co-expressing $NHE1_{HA}$ along with the different form so $CHP3_{myc}$ (WT, G2A, and D123A), were cultured to subconfluence in 6-well plates. Surface membranes proteins were biotinylated, and after removal excess biotin, cells were returned to growth media for the indicated time. Cells cell lysates were obtained, and after a fraction was removed for immunoblotting, the remainder was subject to incubation with NeutrAvidin™-agarose to purify cell surface proteins. All proteins were subject to SDS-PAGE and immunoblotting with mouse monoclonal antibodies specific to the epitopes of $NHE1_{HA}$ and $CHP3_{myc}$ in order visualized total cellular NHE1 and CHP3 as well as cell surface NHE1 (**A**). Densitometry measurements were taken of multiple exposures of fully glycosylated (***fg***) cell surface $NHE1_{HA}$ in order to maintain expression levels within the linear range of exposed film. Results are representative of three separate experiments and data analyzed using ImageJ® and plotted as a function of time as mean ± S.E. (**B**)

N-myristoylation and Ca^{2+}-binding of CHP3 appear to work synergistically in relation to the effect on NHE1 maturation and stability.

Interestingly, the interaction of NHE1 with the N-myristoylation-defective (G2A) and Ca^{2+}-binding deficient (D123A) mutants of CHP3 appear to elicit the same response. The only noticeable difference appears to be the $NHE1_{HA}/CHP3_{myc}$ complex which seems to be influenced by the presence of Ca^{2+} bound to CHP3 (***Fig 2.4***). This would suggest the possibility that the structural integrity of both domains is jointly required for optimal function of CHP3. Indeed, numerous N-myristoylated, EF-hand domain containing proteins function as what is termed Ca^{2+}-myristoyl switch proteins (Ames *et al*, 1997). These include recoverin, a calcium-binding protein found in photoreceptor cells (Zozulya and Stryer, 1992, Ames *et al*, 1995) and neurocalcin, a calcium-sensing protein in the brain (Ladant, 1995). Gutierrez-Ford *et al*, demonstrated that upon binding Ca^{2+}, CHP3 undergoes a conformational change, albeit small in comparison to other EF-hand proteins (Gutierrez-Ford *et al*, 2003). It is plausible that this change may expose the myristoylation site that would otherwise be hidden within the protein. Although there has yet to be detailed structural information of the CHP3 protein, structural studies on the other CHP isoforms (1 and 2) have demonstrated that when complexed with the CHP-binding region of NHE1, the myristoyl moiety is exposed and capable of interacting with the plasma membrane (Ammar *et al*, 2006; Mishima *et al*, 2007).

To further test the interdependence of these two domains, a double mutation of the $CHP3_{myc}$ protein was produced ($CHP3_{myc}$G2A/D123A) and assayed for its ability to bind $NHE1_{HA}$ and to effect change in its biosynthetic maturation, cell surface activity and stability. Co-immunoprecipitation studies were performed on AP-1 cells doubly expressing $NHE1_{HA}$ and wild-type $CHP3_{myc}$ as a control, and the newly constructed double mutant of CHP3 (G2A/D132A). Cell lysates were obtained and after removal of a small fraction for immunoblotting, the remainder of the cell lysates were divided equally. One half of the lysates were precipitated with an antibody specific to the HA-epitope to extract $NHE1_{HA}$ along with any complexed proteins, and the second half was

incubated with an antibody recognizing the myc-epitope to extract $CHP3_{myc}$ protein complexes. After extraction of the complexes with Protein-G-Sepharose, resultant protein complexes were subjected to SDS-PAGE and immunoblotting in order to visualize precipitated and total cell lysate $NHE1_{HA}$ and $CHP3_{myc}$ proteins (***Fig. 2.11***). In both cases, $NHE1_{HA}$ and the double mutant $CHP3_{myc}$G2A/D123A formed a protein complex . While the amount of the $CHP3_{myc}$G2A/D123A that forms a complex with NHE1 seems lower than that of the wild type $CHP3_{myc.,}$ there is a corresponding lower cellular content of $NHE1_{HA}$ in this cell line.

A repeat of the assay by which AP-1 cells expressing $NHE1_{HA}$ were transfected with an increasing ratio of $CHP3_{myc}$G2A/D123A as shown in *Fig.2.5*, results in an increase in both the activity of $NHE1_{HA}$ similar to the effects observed for the individual mutants of CHP3 (G2A and D123A) (***Fig. 2.12A***). Furthermore, this activity increase correlates to an increase in the expression of $NHE1_{HA}$, particularly the fully glycosylated form (***Fig. 2.12B***), without any noticeable difference in the intracellular H^+ (pH_i) affinity of NHE1 (***Fig. 2.12C***).

Finally, the cell surface maturation and stability of $NHE1_{HA}$ in the presence of the $CHP3_{myc}$G2A/D123A was verified by repeating the cell surface biotinylation and a biotinylation pulse chase assay. AP-1 cells cultured on to 10 cm plates were transfected with equal quantities of $NHE1_{HA}$ (8 µg/plate) and an increasing ratio of $CHP3_{myc}$G2A/D123A (0 – 2 µg/plate) to empty vector. Cell surface proteins were biotinylated at 24-hours post-transfection, and after removal of excess biotin, cell lysates were obtained. Cell surface $NHE1_{HA}$ was extracted with NeutrAvidin™-Agarose beads and subjected to SDS-PAGE and immunoblotting for cell surface NHE_{HA} demonstrated that as expression of $CHP3_{myc}$G2A/D123A increases, so does the level of cell surface $NHE1_{HA}$ (***Fig. 2.13***). This result is in agreements with the activity/expression assay in ***Fig. 2.12 A*** and ***B***. The cell surface stability of $NHE1_{HA}$ was assesses in AP-1 cell co-expressing $NHE1_{HA}$/$CHP3_{myc}$G2A/D123A by the surface biotinylation-chase assay as described previously. AP-1 cells co-expressing $NHE1_{HA}$ and $CHP3_{myc}$G2A/D123A were biotinylated to label cell $NHE1_{HA}$, and after removal of excess biotin, cells were chased with growth media supplemented with 10%

Fig. 2.11.

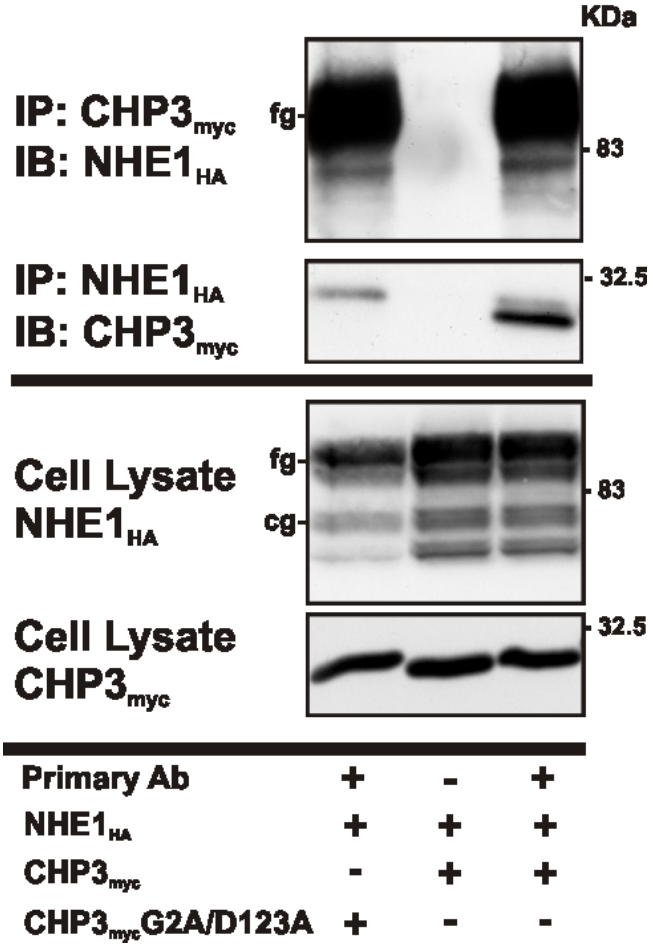


Figure 2.11. **Double mutation of N-myristoylation and EF-hand motif of CHP3 form a complex with NHE1.** Co-immunoprecipitation studies of the NHE1 and a CHP3 mutant deficient in both N-myristoylation and Ca^{2+}-binding ($CHP3_{myc}$G2/D123A) was evaluated in AP-1 cells stably co-expressing both $NHE1_{HA}$ with wild-type $CHP3_{myc}$ as a control, and the $CHP3_{myc}$ mutant. Cell lysates were obtained and after removal of a small fraction protein for Western blotting, the remainder was split into two equal volumes for incubation with either a rabbit polyclonal antibody recognizes the HA-epitope to extract $NHE1_{HA}$, or a rabbit polyclonal antibody recognizing the myc-epitope of $CHP3_{myc}$. Resulting protein complexes were extracted using Protein-G-Sepharose and subject to SDS-PAGE and immunoblotting. Blots were incubated with mouse monoclonal antibodies recognizing the HA-epitope on $NHE1_{HA}$ and the myc-epitope of $CHP3_{myc}$, followed by incubation with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) to visualize immunoreactive bands.

Fig. 2.12.

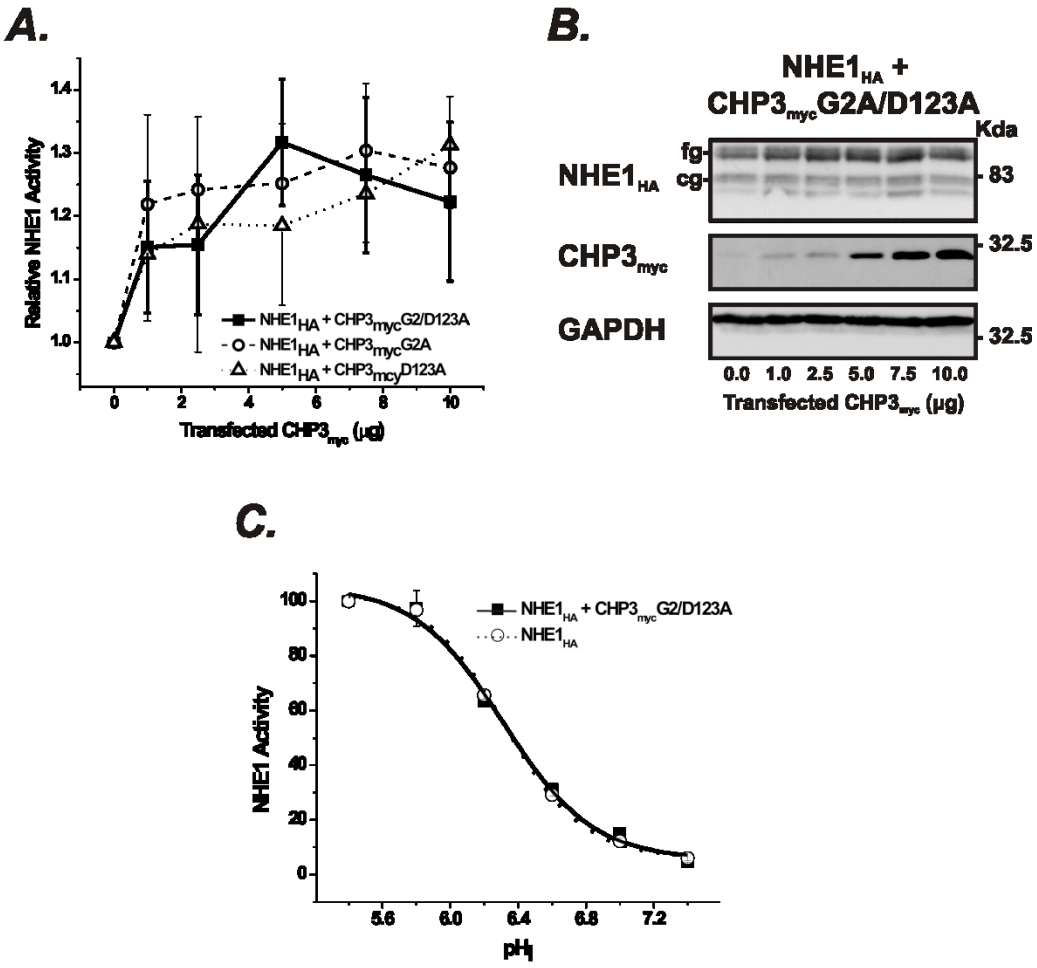

A.
Relative NHE1 Activity
1.4
1.3
1.2
1.1
1.0
0
2
4
6
8
10
Transfected CHP3myc (µg)
NHE1HA + CHP3mycG2/D123A
NHE1HA + CHP3mycG2A
NHE1HA + CHP3mcyD123A
B.
NHE1HA + CHP3mycG2A/D123A
Kda
fg
cg
NHE1HA
83
CHP3myc
32.5
GAPDH
32.5
0.0 1.0 2.5 5.0 7.5 10.0
Transfected CHP3myc (µg)
C.
NHE1 Activity
100
80
60
40
20
0
5.6
6.0
6.4
6.8
7.2
pHi
NHE1HA + CHP3mycG2/D123A
NHE1HA

Figure 2.12. **Activity and expression of NHE1 in response to N-myristoylation defective/Ca^{2+}-binding deficient mutant of CHP3.** Activity of NHE_{HA} in response to an increase in transfected $CHP3_{myc}$ double mutant (G2A/D123A) was evaluated in AP-1 cells stably expressing NHE_{HA} and transfected with $CHP3_{myc}$G2A/D123A as described in *Fig 5*. Transfected cell were split 24 h post-transfection into 24-well plates and at 48 h post-transfection, subject to $^{22}Na^{+}$-influx after NH_4-acid loading, and values were normalized to the activity of cells without transfected $CHP3_{myc}$G2A/D123A (**A**). The resulting uptake values were then plotted in comparison to the values obtained from the single $CHP3_{myc}$ mutations (G2A and D123A) from *Fig. 5A*. The same transfected cells were also plated on 6 well plates in order to obtain cell lysates that were subjected to SDS-PAGE and immunoblotting (**B**). To verify that the affinity for intracellular H^{+} of the exchanger was not affected, AP-1 cells stably co-expressing $NHE1_{HA}$ and $CHP3_{myc}$G2A/D123A were subject to pH-clamp using the K^{+}-nigericin technique as described in *Fig. 6C*, and subject to $^{22}Na^{+}$-influx measurements. Values were normalized to maximum activity at pH_i 5.4 and plotted over the pH_i range (pH_i 5.4 – 7.4) (**C***)*. The values were then overlayed with the values of $NHE1_{HA}$/$CHP3_{myc}$ wild-type expressing cells obtained in *Fig. 6C*.

Fig. 2.13.

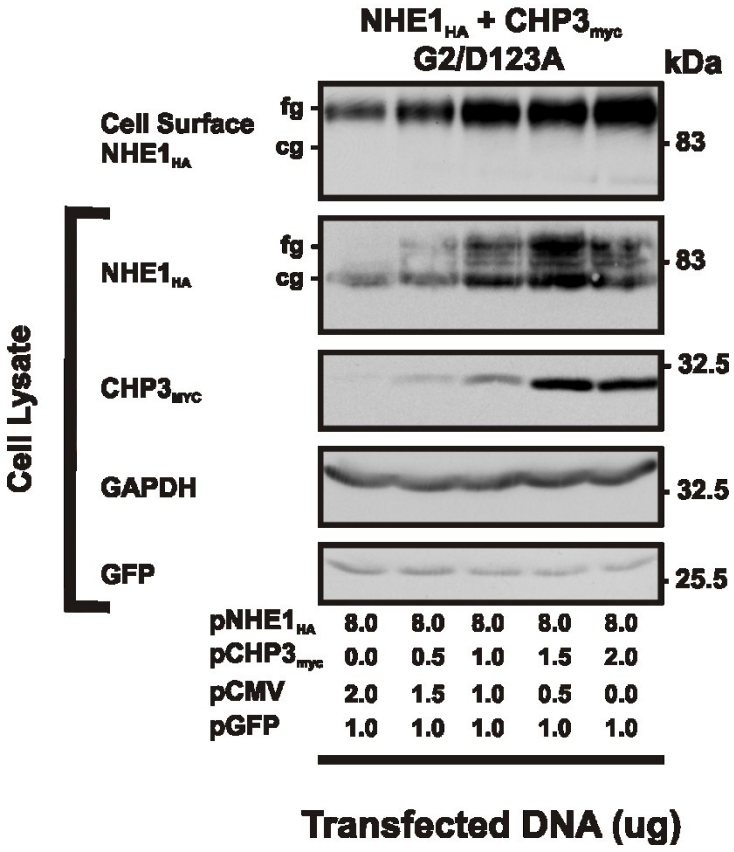


Figure 2.13. **Maturation of cell surface NHE1 in response to CHP3-G2A/D123A expression.** 10 cm dishes of AP-1 cells were co-transfected with a fixed amount of $NHE1_{HA}$ (8 µg/plate) and an increasing ratio of $CHP3_{myc}$G2A/D123A expression vector (0 - 2 µg/plate) to empty vector as described in *Fig 8*. As a control for transfection efficiency, 1 µg of an expression vector containing GFP (pGFP) was also transfected. 24 h post-transfection cell surface proteins were biotinylated for 30 min on ice and after removal of excess biotin, cell lysates were obtained. A small fraction of protein was removed for immunoblotting and the remaining lysates were incubated with NeutrAvidin™-Agarose beads to extract cell surface $NHE1_{HA}$. All proteins were then subject to SDS-PAGE and immunoblotting with monoclonal antibodies to the epitopes of HA and myc to visualize $NHE1_{HA}$ and $CHP3_{myc}$ respectively. As a control for equal loading and transfection efficiency, blots were stripped and reprobed with a monoclonal antibody specific to endogenous GAPDH and GFP.

FBS for up to 48 h. At specific time points cell lysates were obtained and cell surface proteins were purified by incubation with NeutrAvidin™-Agarose beads, followed by SDS-PAGE of cellular proteins and immunoblotting to visualize cellular $NHE1_{HA}$, $CHP3_{myc}$G2A/D123A as well as the decreasing amounts of cell surface $NHE1_{HA}$ (***Fig. 2.14A***). Analysis of the decreasing levels of cell surface $NHE1_{HA}$ was performed by densitometry measurements of band intensity using ImageJ™ software, normalized to maximum expression levels at 0 h, and plotted over time (***Fig. 2.14B***). Overlaying the values for the two single mutations of $CHP3_{myc}$ (G2A and D123A) form *Fig. 2.10*, reveals the half-life of the double mutant (5.0 ± 0.6 h) is not significantly different when compared to the single G2A and D123A mutants (6.1 ± 1.1 h and 4.8 ± 0.8 h respectively). These results suggest that the N-myristoylation and the EF-hand Ca^{2+}-binding domain operate jointly as a calcium-myristoyl switch protein where conformational changes induced upon the binding of Ca^{2+} to CHP3 allow the N-myristoyl group to bind to the inner leaflet of the plasma membrane, thereby stabilizing the NHE1/CHP3 complex at the cell membrane.

Fig. 2.14.

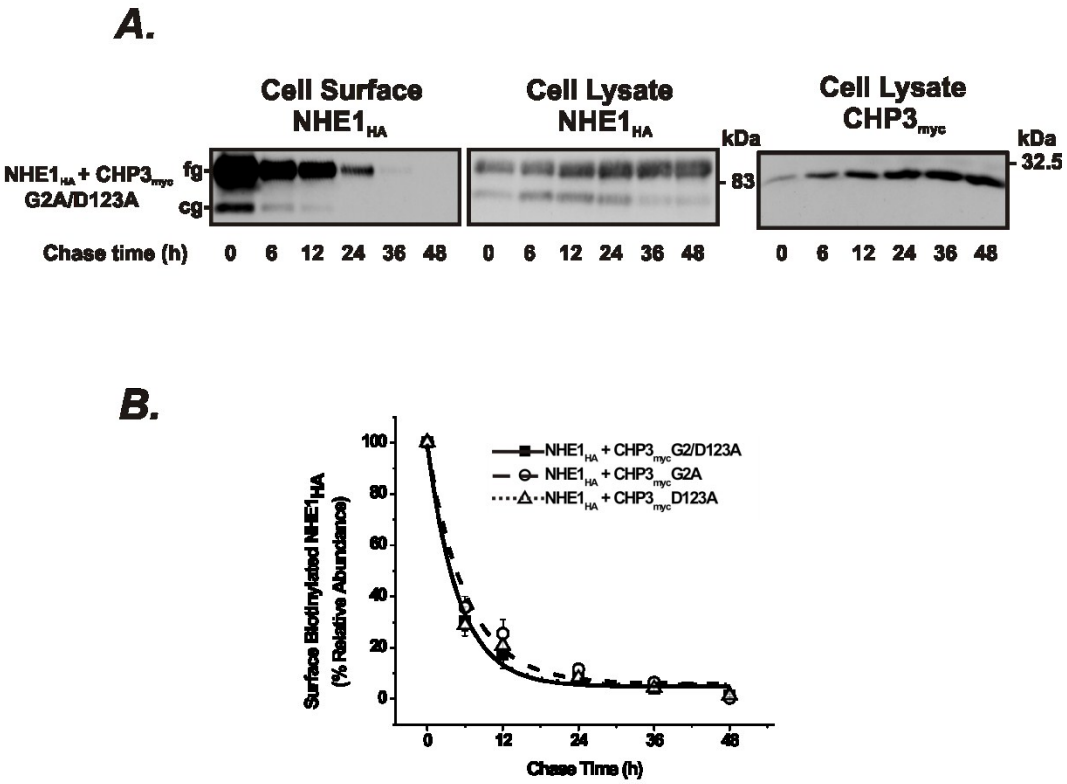


Figure 2.14. **Cell surface stability of NHE1 when co-expressed with CHP3-G2A/D123A.** AP-1 cells doubly expressing $NHE1_{HA}$ and the N-myristoylation-defective/Ca^{2+}-binding deficient $CHP3_{myc}$ (G2A/D123A) were seeded equally in a 6-well plate and cultured to subconfluence. Surface proteins were biotinylated for 30 min on ice, and after removal of excess biotin, cell were returned to growth media supplemented with 10% FBS and antibiotics and incubated at 37ºC, 5% CO_2 in humidified atmosphere. At indicated time-points cell lysates were obtained and after removal of a small fraction for Western blotting, subject to incubation overnight at 4ºC with NeutrAvidin™-agarose beads to extract surface labelled NHE_{HA}. Proteins were separated by SDS-PAGE and immunoblotted to visualize the loss of surface $NHE1_{HA}$ over chase time as described in legend to *fig. 10* (**A**). Band intensities of surface $NHE1_{HA}$ were measured by densitometry using ImageJ™ software, normalized to maximum expression at time 0 h, and plotted as a function of time (**B**). To correlate the stabilization G2A/D123A mutant of $CHP3_{myc}$ on $NHE1_{HA}$, the plot was overlayed with the values for the G2A and D123A mutants obtained in *Fig. 10*.

DISCUSSION:

N-myristoylation of proteins is known to be an important factor in such functions as membrane localization, enzymatic activity, Ca^{2+}-binding, protein-protein interactions as well as thermal stability (Johnson *et al*, 1994; Kakalis *et al*, 1995). EF-hand Ca^{2+}-binding motif is a term first used over 35 years ago to describe graphically the structure of the Ca^{2+}-binding motif formed by the E and F helices in parvalbumin (Kretsinger and Nockolds, 1973). EF-hands consist of a helix-loop-helix structure with 12 crucial amino acids within the loop region that are involved in the coordinating of the Ca^{2+} ions. Proteins containing EF-hand motifs have been shown to have a wide variety of functions that range from calcium buffering and signal transduction to muscle contraction (Lewit-Bentley and Réty, 2000). Previously, we reported that CHP3, an N-myristoylated, EF-hand containing protein, is required for the late stage maturation and stabilization of NHE1 at the cell surface, which results in an optimization of Na^+/H^+ transport by the exchanger (Zaun *et al*, 2008). In this study we further characterize the role that N-myristoylation and Ca^{2+}-binding of CHP3 has and its influence of NHE1 activity, processing, and stability.

The calcineurin B homologous protein 3 (CHP3) is the third member of a family of Ca^{2+}-binding, N-myristoylated proteins that have been shown to interact and enhance the activity of the sodium/protein exchangers (NHEs) (Pang *et al*, 2001, 2002; Mailander *et al*, 2001, Zaun *et al,* 2008). All three CHP proteins contain an N-myristoylated consensus site and at least one functional EF-hand Ca^{2+}-binding domain (Gutierrez-Ford *et al*, 2003; Pang *et al*, 2004) (***Fig. 2.1***). In the case of CHP1, also called p22, N-myristoylation and Ca^{2+}-binding have been shown to influence its association with membranes of the early secretory pathway, particularly the endoplasmic reticulum (Andrade *et al*, 2004). Furthermore, Pang *et al*, demonstrated that the affinity of CHP1 for Ca^{2+} increases upon the formation of a complex with NHE1, and that mutations of both, but not the individual functional EF-hand motifs impaired the interaction between these two proteins. These results suggest that bound Ca^{2+} may be important in stabilizing the

NHE1/CHP1 complex and thereby maintain the physiological activity and H^+-sensitivity of the exchanger (Pang *et al*, 2004).

It had been previously reported that the CHP1 and 2 isoforms bind the sodium hydrogen exchanger in a Ca^{2+}/myristoylation-independent manner (Pang *et al,* 2004, Ammar *et al*, 2006). In fact Pang et al demonstrated that a mutation that prevents N-myristoylation of CHP1 had no apparent effect on plasma membrane expression or exchange activity of NHE1 (Pang *et al*, 2001; 2004). In agreement with these observations, this current study demonstrates, using co-immunoprecipitation (***Fig 2.2***) as well as co-localization by confocal microscopy (***Fig. 2.3***), that N-myristoylation and Ca^{2+}-binding are not required for the interaction of NHE1 and CHP3. However, we do show evidence that suggests that Ca^{2+}-binding to the EF-hand domain of CHP3 does increase the affinity of the interaction between the two proteins (***Fig. 2.4***). Although Pang et al, also showed that removal of Ca^{2+}, with a Ca^{2+}-chelator, reduces the interaction between CHP1 and NHE1, in agreement with the current study, removal of both functional EF-hand motifs abolished the interaction between the two proteins (Pang *et al*, 2004). However, mutating the sole EF-hand motif of CHP3 did not abolish its interaction with NHE1. The fact that removal of Ca^{2+} and the mutation of the sole functional EF-hand motif resulted in such different outcomes in CHP1 is difficult to reconcile, but it is possible that these mutations in CHP1, apart from making Ca^{2+}-binding impossible may also impart a structural deformation of the helix-loop-helix structure of the two motifs (EF-3 and EF-4) of CHP1 resulting in abrogation of the interaction with NHE1.

By analyzing the functional significance of the overexpression of either the N-myristoylated defective (G2A) and Ca^{2+}-binding deficient mutants (D123A) of CHP3, we found that mutation to either of these two sites resulted in a reduction of the maximal rate of NHE1 activity (V_{max}), without affecting the affinity of the exchanger for intracellular H^+ when compared to overexpression of the wild type CHP3 (***Fig. 2.5***), and that this activation correlated with the total expression of the fully glycosylated form of NHE1. These results indicate that both N-myristoylation and Ca^{2+}-binding are required for optimal interaction and

functional regulation of NHE1 by CHP3. Although the results from overexpression of CHP3 with NHE1 in AP-1 cells devoid of endogenous NHE1, indicated that N-myristoylation and Ca^{2+}-binding CHP3 are not crucial for the late stage biogenesis of the NHE1 (***Fig. 2.7, 2.8***), measurements of both the half-life and stability of NHE1 at the cell surface indicate that these post-translational modifications are required for stability of exchanger at the cell surface (***Fig 2.9, 2.10***). Furthermore, the stability of CHP3 itself requires both N-myristoylation and Ca^{+2}-binding as shown by the loss CHP3 when expressed in AP-1 cells and treated with cycloheximide (***Fig. 9A***), and the lower expression in many of the immunoblots compared to wild type CHP3 (*data not shown*).

The majority of EF-hand motif containing proteins can be classified into one of two categories; Ca^{2+}-buffers such as calbindin and parvalbumin, function in modulation of the Ca^{2+} signal by binding free Ca^{2+} temporarily; and Ca^{2+}-sensors, which includes calmodulin and recoverin. Ca^{2+}-sensors undergo a conformational change upon binding Ca^{2+}, in response to an increase of intracellular Ca^{2+}, and translates this into a variety of chemical signals, often through their interaction with target molecules (Gifford *et al*, 2007). CHP3 was shown to bind calcium using both a $^{45}Ca^{2+}$-overlay of GST-huCHP3 (Mailander *et al*, 2001) as well as $^{45}Ca^{2+}$-equilibrium dialysis, that determined that CHP3 binds a single Ca^{2+} ion with submicromolar affinity (0.8 μM) (Gutierrez-Ford *et al*, 2003). Furthermore, Gutierrez-Ford et al, demonstrated that CHP3 undergoes a Ca^{2+}-conformational change allowing for the regulation of protein-protein interactions such as binding NHE1 (Gutierrez-Ford *et al*, 2003), suggesting that CHP3 is most likely a calcium sensor. This type of function has been shown for numerous EF-hand containing proteins including calmodulin that upon Ca^{2+}-binding, undergoes a conformational change to elicit the binding of target proteins that includes NHE1 (Igarashi and Watanabe, 2007; Bertrand *et al*, 1994). In fact, the calcineurin B regulatory subunit (CnB) of the phosphatase, calcineurin, for which the CHP proteins are named, undergoes a conformational change upon binding of Ca^{2+} that allows for the binding to the catalytic subunit calcineurin A (CnA), where it serves a structural role in stabilizing the complex (Rusnak and

Mertz, 2000; Feng and Stemmer, 2001). It should be noted that the functional EF-hand of CHP3 corresponds the third EF-hand of Calcineurin B (EF3) that has been shown to have the highest affinity for Ca^{2+} and plays a structural role in the CnA/CnB complex (Feng and Stemmer, 2001) Although CHP3 contains only one functional EF-hand Ca^{2+}-binding domains, it contains three other helix-loop-helix structural domains that may be involved in the stabilization of the protein through intramolecular EF-hand pairing (Niki *et al*, 1996).

Two decades ago, Recoverin, a Ca^{2+}-sensor involved in vision (Dizhoor *et al*, 1991) was shown to bind the outer segment membrane and other lipids when N-myristoylated, but not unmyristoylated. Furthermore, it was demonstrated that N-myristoylation acts cooperatively with Ca^{2+} binding to two of its four EF-hand domains (Dizhoor *et al*, 1993). This led to the proposal that recoverin possesses a Ca^{2+}-myristoyl switch, in which the binding of Ca^{2+} to the EF-hand domains results in a conformational change that leads to the extrusion of its myristoyl group, allowing for the interaction with a membrane or hydrophobic target (Zozulya and Stryer, 1992). The observation that the unmyristoylated and Ca^{2+}-binding deficient mutants of CHP3 are virtually identical in their regulation of NHE1 compared to wild-type CHP3, suggests that CHP3 may also act as a Ca^{2+}-myristoyl switch protein. Furthermore, the functional differences of these mutants were not compounded in a mutation where both the N-myristoylation site and the EF-hand domain were mutated. Upon binding of Ca^{2+}, CHP3 undergoes a conformational change that may expose the N-myristoylation site and facilitates the interaction with the inner leaflet of the plasma membrane, thereby stabilizing the NHE1/CHP3 complex at the cell surface.

Although several groups had hypothesized that CHP1 and 2 may act as a Ca^{2+}-myristoyl switch protein, the affinity (K_d) of CHP1 for Ca^{2+} is roughly 90 nM and increases to almost 2 nM upon NHE1 binding (Pang *et al*, 2004) Intracellular Ca^{2+} concentration increase from roughly 100 nM (10^{-7} M) to 10 μM (10^{-5} M) in response to stimuli such as membrane depolarization, extracellular signalling molecules, or intracellular messengers, that result in either the influx of extracellular Ca^{2+} or its release from internal stores, particularly the

endoplasmic/sarcoplasmic reticulum (Gifford *et al*, 2007). This suggests that CHP1 may constitutively bind Ca^{2+}, and therefore not act as a Ca^{2+}-myristoyl-switch protein. Furthermore, it has since been demonstrated that the myristoyl moiety of CHP1 and 2 may very well be permanently exposed on the outside of the protein structure and that upon interaction with NHE1, would probably be embedded in the plasma membrane (Ammar *et al*, 2006; Mishima *et al*, 2007). However, unlike CHP1 with its two functional EF-hand Ca^{2+} binding domain, the single EF-hand containing CHP3 has an affinity for Ca^{2+} with a K_d ~ 800 nM (Gutierrez-Ford *et al*, 2003), well within the physiological range of calcium concentration increase upon stimulation. Recoverin, one of the first Ca^{2+}-myristoyl switch proteins characterized has an affinity for Ca^{2+} to be roughly 3 μM (Permyakov *et al*, 2000). Although the structure for CHP1 and 2, bound to the CHP-binding region of NHE1, has been elucidated (Mishima et al, 2007; Ammar *et al*, 2006), currently there have not been any structural studies of the CHP proteins, particularly CHP3, in the absence of Ca^{2+}-binding, or with the EF-hand motifs mutated.

In summary, this study provides further knowledge of the roles of N-myristoylation and Ca^{2+}-binding of the calcineurin B homologous protein (CHP3) in its interaction and regulation of NHE1. CHP3 regulates the processing and stability of cell surface NHE1, and although N-myristoylation and Ca^{2+}-binding are not required for binding and for maturation of the exchanger, they play a crucial role in stabilizing NHE1 and the plasma membrane in order to promote optimal Na^+/H^+ exchange activity.

Preamble to Chapter 3[*]

** This section contains information from a paper published by Aharonovitz et al., 2000, of which the author of this thesis was a contributor. The experiments depicted in figures 1 and 2 of this section were performed by Pascal Lamare, a former student of our lab, and Fig. 3 is by the thesis author. All figures are currently unpublished data.*

Over the past fifteen years there has been much research pertaining to the Na^+/H^+ exchanger isoform 1 and its role in numerous pathophysiological developments within the myocardium. NHE1 activity has been shown to play a major role in many disease states of the heart, particularly cardiac hypertrophy (Karmazyn *et al*, 2008), and myocardial injury associated with ischemia/reperfusion (I/R) (Karmazyn *et al*, 1988, 1999, 2001; Avkiran and Marber, 2002). Studies demonstrating the cardioprotective effects of NHE1 inhibition during these pathophysiological states has been extensive, and although clinical trials involving NHE inhibitors have been generally disappointing (Mentzer *et al,* 2003; Zeymer *et al,* 2001), a large body of evidence continues to support the therapeutic role of NHE pharmacological antagonists (Fliegel, 2009; Avkiran *et al*, 2008; Karmazyn *et al*, 2008).

It has previously been demonstrated that chemically-induced depletion of cellular ATP profoundly reduces the activity of Na^+/H^+ transport by NHE1, even though the exchanger is electroneutral and does not consume metabolic energy (Goss *et al*, 1994; Wakabayashi *et al*, 1994b; Aharonovitz *et al*, 2000). Depletion of intracellular ATP by metabolic inhibitors resulted in both a reduction of the maximal activity of NHE1 (V_{max}) (Goss *et al*, 1994) as well as in an inhibition of the intracellular H^+-affinity of the exchanger (Wakabayashi *et al*, 1994b). Furthermore, this reduction in NHE1 activity is independent of the phosphorylated state of the exchanger (Goss *et al*, 1994); elimination of all putative phosphorylation sites by mutagenesis did not abrogate ATP sensitivity (Goss et al, 1994; Wakabayashi *et al*, 1994b). Further analysis of this effect determined that ATP-dependent sensitivity of NHE1 is mediated by the

interaction of the exchanger with the ubiquitous plasma membrane phosphoinositide, phosphatidylinositol-4,5-bisphosphate (PIP2) (Aharonovitz *et al*, 2000).

Although PIP_2 accounts for less than 1% of the total membrane phospholipid, it has been shown to play a role in many cellular processes, including cytoskeletal organization (Sakisaka *et al*, 1997), vesicular transport, calcium and growth factor signalling as well as regulation of various ion channels and transporters apart from the Na^+/H^+ exchanger (Suh and Hille, 2008; Huang, 2007). Upon metabolic depletion of ATP, PIP_2 undergoes extensive hydrolysis which is accompanied by a corresponding decrease in plasmalemmal PIP_2 content (Aharonovitz *et al,* 2000). In the case of NHE1, we previously demonstrated that elimination of the putative PIP_2 binding sites located in the juxtamembrane region of NHE1 results in a profound reduction in the ATP-dependent component of exchange (Aharonovitz *et al*, 2000). However, even with the reduction of ATP-sensitive Na^+/H^+ exchange, a measurable nucleotide-sensitive component of transport remained, suggesting that there is an additional, PIP_2-independent mechanism that contributes to the effect of ATP on NHE1 (Aharonovitz *et al*, 2000). Although the mechanisms by which PIP_2 is able to confer its effect on the exchanger remains to be determined, evidence suggests the interaction between NHE1 and PIP_2 may align that segment of the C-terminus of the exchanger along the inner leaflet of the plasma membrane; a conformation that supports optimal transport. Perturbations of that conformation, either by depletion of PiP_2 or by mutating the PIP_2 binding sites on NHE1 results in inhibition of the exchanger (Aharonovitz *et al*, 2000).

Within the myocardium, NHE1 is the predominant isoform that is responsible for pH regulation. Confocal fluorescent microscopy has shown that the exchanger is localized primarily to the intercalated disks and transverse tubules, but not along the lateral sarcolemma, suggesting that NHE1 may act to fulfill specialized roles within the heart by regulating the pH within small microenvironments within the heart and contributing to the selective activity of

pH-sensitive proteins such as connexin 43 at the intercalated disks or ryanodine receptor at the sarcoplasmic reticulum cisternae (Petrecca *et al*, 1999).

Although studies have been carried out to determine the subcellular localization of NHE1 within the myocardium, to our knowledge, there have not been any studies on targeting of the sodium hydrogen exchanger during times of ischemia and metabolic inhibition of intracellular ATP. To study the effects of ischemia on the subcellular localization of NHE1 in the myocardium, a previous student in our lab, Pascal Lamare, utilized confocal microscopy on isolated rat heart tissue labelled with antibodies specific to endogenous NHE1. Briefly, rat hearts were isolated and the aorta of the excised heart was immediately cannulated and subjected to nonrecirculating Langendorff perfusion (Hearse *et al*, 1976) at 37ºC with Tyrode's solution (136.9 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$, 10 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.5 mM glucose) at 10 ml/min, oxygenated by 95% O_2 and 5% CO_2. Hearts were either subjected to mimicked ischemia by a reduction of flow rate of Tyrode's solution to 3 ml/min (***Fig. 1***), or by chemical ischemia for 5 min in Tyrode's solution containing 5.5 mM 2-deoxy-D-glucose in replacement of glucose and supplemented with 1 µg/ml of antimycin-A, an inhibitor of oxidative phosphorylation (***Fig. 2***).

Under control conditions of normal Tyrode's solution at a flow rate of 10 ml/min, NHE1 localizes to the intercalated disks and transverse tubules in full agreement with the previous study by Petrecca et al, 1999 (***Fig 1A***). However, when hearts were subjected to low flow ischemia (3 ml/min) for longer than 2 h followed by reperfusion (10 ml/min) for 30 min, there was a redistribution of NHE1 from the intercalated disks and transverse tubules to the lateral sarcolemmal membrane (***Fig 1 C and D***). However, when low flow ischemia was administered for 30 min followed by 30 min reperfusion, no redistribution of the exchanger was observed, suggesting that the consumption of ATP by the cardiac tissue under conditions of low flow ischemia require an extended period of time. This is in agreement with previous studies indicating the ATP levels in hearts undergoing low flow ischemic perfusion required 60 min to drop to roughly 50%

Fig. 1

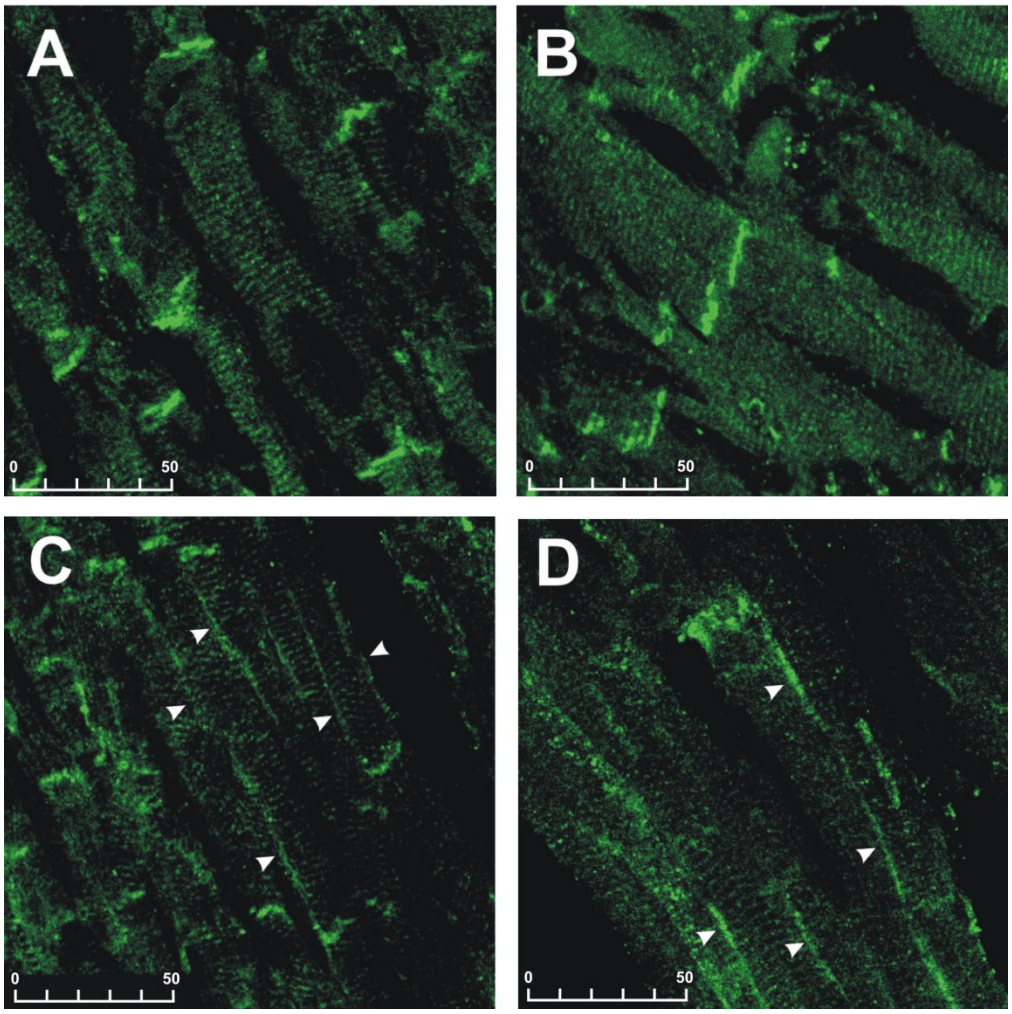


Figure 1. **Effect of low-flow ischemia and reperfusion of the subcellular localization of rat heart NHE1.** Isolated rat hearts (Langendorff preparation) were perfused for 3 h at 10 ml/min (**A**) or at low flow (3 ml/min) for 30 min (**B**), 2 h (**C**) or 3 h (**D**), followed by 30 min of reperfusion (10 ml/min) in normal Tyrode's solution. Tissues were fixed, then incubated with rabbit anti-NHE1 antibody, followed by incubation with a goat anti-rabbit IgG secondary antibody conjugated to oregon-green.

and continued to drop after 30 min or reperfusion (Cave *et al*, 2000; Finnegan *et al*, 1993).

A second method of mimicking ischemia in heart tissue is by chemically depleting ATP levels by perfusion of the heart with Tyrode's solution in which the glucose is replaced with the non-metabolizable analog, 2-deoxy-D-glucose as well as the addition of antimycin-A, which has been demonstrated to rapidly decrease ATP levels (Aharonovitz *et al,* 2000; Goss *et al*, 1994). Under these conditions, NHE1 was rapidly redistributed from the intercalated disk region and transverse tubules (***Fig. 2A***) to the lateral sarcolemma (***Fig. 2B***) after 5 min.

Along with the importance of the juxtamembrane region of NHE1 in regards to the maturation and stabilization of the exchanger through its interaction with CHP3, and ATP-dependence by way of interaction with PiP_2 through two binding regions that flank the CHP-binding site (Aharonovitz *et al*, 2000), this region of the exchanger is also responsible for the interaction of NHE1 with the ERM (ezrin, radixin, moesin) family of actin binding proteins. The ERM proteins preferentially interact with NHE1 at the distal PiP_2site, and this association seems to act as a scaffold assembly that is crucial for regulating the cortical cytoskeleton (Denker *et al*, 2000). Finally, Ikeda et al showed that this region of the NHE1 cytoplasmic domain is crucial for the maintenance of high pH_i-sensitivity of the exchanger (Ikeda *et al*, 1997).

The role of these interactions between the C-terminal tail of NHE1 and CHP3, PIP_2 and ERM, operating either independently or in concert, is thought to promote a stable conformation of the exchanger that is optimal for transport activity and responsiveness to various stimuli. Interestingly, CHP3 expression is predominantly restricted to the heart in adult human (Mailander *et al*, 2001). Furthermore, *in vitro* binding assays of GST-fusion proteins of the juxtamembrane regions of all the plasma membrane NHE isoforms (NHE1 – 5) indicate that CHP3 binds preferentially to NHE1 (***Fig. 3.***). This is seemingly in contrast to CHP1 which has been reported to interact with several plasma membrane-type NHE isoforms (Pang *et al*, 2001). The fact that CHP3 acts to stabilize the exchanger and thereby promotes optimal exchange activity, suggest

Fig. 2.

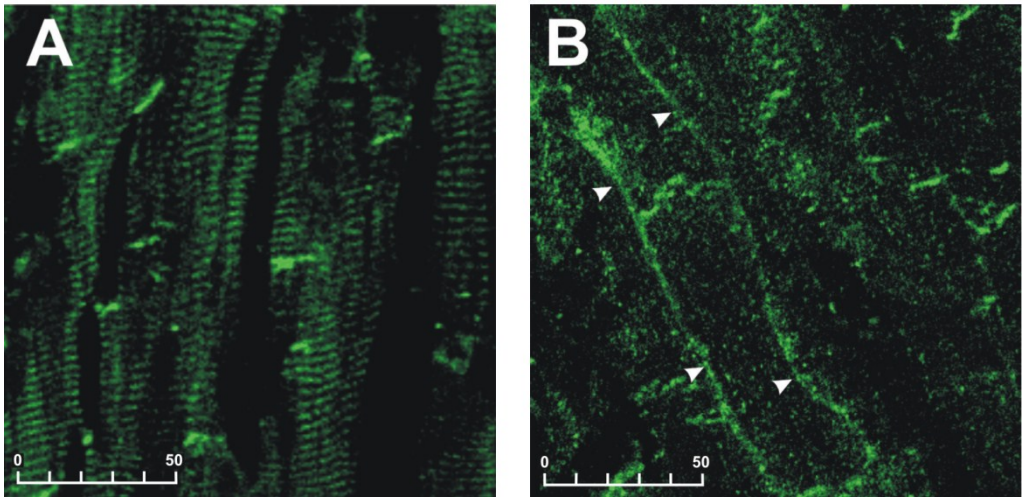


Figure 2. **Effect of chemically-induced ATP depletion on the subcellular distribution of rat heart NHE1.** Isolated rat hearts (Langendorff preparation) were perfused in normal Tyrode's solution (**A**), or in Tyrode's solution contain antimycin A (1 µg/ml) and 2-deoxy-D-gluces (5.5 mM) (**B**) for 5 min at 10 ml/min. Tissues were fixed, then incubated with rabbit anti-NHE1 antibody, followed by incubation with a goat anti-rabbit IgG secondary antibody conjugated to oregon green.

Fig. 3.

A.

```
GST-NHE1(505-540): 506 ---LVDLLAVKKKQETKRSENEEIHTQFLDHLLTGIED    - 540
GST-NHE2(479-515): 479 IRPLVEEFLDVKRSNKKQQAVSEEIHCRFFDHVKTGIE     - 515
GST-NHE3(456-489): 456 ---LVQWLKVKRSEQREPKLNEKLHGRAFDHILSAIE     - 489
GST-NHE4(463-496): 463 --PLVRYLDV-RKTNKKESINEELHIRLMDHIKAGIE     - 496
GST-NHE5(451-496): 451 --PLVKWLKVKRSEHHKPTLNQELHEHTFDHILAAVEDVVG - 489
```

B.

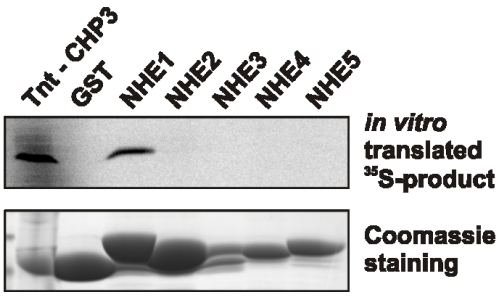


Figure 3. In vitro* interaction of the carboxyterminal regulatory domains of plasma membrane Na^+/H^+ Exchangers isoforms (NHE1 – 5) with CHP1 and CHP3.** It has previously been demonstrated that the CHP proteins bind the juxtamembrane region of the Na^+/H^+ exchanger isoform 1 (NHE1) (Pang *et al,* 2001; 2002, Zaun *et al,* 2008), and that CHP1 also interacts with several of the NHE isoforms (NHE2 and -3) (Pang *et al,* 2001). The juxtamembrane region of all five plasma membrane NHE isoforms (NHE1 through NHE5) (**A**) were obtained using PCR, subcloned into the GST-expression vector pGEX-2T (Amersham Bioscience) and transformed into the Epicurian Coli® BL-21 CodonPlus™ (Stratagene, Cedar Creek, TX). Bacterial lysates containing GST-fusion proteins of the NHE tail constructs were purified with Glutathione-Sepharose (Amersham Bioscience) and incubated with CHP3 proteins that were *in vitro* translated, and ^{35}S-labelled CHP1 using rabbit reticulocyte lysates (Promega, Madison, WI). After extensive washing GST-fusion protein complexes were extracted with Glutathione-Sepharose and subjected to duplicate SDS-PAGE. One gel was dried and bound radioactive CHP proteins were visualized using a PhosphorImager (**B, *upper panel), and the second was subjected to coomassie-blue staining to verify protein quantity (**B, *lower panel***).

that this interaction may be affected during periods of ATP depletion and ischemia/reperfusion and contribute to the decline in the membrane stability and

Chapter 3:

Regulation of the Na^+/H^+ Exchanger Isoform 1 (NHE1) During Episodes of Metabolic Stress: Assessment of the Role of Calcineurin B Homologous Protein 3 (CHP3)

Hans-Christian Zaun, Alvin Shrier, John Orlowski.

ABSTRACT

Restoration of cardiac intracellular pH following metabolically-induced acidification is of crucial importance for myocardial contractility. One of the primary regulators of pH homeostasis in the heart is the sodium/proton exchanger isoform 1 (NHE1) which functions to protect the myocardium from excess acid accumulation by extruding intracellular protons (H^+) in exchange for extracellular sodium (Na^+). Dysregulation of NHE1 during periods of myocardial ischemia and reperfusion is linked to arrhythmias, tissue damage and ultimately heart failure. Previous studies in our lab have determined that during times of low-flow ischemia/reperfusion and chemically-induced metabolic depletion of ATP in rat myocardium, NHE1 is redistributed from the intercalated disks and transverse tubules to the lateral sarcolemma (Lamare *et al*, *unpublished data*). Furthermore, acute ATP depletion in cultured fibroblastic cells severely impairs NHE1 activity and has been attributed, at least partially, to a loss of the interaction between the juxtamembrane region of the regulatory cytosolic tail of NHE1 with phosphatidylinsoitol-4,5-bisphosphate (PIP_2) located in the inner leaflet of plasmalemma (Aharonovitz *et al*, 2000). The calcineurin B homologous protein isoform 3 (CHP3) is a heart predominant member of the family of EF-hand Ca^{2+}-binding proteins that interacts with NHE1 in the immediate vicinity of the PIP_2 binding sites and has been demonstrated to stabilize NHE1 at the plasma membrane (Zaun et al, 2008). In this report, we assessed the consequences of ATP depletion on the physical and function interaction between NHE1 and CHP3. The results show that in Chinese hamster ovary fibroblasts, NHE1 cell surface abundance and subsequent activity is lost upon ATP depletion. Overexpression of CHP3 cannot compensate for these changes in NHE1 function despite the fact CHP3 remains bound to NHE1 throughout the period of cellular ATP depletion. Thus, during severe metabolic stress, the membrane redistribution and decline in NHE1 activity cannot be attributed to disruption in the interaction between the exchanger and CHP3.

INTRODUCTION:

The sodium/protein exchanger isoform 1 (NHE1) is a member of a family of eleven electroneutral (glyco)phosphoproteins that regulate intracellular pH (pH_i) homeostasis and cell volume through the translocation of intracellular H^+ for extracellular Na^+ (Orlowski and Grinstein, 2004). It is ubiquitously expressed and is the primary isoform found within the myocardium where it protects the heart from acidification, a conditions that has been shown to have a profound detrimental effect on myocardial contraction (Orchard and Kentish, 1990). NHE1 consists of two domains; an N-terminal transmembrane domain of approximately 500 amino acids which is composed of 12 transmembrane segments, that is responsible for ion translocation, and a large C-terminal cytoplasmic tail that is responsible for the regulation of the exchanger through phosphorylation by various protein kinases as well as interaction with numerous proteins and biomolecules (Orlowski and Grinstein, 2004).

Among the numerous interacting partners of the exchanger are a family of EF-hand Ca^{2+}-binding proteins termed the calcineurin B homologous proteins (CHPs), due to their similarity to the regulatory B subunit of the serine/threonine phosphatase, calcineurin, as well as their ability to regulate calcineurin activity (Lin *et al*, 1999; Gutierrez-Ford *et al*, 2003, Li *et al*, 2008). All three isoforms have been shown to stimulate NHE1 activity and bind the exchanger at the juxtamembrane region of the C-terminal tail (Pang *et al*, 2001, 2002; Zaun *et al*, 2008) There are three isoforms of the CHP proteins characterized to date (CHP1 through 3) that vary in their cellular distribution. CHP1 is ubiquitously expressed and is shown to interact with several isoforms of the plasmalemmal NHEs where it has been shown to be involved in setting the resting pH_i-sensitivity of the exchanger (Pang *et al*, 2001) and is involved in the stabilization of NHE1 at an early stage of biogenesis (Matsushida *et al*, 2007). CHP2 expression is limited to intestinal epithelia (Inoue *et al*, 2003) as well as various malignant cell lines where it constitutively activates the exchanger in a serum-independent manner (Pang *et al*, 2002). Although originally isolated from developing mouse testis,

CHP3's (also called Tescalcin) distribution in adult tissue is largely restricted to the heart in humans (Mailander *et al,* 2001), although it is also expressed in brain and stomach of mouse (Gutierrez-Ford *et al*, 2003). Furthermore, unlike the other CHP isoforms that interact with several NHE isoforms, (Pang *et al,* 2001, 2002) *in vitro* binding studies seem to indicate that CHP3 interacts preferentially with NHE1 (*unpublished date, see preamble to chapter 3*), where it promotes both late stage maturation as well as cell surface stability of NHE1 (Zaun *et al*, 2008).

The importance of the role of NHE1 in the myocardium has been exemplified over the years through numerous studies implicating the exchanger in both myocardial hypertrophy as well as damage sustained by the heart during ischemia/reperfusion (I/R) (Fliegel, 2009; Karmazyn *et al*, 1999). NHE1 has been shown to be elevated in a variety of cardiac disorders, including hypertrophied and diabetic hearts, as well as myocardium that has been subjected to ischemia or chronic acidosis (Jandeleit-Dahm *et al*, 2000; Gan *et al*, 1999; Dyck *et al*, 1995). Although the exact mechanisms by which the exchanger expression is induced, several studies have shown that it occurs at the transcriptional level in some, but not all cell types (Dyck *et al*, 1995; Gan *et al*, 1999). However, post-translational modifications have also been reported, including phosphorylation by various protein kinases that are activated by ischemia/reperfusion in intact hearts and isolated myocardiocytes (Moor *et al*, 2001). Furthermore multiple studies over the past several decades have demonstrated that inhibition of NHE1 alleviates both hypertrophy as well as damage from I/R (Karmazyn *et al*, 2008; Avkiran *et al,* 2008; Cingolani *et al*, 2003; Karmazyn *et al*, 2005).

Another interacting partner of NHE1 is the plasma membrane phosphoinositide, phosphatidylinositol-4,5-bisphosphate (PIP_2) which confers ATP sensitivity on the exchanger (Aharonovitz *et al*, 2008). Chemically-induced metabolic depletion of ATP inhibits the exchanger by both reducing the absolute activity of the exchanger (V_{max}) as well as inhibiting the exchangers affinity for intracellular H^+ (pH_i) (Wakabayashi *et* al, 1994b) Furthermore unpublished data from our lab using isolated rat hearts demonstrated that extended low-flow ischemia followed by reperfusion, or the rapid depletion of ATP, results a

redistribution of the exchanger from the intercalated disks and transverse tubules to the lateral sarcolemmal membrane (Lamare *et al*, *unpublished data*). The ability of the heart predominant CHP3 to stabilize NHE1 at the cell surface in mammalian cell line and thereby enhancing exchange activity makes it an attractive choice for study of NHE regulation during times of ATP-depletion. In this study, we focus on the regulation of NHE1 in mammalian cells subjected to ATP-depletion and the possible roles that CHP3 may play. Our results indicate that during ATP-depletion there is a loss of cell surface NHE1 that seems to be independent of CHP3 expression.

MATERIALS AND METHODS

Materials and Solutions:

Minimum essential medium (αMEM), fetal bovine serum (FBS), penicillin/streptomycin, geneticin (G418), trypsin/EDTA and lipofectamine™-2000 were all purchased from Invitrogen (Grand Island, NY). Claycomb medium was purchased from JRH bioscience (Lenexa, KS), norepinephrine, L-glutamine. gelatine and fibronectin were obtained from Sigma-Aldrich, as was Nigericin, antimycin A, amiloride hydrochloride and 2-deoxyD-glucose. Carrier free $^{22}NaCl$ was purchased from PerkinElmer.

Monoclonal antibodies to the influenza virus hemagglutinin (HA) peptide was obtained from Convance Inc. (Berkeley, CA) and the c-myc proto-oncogene monoclonal antibody was from Millipore (Temecula, CA). Polyclonal antibodies to the same epitopes as well as antibodies specific to glyceraldehyde 3-phosphate (GAPDH) were obtained from Abcam Inc. (Cambridge, MA). Secondary antibodies conjugated to oregon-green was purchased from Jackson Laboratories (Bar Harbour, ME) as were the goat anti-mouse and anti-rabbit IgG antibodies conjugated to horseradish peroxidase. Cy-3, FITC, AlexaFluor™-488 and AlexaFluor-569 secondary antibodies were obtained from Molecular Probes (Eugene, OR).

All other chemical and reagents, unless otherwise stated were purchased from either Fisher Scientific or Sigma Aldridge and were of highest grade available.

Cell lines, Cell Culture.

Chinese hamster ovary cells devoid to endogenous plasma membrane NHE activity (AP-1) (Rotin and Grinstein, 1989) were cultured in α-minimum essential medium supplemented with 10% fetal bovine serum and 100 U/ml of penicillin and 100 µg/ml streptomycin and 25mM $NaHCO_3$ (pH 7.4). HL-1 mouse atrial cardiomyocyte cell line was maintained in Claycomb medium supplemented with 10% fetal bovine serum, 100 U/ml and 100 µg/ml of penicillin and

streptomycin respectively, 0.1 mM norepinephrine and 2mM L-glutamine as described by Claycomb *et al*, 1998, and grown on culture dishes pre-treated with 0.02% gelatine and 25 µg/ml fibronectin (Sigma-Aldrich). All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC and passaged every 48-72 hours before cell reached total confluency.

Depletion of Intracellular ATP.

Depletion of intracellular pH was carried as described by Goss et al. (*Goss et al,* 1994). AP-1 cells stably expressing either $NHE1_{HA}$ alone or co-expressing $NHE1_{HA}$ with $CHP3_{myc}$ were washed twice and then incubated in ATP depletion solution (100 mM K^+-glutamate, 30 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 5 mM 2-deoxy-D-glucose and between 1 µg/ml antimycin A). The high K^+, low Na^+ and Ca^{2+}-free solution was used in order to prevent Na^+ and/or Ca^{2+} loading by the Na^+/K^+- or Ca^{2+}-pumps upon inhibition and the use of glutamate as a substitute for Cl^- was to minimize cell swelling. Control cells were incubated in either in the same solution with the exception of 5 mM D-glucose replacing the 2-deoxy-D-glucose and without antimycin-A, or left untreated (Time course experiments)

Cell surface expression of the $NHE1_{HA}$ in AP-1 cells in the presence and absence of $CHP3_{myc}$ expression.

AP-1 cell expressing either $NHE1_{HA}$ exclusively or co-expressing $NHE1_{HA}$ along with $CHP3_{myc}$ were established as described previously (Zaun *et al*, 2008) and cultured to confluence on a series of 10 cm culture dishes. Cells were either incubated with ATP depletion solution as described above for up to 60 min or left untreated. At particular time points, cells were placed on ice and biotinylated with NHS-SS-biotin (Thermo Scientific, Rockford, IL) for 30 min on ice, followed quenching twice in buffer supplemented with 20 mM glycine for 7 min each to remove excess biotin. Cells were then washed extensively and cell lysates were obtained by the addition of ice-cold Radioimmunoprecipitation buffer (RIPA) (150 mM NaCl, 50 mM Tris (pH 8.0) 1 mM EDTA, 2.5 % deoxycholate, 0.5%

NP-40 and protease inhibitors (Roche Diagnostics, Indianapolis, IN), scrapping and incubating at 4ºC for 20 min on ice, followed by centrifugation for 20 min at 4ºC to pellet cellular debris. A small fraction of the supernatant was removed for immunoblotting and the remainder was incubated with NeutrAvidin™-Agarose (Thermo Scientific, Rockford, IL) in order to extract biotinylated cell surface proteins. After extensive washing, cell surface proteins and total cell lysates were subject to SDS-PAGE and immunoblotting with mouse monoclonal antibodies specific to the HA-epitope to recognize $NHE1_{HA}$, and myc-epitope, for $CHP3_{myc}$. This was followed by incubation with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase and immunoreactive bands were visualized by Western Lightning® Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Waltham, MA).

Coimmunoprecipitation:

Immunoprecipitation was carried out on a series of 10-cm plates of AP-1 cells co-expressing NHE1 containing a C-terminal HA-epitope ($NHE1_{HA}$) and CHP3 with a C-terminal myc-epitope ($CHP3_{myc}$). Cells were treated with ATP-depletion solution for different times ranging from 0 to 60 min after which cell lysates were obtained by washing cell twice with ice cold PBS, followed by the addition of 1 ml of ice-cold RIPA buffer (see above). Cell lysates were obtained by scraping and incubating the cell for 30 min. at 4ºC with gentle rocking, followed by centrifugation for 30 min at 4ºC to pellet cellular debris. Supernatants were pre-cleared with 100 µl of Protein G-Sepharose (GE Healthcare, Piscataway, NJ) slurry in RIPA buffer for a minimum of 2 h at 4ºC, followed by a brief centrifugation to remove slurry from cell lysate. A fraction of each lysate was removed for immunoblotting and the remainder was incubated with either 5 µl of a rabbit polyclonal antibody against the myc-epitope of CHP3, or 10 µl of a rabbit polyclonal antibody recognizing the HA-epitope of NHE1 and allowed to incubate overnight at 4ºC with gentle rocking in order to maximize antibody binding. Antibody-protein complexes were extracted by the addition of 100 µl of a 50% Protein-G-Sepharose slurry to each tube and incubating at 4ºC for several

hours with gentle rocking. Following repeated washes with RIPA buffer, proteins complexes were eluted by SDS-sample buffer containing 10 mM DTT and incubating at room temperature with gentle rocking for 30 min, without boiling in order to minimize aggregation of $NHE1_{HA}$. All samples were then subjected to SDS-PAGE and immunoblotting as described previously. Anti-myc and anti-HA immunoprecipitated proteins as well as protein lysates, were visualized by incubating blots with mouse monoclonal antibodies recognizing the HA- and myc-epitopes of $NHE1_{HA}$ and $CHP3_{myc}$ respectively, followed by incubation with goat anti-mouse secondary antibodies conjugated to horseradish peroxidase. All blots were visualized with Western Lightning® Plus-ECL enhanced chemiluminescence substrate.

Measurement of Na^+/H^+ exchange activity.

The activity of the Na^+/H^+ exchanger in AP-1 cells expressing either $NHE1_{HA}$ or the CHP3-binding defective mutant of $NHE1_{HA}$ (FL-A) (Zaun *et al*, 2008) as well as cells co-expressing both $NHE1_{HA}$ and $CHP3_{myc}$ in response to ATP depletion was assessed using a $^{22}Na^+$-influx assay as described previously (Aharonovitz *et al,* 2000). Briefly, cells were grown to confluency on 24-well plates and subject to ATP-depletion by incubating cells with ATP-depletion solution for different time points. At appropriate times, the cells were washed rapidly twice with an isotonic choline chloride solution (125 mM choline-Cl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM 2-deoxy-D-glucose, 20 mM HEPES (pH 7.4)), and the intracellular pH (pH_i) of the cells was adjusted to pH 6.2 by the addition of a solution that imposes a $[K^+]$ gradient in the presence of the K^+/H^+ ionophore, nigericin (8.8 mM KCl, 155 mM NMG-methanosulfonate, 2 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES (pH. 7.4), 10 μM nigericin, 1 mM ouabain), for 4 min at room temperature. $^{22}Na^+$-influx measurements were performed in the same K^+/nigericin solution containing carrier-free $^{22}Na^+$ in the presence and absence of amiloride for 10 minutes at room temperature.

The influx of $^{22}Na^+$ was terminated by the addition of 4 volumes of ice cold NaCl stop solution (130 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM

HEPES (pH 7.4)) followed by two additional washing steps in the same solution. Radiolabel was then extracted by solubilizing the cells with 0.25 ml of 0.5 N NaOH, which was transferred to scintillation vials. All the wells were washed with an additional 0.25 ml 0.5 N HCl, which was transferred to the same respective vials, followed by the addition of 5 ml scintillation fluid, and radioactivity was assessed by liquid scintillation spectroscopy. Measurements of $^{22}Na^{+}$ influx specific to NHE1 was determined as the difference between the initial rates of H^{+}_{i}-activated $^{22}Na^{+}$ influx in the absence and presence of 2 mM amiloride. Results were normalized to total protein content as determined using the Bio-Rad DC protein assay kit (BioRad, Hercules, CA) according to the manufacturers protocol. Each data point represents a minimum of 3 experiments done in triplicate and expressed as the mean ± S.E.

To assess the role of ATP-depletion on NHE1's affinity for intracellular H^{+}-concentration, cells grown to confluency on 24-well plates were subject to ATP-depletion by incubation with ATP-depletion solution or treated with control solution for 30 min, followed by clamping the intracellular pH (pH_i) at a concentration range of 5.4 – 7.4 by the addition of solution of varying K^{+} concentration with the addition of nigericin, followed by $^{22}Na^{+}$-influx as described above and by Aharonovitz et al (Aharonovitz *et al*, 2000).

Immunofluorescent confocal microscopy:

To determine the localization of $NHE1_{HA}$ and $CHP3_{myc}$, AP-1 cells stably expressing $NHE1_{HA}$ were plated onto glass coverslips, cultured to ~80% confluency, and transfected with $CHP3_{myc}$ using Lipofectamine™-2000 following the manufacturers recommended protocol. The targeting of these two proteins was also analyzed in the mouse atrial cardiomyocyte cell line, HL-1, by co-transfecting 5 μg of expression vectors containing cDNA of $NHE1_{HA}$ and $CHP3_{myc}$ using Lipofectamine™-2000. 24 h post-transfection cells were fixed in a PBS containing 2% paraformaldehyde for 20 min, and after washing, permeabilized in 0.1% triton in PBS for 30 min. This was followed by blocking for 1 hour in PBS containing 5% nonfat milk, incubation with a mouse

monoclonal antibody specific to the HA-epitope of NHE_{HA} and a rabbit polyclonal antibody recognizing the myc-epitope of $CHP3_{myc}$. Cells were incubated with secondary antibodies conjugated either to FITC (green) or to cy-3 (red) and mounted onto glass slides with ImmunoFluor™ mounting media (ICN Biochemicals, Aurora, OH).

The effect of ATP depletion on the immunofluorescent localization of NHE_{HA} and $CHP3_{myc}$ was studied in AP-1 cell stably co-expressing both $NHE1_{HA}$ and $CHP3_{myc}$ by culturing cells onto glass coverslips treated with fibronectin (1.5 μg/ml) (Sigma-Aldrich). Cells were fixed with PBS/2% paraformaldehyde for 20 min, permeabilized and with 0.2% saponin in PBS and after several washes with 10 mM glycine/0.01% saponin in PBS, cells were incubated in blocking solution (10% goat serum/0.01% saponin/PBS) for 1 hour. Cells were then incubated with a primary mouse monoclonal antibody specific to the HA-epitope and rabbit polyclonal antibody recognizing the myc-epitope at dilutions of 1:1000 and 1:500, respectively, in 0.01% saponin/10 % goat serum/PBS, for 1 hour at room temperature. After several washes in PBS/0.01% saponin, secondary goat anti-mouse and anti-rabbit antibodies conjugated to either AlexaFluor™-488 or AlexaFluor™-569 at a dilution of 1:2000 was added to the cells and incubated for 1 hour at room temperature. Coverslips were washed extensively in 0.01% saponin/PBS and mounted onto glass coverslips with ImmunoFluor™ mounting media.

All coverslips were analyzed using a Zeiss 510 laser scanning confocal microscope. The images presented are representative of multiple images of three separate experiments.

RESULTS

Co-localization of NHE1 and CHP3 in mammalian fibroblasts and myocytes:

It has previously been shown by our lab and others that NHE and CHP3 co-localize at the cell membrane when transfected into AP-1 Chinese hamster ovary fibroblasts (Zaun *et al*, 2008) and mouse LAP1 mouse fibroblasts (Mailander *et al*, 2001). In order to verify localization and targeting of NHE_{HA} and $CHP3_{myc}$ in cardiac cells, we studied the subcellular localization of the two proteins by confocal microscopy on a confluent monolayer of the mouse atrial cardiomyocyte cell line HL-1, an established cardiac myocyte cell line that retains a differential cardiac monolayer phenotype (Claycomb *et al*, 1998). HL-1 cells transiently co-expressing epitope-tagged $NHE1_{HA}$ and $CHP3_{myc}$ were labelled with primary mouse monoclonal anti-HA and rabbit polyclonal anti-myc antibodies, followed by secondary goat anti-mouse and anti-rabbit antibodies conjugated to either FITC (green), Cy-3 (red) or Cy-5 (red) (***Fig 3.1A and 3.1B***). Though both proteins can be readily seen throughout the cellular cytoplasm, there is strong co-localization at the plasma membrane, particularly in areas of cell-cell interaction (***Fig 3.1C***).

As a transfection and expression control, we also transiently co-transfected AP-1 cells with NHE_{HA} and $CHP3_{myc}$ as described above, with fixing, permeabilization and antibody labelling performed identically to HL-1 cells. As with the HL-1 cells both proteins showed protein showed labelling throughout the cell due to overexpression (***Fig 3.1D and 3.1E***), but with strong co-localization signal at the plasma membrane, particularly along the leading edge of the plasma lamellipodia (***Fig. 3.1F***).

Metabolic depletion of ATP reduces the amount of cell surface $NHE1_{HA}$ abundance without effecting total cell NHE protein level.

Previously, our lab demonstrated that when cardiac tissue was subject to either low flow ischemia followed by reperfusion or to depletion of ATP by

Fig. 3.1.

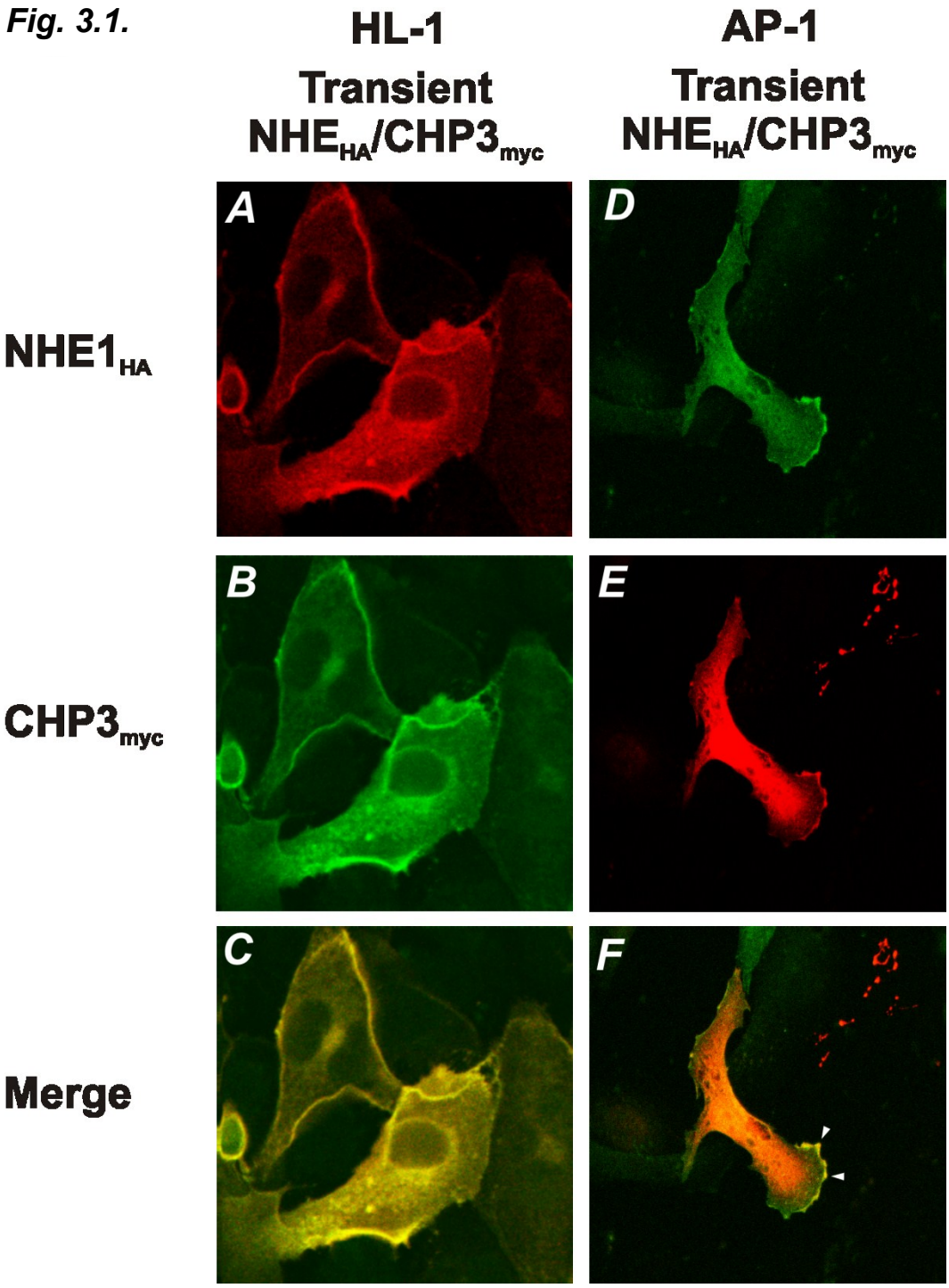

HL-1
Transient
NHE$_{HA}$/CHP3$_{myc}$
AP-1
Transient
NHE$_{HA}$/CHP3$_{myc}$
NHE1$_{HA}$
CHP3$_{myc}$
Merge
A
B
C
D
E
F

Figure 3.1. **Colocalization of wild type NHE1 and CHP3 in mammalian cells.** Subcellular distribution of transfected NHE_{HA} and $CHP3_{myc}$ was analyzed in a confluent monolayer of the mouse atrial cardiomyocyte cell line, HL-1 (**A – C**) and exchanger deficient Chinese hamster ovary fibroblasts, AP-1 (**D – F**). Cells co-transfected with $NHE1_{HA}$ and $CHP3_{myc}$ were incubated with mouse monoclonal and rabbit polyclonal antibodies specific to the HA- and myc-epitopes of NHE1 and CHP3 respectively, followed by incubation with secondary anti-mouse and anti-rabbit antibodies conjugated to either oregon green (green) or cy-3 (red) in order to visualize NHE1 and CHP3 expression. Within HL-1 cells, there is strong co-localization along the plasma membrane and cell-cell junctions (**C**) whereas AP-1 cells show strong colocalization at the leading edge of the cellular lamellipodia (**F, *white arrows***).

antimycin A and 2-deoxy-D-glucose, there is a redistribution of NHE1 from the intercalated disc region and transverse tubules to the lateral sarcolemmal membrane (Lamare *et al, unpublished data*). In order to examine the cell surface stability of $NHE1_{HA}$ in response to metabolic depletion of ATP in CHO/AP-1 cells overexpressing either $NHE1_{HA}$ or $NHE1_{HA}/CHP3_{myc,}$ and whether there is a stabilization effect that has been shown to occur when $CHP3_{myc}$ is co-expressed in mammalian fibroblasts, we undertook a cell surface labelling assay of $NHE1_{HA}$ over varying of times of ATP depletion. Cells were subjected to ATP depletion by incubation with ATP-depletion solution for a period time ranging from 5 – 60 min (controls cells at time point 0, were left untreated). At time points indicated, cell surface $NHE1_{HA}$ was biotinylated as described in "Materials and Methods", and biotinylated cell surface proteins were extracted with NeutrAvidin™-Agarose beads. Cell lysates and cell surface proteins were subjected to SDS-PAGE and immunoblotting to visualize cell surface $NHE1_{HA}$, total cell $NHE1_{HA}$, $CHP3_{myc}$, as well as endogenous GAPDH as a control for total protein (***Fig. 3.2***). In both the exclusively $NHE1_{HA}$ expressing cells as well as with the cells co-expressing $NHE1_{HA}/CHP3_{myc}$, cell surface NHE1 rapidly diminishes to minimal levels at 15 min. However, total $NHE1_{HA}$ appears much more uniform among all the time points, demonstrating that the cell surface exchanger seems to redistribute away from the cell membrane upon ATP-depletion without affecting the total cellular level of $NHE1_{HA}$. Interestingly both CHP_{myc} and endogenous GAPDH showed a noticeable decrease in total protein levels in relation to ATP-depletion time (***Fig. 3.2***). This may be a result of cell toxicity due to exposure to 2-deoxy-D-glucose and antimycin-A over an extended period of time. However, studies have shown that 2-deoxy-D-glucose stimulates autophagy which may also affect the decrease in cytoplasmic proteins such as GAPDH and CHP3 (Xi *et al*, 2010). Although it appears as if cell surface NHE_{HA} is not expressed at later time points of ATP depletion, the blot in *Fig.* 2 was used to represent cell surface depletion of $NHE1_{HA}$ without over-saturation the 0 and 5 min time points. Allowing for longer exposure of these blots did result in the visualization of cell surface $NHE1_{HA}$ at all time points indicated (*data not shown*). These results suggests that the CHP3

Fig. 3.2.

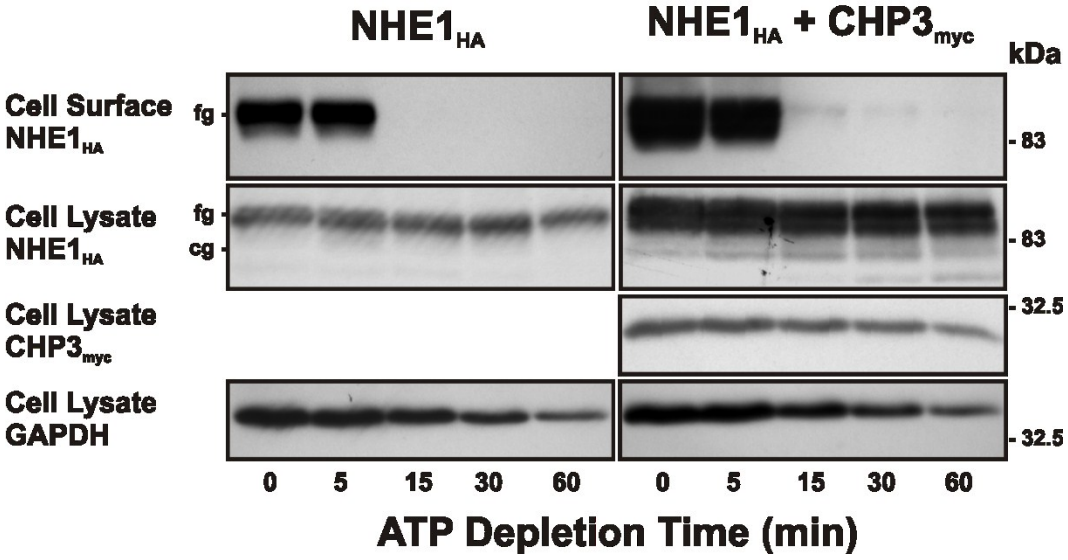


Figure 3.2. **ATP-depletion decreases cell surface NHE1 independent of CHP3 expression.** Endogenous Na^+/H^+ exchanger deficient AP-1 cells stably over-expressing $NHE1_{HA}$ or co-expressing $NHE_{HA}/CHP3_{myc}$ were subjected to ATP-depletion of a period of time from 0 to 60 min. At appropriate time points cells were treated with Sulfo-NHS-SS-biotin to label cell surface proteins, followed by extraction with NeutrAvidin™-agarose. After purification, cellular proteins were subjected to SDS-PAGE and immunoblotting with mouse monoclonal antibodies specific to the epitopes of $NHE1_{HA}$ and $CHP3_{mcy}$, followed by a secondary goat anti-mouse antibody conjugated to horseradish peroxidase in order to visualize expression of Cell surface $NHE1_{HA}$ along with total cellular NHE_{HA}, $CHP3_{myc}$. Immunoreactive bands of NHE1 appear as two bands representing the mature fully glycosylated (**fg**) and core-glycosylated (**cg**) form. Blots were also incubated with monoclonal antibody recognizing endogenous GAPDH to analyze total protein quantity.

expression fails to stabilize the NHE1/CHP3 complex at the cell surface upon metabolic depletion of ATP.

Co-immunoprecipitation of $NHE1_{HA}$ and $CHP3_{myc}$ under conditions of ATP-depletion:

Since the expression of $CHP3_{myc}$ did not seem to have an effect on the cell surface stability of $NHE1_{HA}$, we hypothesized that metabolic depletion of ATP may affect the interaction between the two proteins, resulting in a disassociation of CHP3 from NHE1, and thereby negating CHP3-dependent stabilization of NHE1 at the cell membrane. To test this hypothesis AP-1 cells co-expressing both $NHE1_{HA}$ and $CHP3_{myc}$ were cultured to confluence on a series of 10 cm tissue culture dishes. All cells were subjected to ATP-depletion by incubating with ATP-depletion solution as described above. At particular time points ranging from 0 (untreated cells) to 60 min, cell lysates were obtained and subjected to co-immunoprecipitation as described in "Materials and Methods". Briefly, after preclearing with protein G-sepharose, cell lysates were incubated with a rabbit polyclonal antibody specific to the myc-epitope of $CHP3_{myc}$. CHP3-protein complexes were then extracted with protein G-sepharose, followed by SDS-PAGE and immunoblotting with a monoclonal antibody recognizing the HA-epitope of NHE_{HA} (***Fig. 3.3***). Results demonstrated that both $NHE1_{HA}$ and $CHP3_{myc}$ remain in complex during ATP-depletion for up to 60 min, and that metabolic depletion does not result in a dissociation of the two proteins. In order to verify the interaction, co-immunoprecipitation experiments were also performed using a polyclonal antibody to extract $NHE1_{HA}$, which resulted in $CHP3_{myc}$ co-immunoprecipitating with $NHE1_{HA}$ throughout the ATP-depletion time indicated (*data not shown*).

Targeting and subcellular localization of $NHE1_{HA}$ and $CHP3_{myc}$ in mammalian cells subjected to ATP-depletion.

AP-1 cells stably expressing either $NHE1_{HA}$ alone or co-expressing $NHE1_{HA}$ along with $CHP3_{myc}$ were analyzed for subcellular localization of NHE1

Fig. 3.3.

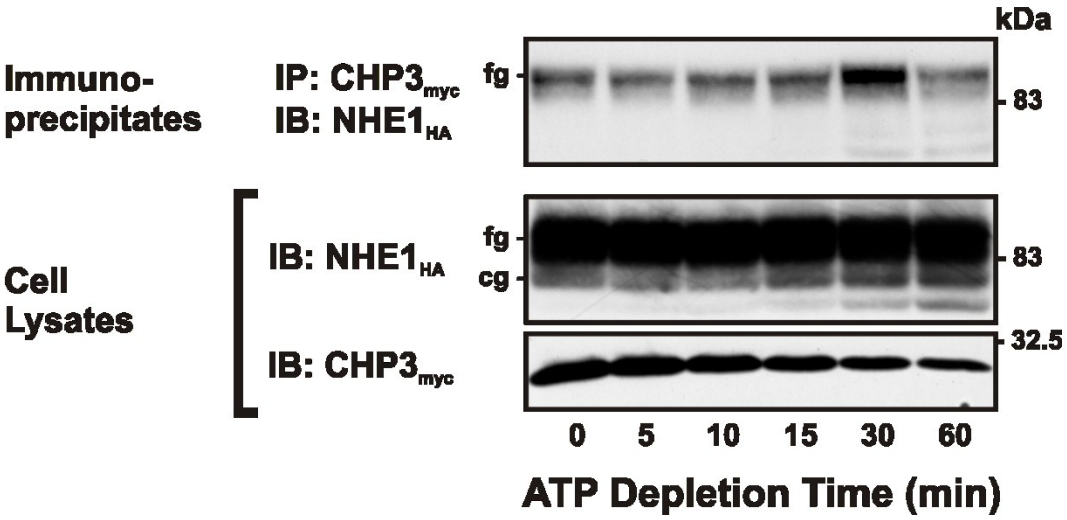


Figure 3.3. **ATP-depletion does not inhibit or dissociate NHE1/CHP3 co-immunoprecipitation.** Chinese hamster ovary AP-1 cells doubly expressing $NHE1_{HA}$ and $CHP3_{myc}$ were subjected to ATP depletion for different time points ranging from 0 to 60 min. At time points indicated, cells were lysed in RIPA buffer and cellular proteins harvested. After removal of a small quantity for Western blotting, cellular extracts were incubated with a rabbit polyclonal antibody specific for the myc-epitope followed by incubation with protein G-sepharose in order to immunoprecipitate (**IP**) $CHP3_{myc}$ and any complexed proteins. Immunoprecipitated complexes as well as cell lysates were subjected to SDS-PAGE and western blotting (**IB**) with monoclonal antibodies specific to the HA-epitope of $NHE1_{HA}$, indicating that $NHE1_{HA}$ remains complexed to CHP3 after ATP-depletion up to 60 min. Cell lysates were also immunoblotted with mouse monoclonal antibody recognizing the myc-epitope of CHP3. Blots are representative of three separate experiments.

and CHP3 under conditions of ATP-depletion over time using fluorescent confocal microscopy. In agreement with previous studies by us (Zaun *et al*, 2008) and others (Mailander *et al*, 2001), control cells (0 min) showed strong localization of $NHE1_{HA}$ to the cell membrane, and in cells co-expressing NHE_{HA} and CHP_{myc}, both protein co-localized to the cell membrane (***Fig. 3.4, 0 min***). As ATP depletion time increased, the membrane localization of NHE1, particularly in the cells expressing the exchanger in the absence of CHP3, appeared to become less distinct at 15 min onwards, in agreement with the cell surface stability assay performed previously (*Fig. 3.2*). Interestingly the loss of cell membrane localized $NHE1_{HA}$ coincided with a loss of $CHP3_{myc}$ at the plasma membrane as well, particularly at 15 min time point and onward (***Fig. 3.4, 15 – 60 min***). Although the loss of co-localized $NHE1_{HA}/CHP_{myc}$ signal at the 15 min and longer seems to disagree with the co-immunoprecipitation studies performed in Fig. 3, this observation may reflect the overexpression of both proteins within the cell. At all time points, there is an abundance of signal from either protein that does not overlap, and as two proteins redistribute away from the plasma membrane and disperse into the cytoplasm, it may become more difficult to visualize the interaction between the two.

Activity of $NHE1_{HA}$ in presence of absence of CHP3 overexpression when subjected to ATP-depletion.

Previous studies demonstrate that the inhibitory effect of ATP-depletion on NHE1 acts on both absolute activity of the NHE1 (V_{max}) as well as inhibiting its sensitivity to intracellular H^+ (pH_i) (Goss *et al*, 1994; Wakabayashi *et al*, 1994b). Both Pang et al and our lab demonstrated that when the crucial Phe and Leu residues of the CHP-binding domain of NHE1 were mutated to either Arg or Gln both the maximal activity as well as the H^+-sensitivity of the exchanger was inhibited, resembling that of ATP-depletion (Pang *et al*, 2001; Zaun *et al*, 2008). This suggests the possibility that the binding of CHP proteins to the juxtamembrane region of NHE1 at a site in between the two regions responsible for binding PiP_2, may influence the ATP-sensitivity of the exchanger. In order to

Fig. 3.4.

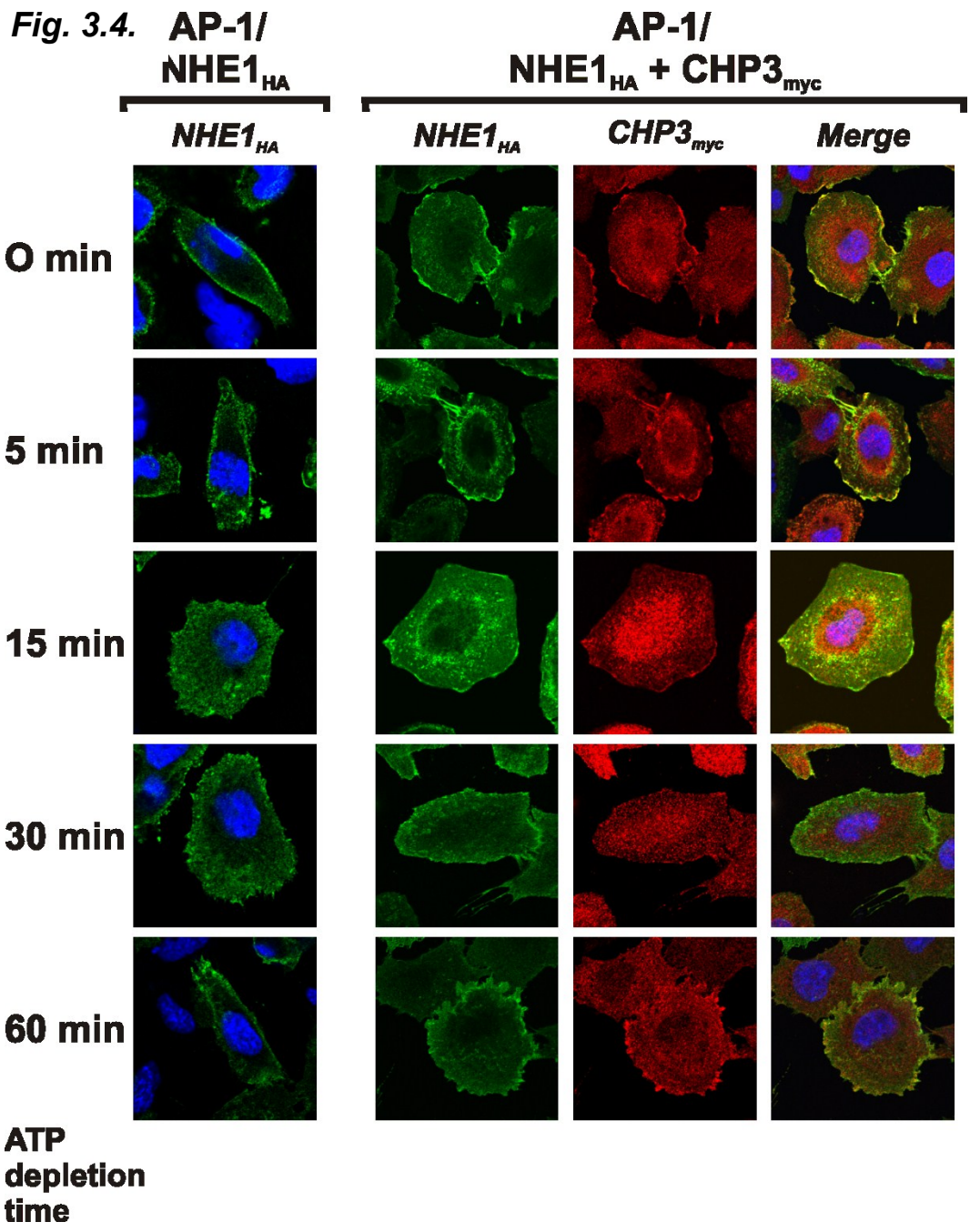

AP-1/ NHE1HA
AP-1/ NHE1HA + CHP3myc
NHE1HA
NHE1HA
CHP3myc
Merge
O min
5 min
15 min
30 min
60 min
ATP depletion time

Figure 3.4. **Cellular distribution and colocalization of NHE and CHP3 in mammalian cells subjected to ATP-depletion.** The intracellular distribution as well as colocalization of NHE1 and CHP3 was analyzed by fluorescent confocal microscopy on AP-1 cells overexpressing $NHE1_{HA}$ alone or in conjunction with $CHP3_{myc}$. Cells were cultured on glass coverslips treated with fibronectin to subconfluency and ATP-depleted for different time points ranging from 0 to 60 min. After fixing and permeabilization, cells were incubated with mouse monoclonal antibody specific to the HA-epitope on $NHE1_{HA}$ and rabbit polyclonal antibody recognizing the myc epitope of $CHP3_{myc}$. Cells were then incubated with goat anti-mouse and anti-rabbit secondary antibodies conjugated to AlexaFluor™-488 and AlexaFluor™-569 respectively to facilitate visualization of protein expression. At 0 min time point $NHE1_{HA}$ showed strong localization to the plasma membrane in both NHE_{HA} over-expressing AP-1 cells as well as cells over-expressing both $NHE1_{HA}$ and $CHP3_{myc}$. CHP3 showed a strong co-localization at the cell surface with NHE1, in agreement with previous findings (Mailander *et al*, 2001; Zaun *et al*, 2008) as well as localization studies in *Fig. 1*. At 15 min ATP-depletion, the expression of NHE at the plasma membrane began to decrease as did the co-localizing CHP3 protein.

test whether CHP3 or the binding of the CHP proteins influences the activity or H^+_i-sensitivity of $NHE1_{HA}$ subjected to ATP depletion, AP-1 cells stably expressing $NHE1_{HA}$ or $NHE1_{HA}$ along with $CHP3_{myc}$ were subjected to $^{22}Na^+$-influx measurements after ATP-depletion for up to 60 min (***Fig. 3.5***), as well as a function of intracellular pH following ATP-depletion ***(Fig. 3.6***) as described previously in "Materials and Methods". AP-1 cells expressing $NHE1_{HA}$ containing mutations of the crucial CHP-binding residues (^{530}**FL**DH**LL**^{535}) mutated either to Ala (^{530}**AA**DH**AA**^{535}, FL-A), Gln (^{530}**QQ**DH**QQ**^{535}, FL-Q) or Arg (^{530}**RR**DH**RR**^{535}) were also assayed for effects of ATP-depletion. These mutations have been described previously and are devoid of the ability to bind the CHP proteins (Pang *et al*, 2001; Zaun *et al,* 2008). NHE1 activity of these mutants was considerably reduced to ~26%, ~2% and ~4% for FL-A, FL-Q and FL-R mutants respectively and although the FL-A mutant displayed an affinity for intracellular H^+ that was equivalent to the of wild-type NHE1, the FL-Q and FL-R mutations exhibited markedly reduced H^+-affinity (Pang *et al,* 2001, Zaun *et al*, 2008).

24-well plates containing a confluent monolayer of cells were subjected to ATP-depletion by incubating with ATP-depletion solution for indicated time points, and assayed for activity by ^{22}Na-influx at an intracellular pH_i-clamp at 6.2 using the K^+-nigericin method as described in the presence and absence of the NHE inhibitor, amiloride, as described in "Materials and Methods". A small fraction of dissolved cells was taken and a protein quantification assay was performed using the BioRad DC protein assay kit as recommended by the manufacturer. Total activity at each time point was then measured by liquid scintillation spectroscopy of dissolved cells and measured as total amiloride inhibitable uptake and plotted as a function of ATP-depletion time, normalized for equal total cellular protein (***Fig. 3.5A***). The results indicate that $NHE1_{HA}$, CHP-binding defective NHE_{HA} (FL-A), as well as cells co-expressing $NHE1_{HA}/CHP3_{myc}$ are inhibited by ATP-depletion quite rapidly at 15 min. Furthermore there is no discernable difference between the different cell lines suggesting that CHP3 does not influence the inhibition of NHE1 by ATP-

Fig. 3.5.

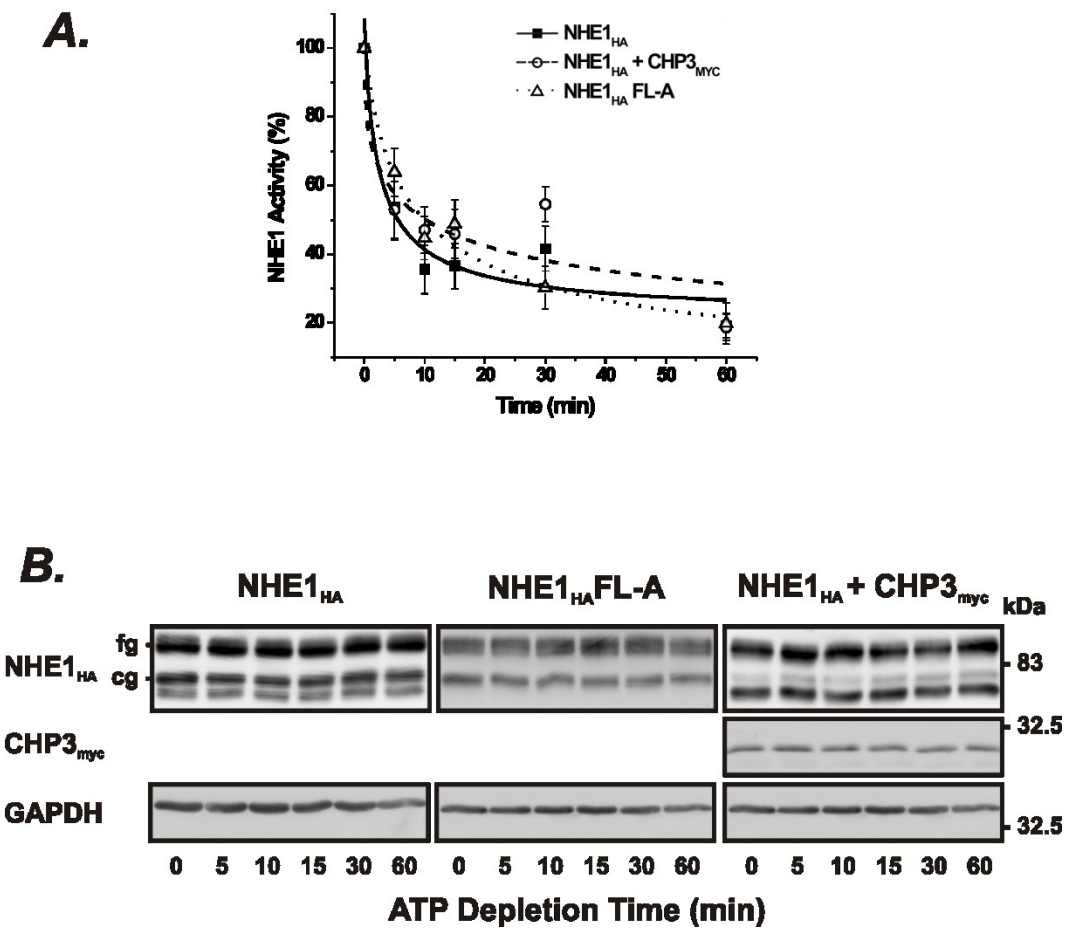

A.
NHE1 Activity (%)
Time (min)
NHE1HA
NHE1HA + CHP3MYC
NHE1HA FL-A
B.
NHE1HA
NHE1HAFL-A
NHE1HA + CHP3myc
kDa
NHE1HA
fg
cg
CHP3myc
GAPDH
83
32.5
32.5
0 5 10 15 30 60 0 5 10 15 30 60 0 5 10 15 30 60
ATP Depletion Time (min)

Figure 3.5. **Comparative analysis of CHP3 over-expression on NHE1 activity as well as activity of CHP-binding defective NHE1 mutant in response to ATP depletion.** AP-1 cells either expressing wild type or CHP-binding defective mutant of NHE1 (FL-A) alone or co-expressing wild type NHE1 and CHP3 were analyzed for alterations in activity in response to ATP-depletion over time. Cells cultured to confluence on 24-well plates were subjected to ATP depletion over a period of time ranging from 0 – 60 min. At given time points NHE1 activity was assessed by $^{22}Na^{+}$-influx using the K^{+}/nigericin technique with an intracellular pH clamped at 6.2, as described in "Materials and Methods". To allow for comparison of effects of mutating the CHP-binding site as well as over-expression of CHP3 on NHE1 activity during ATP-depletion, values were normalized to their respective maximal uptake at 0 min (**A**). To verify that inhibition of activity was not due to loss of NHE1 expression, cell were plated onto 6-well plates, cultured to confluency and ATP-depleted in concert with the 24-plates used for activity assessment. After ATP-depletion for given time points, cell lysates were obtained and equal quantities were subjected to SDS-PAGE and immunoblotting with mouse monoclonal antibodies recognizing the HA-epitope of NHE1, the myc-epitope on CHP3 as well as endogenous GAPDH to verify equal loading (**B**). Values represent the mean ± S.E. of a minimum of three separate experiments performed in triplicate.

depletion. Cells expressing the CHP-binding defective mutants FL-Q and FL-R, were unable to be assayed due to the very low activity of these mutants.

In order to verify the inhibition of NHE activity is not due to loss of NHE1 protein quantity, 6-well plates were also cultured to confluence in and subjected to ATP-depletion over the same time course. Cell lysates were obtained and equal cellular protein quantities were determined using the BioRad DC protein assay kit. Lysate were equally loaded and subjected to SDS-PAGE and immunoblotting with a mouse monoclonal antibody specific to the HA-epitope of $NHE1_{HA}$ to assess total amount of NHE1 for each time point (***Fig. 3.5B***). The immunoblots indicate there is no loss of the total quantity of NHE1 in the cells over time. This is in agreement with the previous assay of loss of cell surface $NHE1_{HA}$ subjected to ATP depletion (Fig. 2), that demonstrated that although cell-surface $NHE1_{HA}$ is lost with ATP-depletion, total NHE1 remains relatively unchanged.

These cell lines were also subjected to an intracellular pH-sensitivity assay by measuring the amiloride-inhibitable $^{22}Na^+$-influx over a pH_i range from 5.4 to 7.4 as described in "Materials and Methods". Cell expressing $NHE1_{HA}$, NHE_{HA}-CHP binding deficient mutants (FL-A, FL-Q, FL-R) and co-expressing $NHE_{HA}/CHP3_{myc}$ were cultured to confluency on 24-well plates, and assayed for activity throughout the pH range using the K^+/nigericin methods with and without metabolic depletion of ATP.. These cells were also subjected to ATP depletion by rapid washing and incubation for 30 min with isotonic Choline-Cl containing 2-deoxy-D-glucose and supplemented with antimycin A (1 µg/ml) or in a control solution of choline chloride. The intracellular pH of the cells was then adjusted by pH-clamping in K^+/nigericin pH-clamp solution for 4 min at indicated pH_i-range, and activity was plotted as a function of intracellular pH (pH_i) normalized to maximal activity at pH 5.4 for each construct (***Fig. 3.6***). In the case of cells expressing NHE1 alone or in conjunction with CHP3, both showed a decrease in pH_i sensitivity in response to ATP depletion which is in agreement with an earlier report (Wakabayashi *et al,* 1992). Due to the minimal activity of the CHP-binding deficient mutants of $NHE1_{HA}$ (FL-Q and FL-R), H^+-affinity

Fig. 3.6.

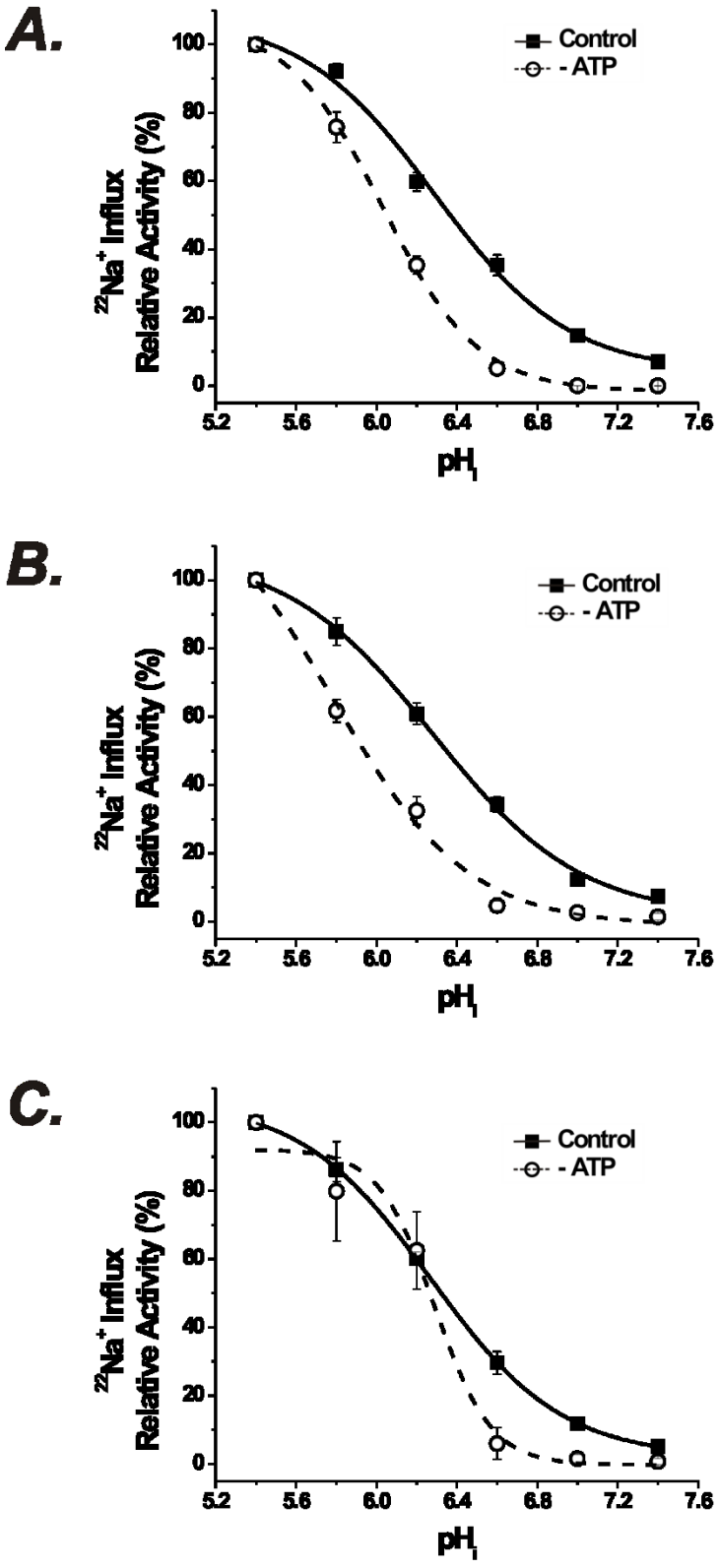

A.
B.
C.
Control
- ATP
$^{22}Na^+$ Influx
Relative Activity (%)
pH_i
100
80
60
40
20
0
5.2
5.6
6.0
6.4
6.8
7.2
7.6

Figure 3.6. **Effect of ATP-depletion on the intracellular proton affinity (pH_i) of AP-1 cells over-expression either wild type NHE1, CHP-binding deficient mutant of NHE1 (FL-A) and wild type NHE1 co-expressed with CHP3.**
AP-1 cells expressing either wild type $NHE1_{HA}$ (**A**), wild type NHE_{HA} co-expressed with $CHP3_{myc}$ (**B**), or CHP-binding deficient NHE1 (FL-A) (**C**) were cultured on 24-well plates and $^{22}Na^+$-influx was measured at various intracellular pH values ranging from 5.4 to 7.4 following incubation in either ATP-depletion solution or control solution as described in "Materials and Methods". pH_i was adjusted by incubating the cells in the appropriate K^+-nigericin pH-clamping solution for 4 minutes, followed by 10 min in the same solution supplemented with carrier-free $^{22}NaCl$ (1 μCi/ml) for 10 min to facilitates influx as described in "Materials and Methods". All values were normalized to their respective maximal rates of uptake at pH_i 7.4. in order to allow for comparison of the effects of ATP-depletion. Values represent the mean ± S.E. of a minimum of three separate experiments performed in triplicate.

measurements were unable to be obtained, although Pang *et al*, 2004, demonstrated that there was no further inhibition of intracellular H^+-sensitivity in cells subjected to ATP-depletion. However, the CHP-binding defective NHE1 mutant, FL-A, which was previously shown to be unable to interact with CHP3, but retained the pH_i-sensitivity of wild type NHE1 (Zaun *et al*, 2008) demonstrated a resistance to ATP-depletion induced inhibition of pH_i-sensitivity of the exchanger at the acidic range ($pH_i \leq 6.2$). However, inhibition at more neutral range ($pH_i \geq 6.6$) did occur, suggesting that the NHE1/CHP interaction may influence the H^+-affinity of the exchanger undergoing ATP depletion during severe acidification.

DISCUSSION

In this current study we examined the distribution of NHE1, the predominant isoform in the myocardium, under conditions of mimicked chemical ischemia through the metabolic depletion of intracellular ATP. We also looked at the possible roles that the calcineurin B homologous protein, CHP3, may play in regulating the exchanger under these conditions. CHP3 is also predominantly expressed in the heart in adult humans and previously demonstrated to upregulate NHE1 through enhancing the exchangers biosynthetic maturation and cell surface stability (Zaun *et al*, 2008). The role of NHE1 within the myocardium, as well as its role in cardiac pathology has been extensively studied for several decades (Fliegel, 2009; Karmazyn *et al*, 2008). It is well known that during episodes of ischemia/reperfusion, NHE1 activity exacerbates the tissue injury that occurs (Karmazyn *et al,* 1999). NHE1 has also been demonstrated to be in a hyperactivated state in the myocardium of both spontaneous hypertensive rats (Perez *et al,* 1995; Schussheim and Radda, 1995) as well as other cardiac hypertrophy models (Ennis *et al*, 2003; Chen *et al*, 2001; Karmazyn *et al*, 2003). There has been an overwhelming amount of evidence demonstrating that inhibition of the exchanger not only reduces the damage caused by ischemia/reperfusion, but also abrogates hypertrophy, in numerous animal models (Fliegel, 2009; Karmazyn *et al*, 2008; Avkiran *et al*, 2008).

It has been previously demonstrated that by mimicking the effects of global ischemia through ATP depletion, the activity of NHE1 is markedly reduced and this effect is mediated in part by the interaction with phosphotidylinositol-4,5-bisphosphate (PIP2) (Aharonovitz *et al*, 2000), independent of phosphorylation of the exchanger (Goss *et al*, 1994). Depletion of intracellular ATP seems to elicit regulation of NHE1 by reducing both the maximal activity of the exchanger (V_{max}) as well as inhibiting the affinity of the exchanger for intracellular H^+ (pH_i) (Wakabayashi *et al*, 1994b). Additional unpublished data from our lab demonstrated that during episodes of ATP depletion and/or mimicked ischemia/reperfusion, there is a redistribution of NHE1 from the intercalated disks

and transverse tubules to the lateral sarcolemmal membrane (Lamare *et al, unpublished data*). Furthermore, we previously demonstrated that CHP3 act to stabilize NHE1 at the plasma membrane (Zaun *et al*, 2008) and hypothesized that this may play a role in stabilizing the exchanger during ATP depletion, thereby maintaining the exchanger in an active state.

In partial agreement with the localization studies by Lamare, (*see chapter 3 preamble*), in AP-1 fibroblasts over-expressing $NHE1_{HA}$, biotinylated cell surface $NHE1_{HA}$ levels decline rapidly (within 15 min) both in the presence and absence of CHP3 overexpression (***Fig. 3.2***). Since over-expression of CHP3 does not seem to affect the stability of NHE at the cells surface, we tested the hypothesis that ATP-depletion may disrupt the interaction between NHE1 and CHP3. By utilizing co-immunoprecipitation assays as well as fluorescent confocal microscopy, our results indicated that the two proteins remain in complex (***Fig 3.3 and 3.4***) though results from confocal microscopy seem to indicate that there is a slight increase in the cell surface expression of NHE1 in the presence of CHP3 after 15 min ATP-depletion, as well as a diminishment of the co-localization signal (***Fig. 3.4***). However, it is possible that as the two proteins are redistributed away from the plasma membrane, their respective signals become more diffuse, therefore making it difficult to visualize their colocalization. Activity analysis of $NHE1_{HA}$ in the presence and absence of $CHP3_{myc}$, over-expressed in AP-1 cells results in a decrease of both absolute activity as well as a decrease in the intracellular H^+-affinity of the exchanger regardless of CHP3 involvement. These results that are in agreement with previous findings both in this study as well as others (Goss *et* al, 1994; Wakabayashi *et* al, 1994b; Aharonovitz *et al*, 2000). Collectively these results demonstrate that ATP depletion results in a loss of cell surface NHE1 independent of CHP3 interaction.

It has been hypothesized that the interactions of the CHP proteins with the C-terminal domain of NHE1 may act to stabilize the structural integrity of the NHE1 tail, which is also the region that binds PiP_2 to confer ATP-sensitivity of the exchanger (Ammar *et al*, 2006; Mishima *et al*, 2007). We therefore tested whether this inhibition of NHE1 activity may be influenced by the binding of

either endogenous CHP proteins, by assaying CHP3-binding defective mutants of NHE1 (FL-A, FL-Q, FL-R) (Zaun *et al*,2008). It is noteworthy that the mutations to the juxtamembrane region of NHE1 at the CHP binding site involving the Phe and Leu residues involved in the interaction between the two proteins induces t shift in the pH_i dependence of the exchanger that mimics the effects of ATP depletion when mutated either to Gln or Arg (FL-Q and FL-R) (Zaun *et al*, 2008; Pang *et al* 2001; Aharonovitz *et al*, 2000). Others demonstrated that in PS120 cells over-expressing these mutants of NHE1, there was no further change to the pH_i sensitivity upon ATP depletion (Pang *et al*, 2004). However, a CHP3-binding deficient mutation of the crucial Phe and Leu residues to Ala (FL-A) does not result in any discernable change in the pH_i-sensitivity of the exchanger under normal conditions (Zaun *et al*, 2008). When subjected to ATP depletion, the FL-A mutation did not show a shift in the pH sensitivity at the extreme acidic range (pH 5.4 – 6.2), but did show an inhibition to intracellular H^+ at the more neutral range (pH 6.6 – 7.4) (***Fig. 3.6C***) which more closely represents physiological pH_i in cell undergoing metabolic inhibition or ischemic hearts (~6.2 – 6.6) (Yan and Kleber, 1992; Garlick *et al,* 1979). This suggests that rather than maintaining the pH_i sensitivity of the exchanger under conditions of metabolic inhibition, the FL-Q and FL-R mutation may cause a much more dramatic change in the overall structure of the juxtamembrane region of NHE1 that results in a disruption of the interaction between PIP_2 at the two binding sites that flank the CHP-binding sites on NHE1.

From these results we conclude that CHP3 expression does not seem to play a major role in the regulation of NHE1 during ATP-depletion. However, we cannot exclude a cooperative role that the CHP/NHE1 interaction may play in stabilizing the structure of the juxtamembrane region of NHE1 in concert with the interaction of PIP_2 or the ERM family of acting binding proteins, as evidence by the ability of the CHP-binding deficient mutant (FL-A) to resist the H^+-affinity inhibitory effect of ATP-depletion at the acidic range. Indeed Ikeda et al, demonstrated that the juxtamembrane region of the NHE1 regulatory domain is required for maintaining the high affinity for intracellular H^+ of the exchanger

(Ikeda *et al,* 1997). Furthermore, the expression of CHP3 which has been demonstrated to be almost exclusively restricted to the heart in adult human, and seem to interact exclusively with NHE1 *in vitro* (*unpublished data*), would suggest that it may play an important physiological or pathophysiological role within cardiac tissue.

All three of the CHP proteins have been shown to regulate calcineurin activity. Calcineurin is a calcium/calmodulin-dependent serine/threonine protein phosphatase that is ubiquitously expressed and is known to be involved in a number of physiological processes that includes cardiac hypertrophy (Molkentin *et al* 1998; Sussman *et al*, 1998). However, where CHP1 and 3 have been shown to inhibit calcineurin activity (Lin *et al*, 1999; Gutierrez-Ford *et al,* 2003), CHP2 activates its activity (Li *et al*, 2008). This discrepancy between the CHP family members may well be related to the roles of each protein. Upregulation of calcineurin results in a dephosphorylation of the Nuclear Factor of Activated T cells (NFAT), which subsequently translocates from the cytoplasm to the nucleus where it binds and induces transcription of various target genes (Horsley and Pavlath, 2002; Hogan *et al*, 2003). Although the best known targets are involved in the activation and effector function of immune cells (Macian, 2005), NFAT is also known to be involved in the transcriptional activation of many genes involved in cell cycle progression and differentiation (Horsley and Pavlath, 2002; Hogan *et al*, 2003) and activation of NFAT members is important in oncogenic transformation of several human malignancies (Konig *et al,* 2010). CHP2 is highly expressed in malignant cell lines where its role in the serum-independent activation of NHE activity assists in increasing viability of tumour cells through the maintenance of a high intracellular pH (pH_i) (Pang *et al,* 2002) and overexpression of CHP2 has been shown to enhance tumour cell progression, invasion and metastasis in ovarian cancer (Jin *et al*, 2007), and accelerating tumourigenesis of HEK293 cells in nude mice (Qian *et al*, 2009). Therefore, CHP2-induced activation of the calcineurin/NFAT signalling pathway may well compound its role in tumour cell progression. However, in the myocardium, where CHP3 is the predominant isoform, calcineurin/NFAT activation is pro-

hypertrophic. Indeed, recent evidence suggests that calcineurin suppression may have a role in cardioprotection. Calcineurin A beta (CnAβ) gene-targeted mice have a greater tendency for the loss of viable myocardium due to cardiomyocyte apoptosis following acute ischemia/reperfusion injury (Beuno *et al*, 2004), suggesting that the inhibitory role of CHP3 on calcineurin may act as a cardioprotective mediator. However, CHP3 ability to inhibit calcineurin has only been measured *in vitro* (Gutierrez-Ford *et al,* 2003) and a physiological role for this interaction has yet to be established.

Levay and Slepak demonstrated that in HL-60 cells, CHP3 can either be up- or down-regulated by the ERK during differentiation to a granulocyte or macrophage lineage respectively, and it seems that the kinetics of ERK activation determines the direction CHP3 is regulated. Rapid PKC-mediated activation of the Raf/MEK/ERK pathway of the MAPK cascade results in a very quick disruption of transcription of the CHP3 gene whereas a delayed activation resulted in CHP3 upregulation in HL-60 cells (Levay and Slepak, 2010). This same pathway results in phosphorylation and activity increase of the cardiac NHE1 isoform, particularly at time of ischemia/reperfusion (Haworth *et al*, 2006; Armstrong, 2004). Moor et al, demonstrated that MAPK-dependent pathways that include ERK1/2 and $p90^{RSK}$ are important in regulating NHE1 through a dramatic increase in their activity toward the exchanger, yet may have an important cardioprotective inhibitory effect on Na^+/H^+ exchanger activity during reperfusion following ischemia (Moor *et al*, 2001). However, whether the up- or down-regulation of CHP3, which may either increase or decrease the activity of NHE1 through accumulation and stability of the exchanger at the plasma membrane has not yet been investigated. Although it would seem unlikely that transcriptional regulation of CHP3 would allow for any rapid response during early ischemic episodes, it does not discount CHP3 regulation of the exchanger over longer ischemic periods. To date there have not been any studies on endogenous CHP3 within the myocardium, or whether the regulation of the protein is affected by the regulation of the ERK pathway, as it does in hematopoietic progenitor cells.

In summary, the current study demonstrates that apart from inhibiting the pH_i-sensitivity of NHE1, depletion of intracellular ATP also acts in decreasing the amount exchanger at the cell surface. This is in agreement with previous studies in our lab that show a redistribution of NHE1 in cardiac tissue away from the intercalated disk and transverse tubules to the lateral sarcolemmal membrane (Lamare *et al, unpublished*), although the molecular mechanisms responsible for this redistribution have not yet been elucidated. Furthermore, although it has previously been demonstrated that CHP3 acts to stabilize the exchanger at the cell surface (Zaun *et al,* 2008), loss of cell surface NHE1 is CHP3-independent. However, the absence of CHP proteins bound to the exchanger do influence the sensitivity of the exchanger to intracellular H^+ at acidic intracellular pH (5.4 – 6.2). However a role for CHP3 in the myocardium, particularly under pathophysiological conditions cannot be ruled out, further studies of the protein within the heart are most definitely warranted.

General Discussion and Conclusions:

The results of this thesis extend our knowledge of the regulation of the Na^+/H^+ exchanger isoform 1 (NHE1) by calcineurin B homologous protein 3 (CHP3), and assists in clarifying how the exchanger is regulated within the myocardium, particularly during times of metabolic stress through ATP-depletion. In chapter 1 of this thesis, we demonstrated that CHP3 binds NHE1 at identical residues to that of the other CHP isoforms (CHP1 and 2), and that over-expression of CHP3 results in an increase of cell surface NHE1 through the biosynthetic maturation and cell surface stability of the exchanger at the plasma membrane. Whereas the regulation of NHE1 is often attributed to a change in the affinity of the exchanger to intracellular H^+ (pH_i), the interaction of the exchanger with the CHP3 establishes a separate mechanism by which the exchanger is regulated. However, the physiological relevance of this interaction and the subsequent regulation it plays within the body, particularly the heart, must be further clarified.

CHP3 is a member of a family of an N-myristoylated, EF-hand Ca^{2+}-binding proteins that share similarity to the regulatory B subunit of the serine/threonine phosphatase, calcineurin. In the second chapter, we show that N-myristoylation and Ca^{2+}-binding of CHP3 are not required for binding NHE1 or late stage maturation of the exchanger to the plasma membrane, but are crucial for cell surface stability of the complex. Furthermore, the results suggest that CHP3 may act as a Ca^{2+}-myristoyl switch protein, by which the N-myristoylation and EF-hand domain act cooperatively in regulating NHE1. Increasing levels of intracellular Ca^{2+} have been previously demonstrated to increase Na^+/H^+ exchange activity primarily through the interaction of NHE1 with the Ca^{2+}-binding protein, calmodulin (Bertrand *et al*, 1994; Wakabayashi *et al*, 1994), and the NHE1/CHP3 interaction represents another mechanism by which intracellular Ca^{2+} levels regulate Na^+/H^+ exchanger.

Finally, in chapter 3, we further characterize the role of NHE1 during metabolic stress through the depletion of ATP in CHO/AP-1 cells overexpressing NHE1. Apart from the inhibition of NHE1 during periods of ATP depletion due to

reduction of plasmalemmal phosphatidylinositol-4,5-bisphosphate (PIP2) through dephosphorylation of the phospholipid (Aharonovitz *et al*, 2000), we demonstrate that ATP depletion also reduces that amount of NHE1 at the plasma membrane without affecting total cellular NHE1 content. Furthermore, the reduction is independent of the binding of NHE1 with CHP3 although our results suggest that this interaction may regulate the H^+ affinity of the exchanger at lower pH_i values (5.4 – 6.2) during episodes of ATP depletion.

A large body of evidence is consistent with the hypothesis that myocardial ischemic/reperfusion injury results in part from increases in intracellular Na^+ (Na_i) by NHE1 that in turn promotes the Na^+/Ca^{2+} exchanger-mediated increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and Ca^{2+}-dependent cell damage. During myocardial ischemia, mitochondrial ATP synthesis ceases culminating in the depletion of ATP and a decrease in intracellular pH which activates the NHE1 resulting in the extrusion of H^+ and the influx of Na^+. Upon reperfusion, extracellular H^+ rapidly decreases, increasing the intracellular to extracellular H^+ gradient. This large H^+ gradient hyperactivates NHE1 which increases intracellular Na^+ and due to inactivation of the Na^+/H^+ ATP-pump, leads to accumulation of intracellular Ca^{2+} through the Na^+/Ca^{2+} exchanger. The accumulation of Ca^{2+} contributes to cellular damage resulting in arrhythmias and myocardial contracture (***Fig. 1***). Furthermore, Ca^{2+} is a major pro-hypertrophic signal within the myocardium that activates several intracellular pathways including the calcineurin/NFAT pathway. Indeed, a vast amount of research utilizing animal and cellular models has demonstrated the cardio-protective effects of NHE1 inhibition in reducing ischemia/reperfusion injury as well as cardiac hypertrophy and heart failure in the myocardium (Karmazyn, 2001; Engelhardt *et al* 2002).

NHE1 has been demonstrated to be in a hyperactivated state in the myocardium of both spontaneous hypertensive rats (Perez *et al,* 1995; Schussheim and Radda, 1995) as well as other cardiac hypertrophy models (Ennis *et al*, 2003; Chen *et al*, 2001; Karmazyn *et al*, 2003). A recent study by Ennis *et al*, suggests that cardiac hypertrophy regression induced by NHE1 inhibition involves the inactivation of the calcineurin/NFAT (CaN/NFAT) signalling pathway (Ennis *et*

Fig. 1.

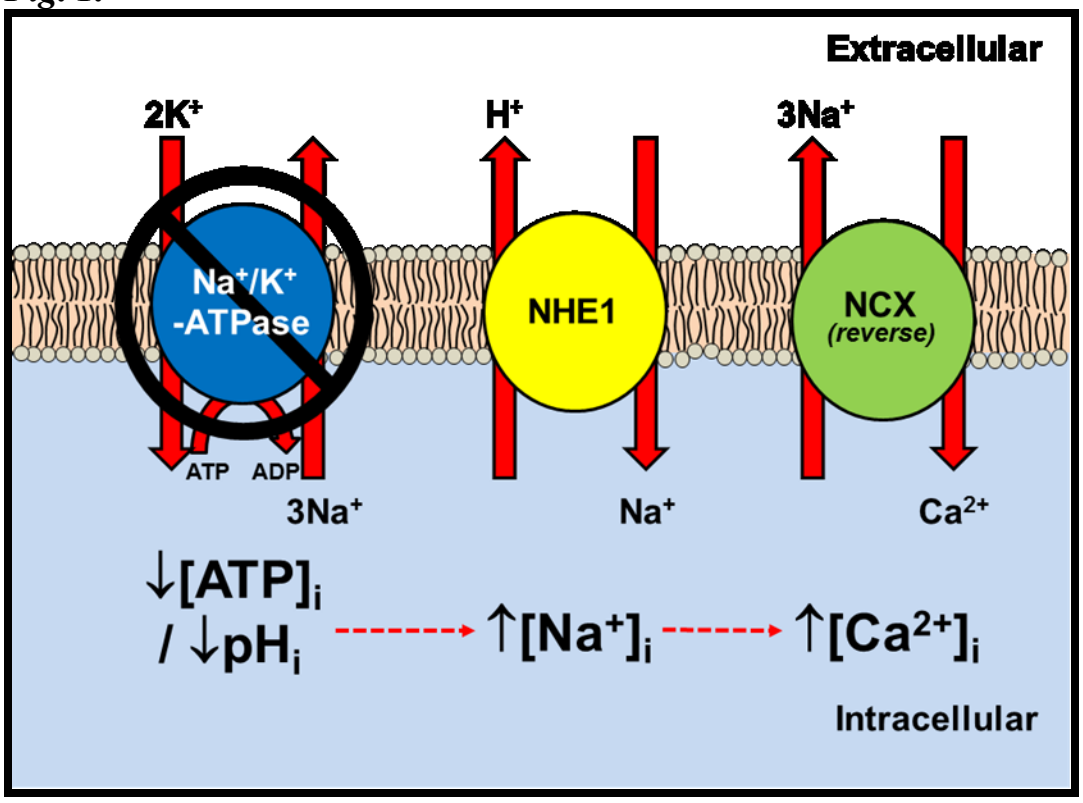


Figure 1. Schematic representation of Hypothesized mechanisms of myocardial ischemia/reperfusion injury: Loss in intracellular ATP results in an inactivation of the **Na^+/K^+-ATPase** and a drop in pH_i. Upon reperfusion, activation of the Na^+/H^+ exchanger (**NHE1**) results in a rapid influx of Na^+. This increase in intracellular Na^+ induces the sodium/calcium exchanger (**NCX**) to operate in reverse, thereby increasing the intracellular levels of Ca^{2+}, and subsequent cellular damage.

al, 2007). Since there is much evidence that calcineurin plays a crucial role in many pathological models of cardiac hypertrophy (Molkentin *et al*, 1998; Taigen *et al*, 2000; Bueno *et al*, 2002; Ritter *et al,* 2002), it is attractive to speculate a role for CHP3 in the CaN/NFAT signalling pathway involved in cardiac hypertrophy. However, whether the CHP3 inhibitory effect on the calcineurin phosphatase activity occurs under physiological conditions remains unclear.

It has also been shown that CHP3 upregulation results in the expression of members of the Ets family of transcription factors (Levay and Slepak, 2007), particularly Ets-1 and Ets-2 which are crucial for proper coronary vascular formation and myocardial development in chick embryos (Lie-Venima *et al,* 2003). Ets-1 has also been shown to be possibly involved in the transcriptional regulation of inducible nitric oxide synthase (iNOS) in chick embryonic ventricle myocytes (Takahashi *et al*, 2001). Although absent in healthy heart, the expression of iNOS is induced by pro-inflammatory mediators (Umar and van der Laarse, 2010). Though some studies have pointed to the detrimental effects of iNOS leading to initiation of cardiac remodelling that is characterized by ventricle hypertrophy, dilatation and sudden cardiac death (Mungrue *et al*, 2002), others have argued for a cardioprotective effect of iNOS limiting post-ischemic myocardial damage (Jones and Bolli, 2006).

The mechanism by which CHP3 promotes the expression of these transcription factors is currently unknown. Since CHP3 does not possess a DNA-binding domain, it may regulate the expression of transcription factors through an indirect mechanism (Levay and Slepak, 2007). Beyond its interaction with NHE1 and regulation of calcineurin, little is known about other potential interacting partners. A search of novel interacting partners, either by yeast-two hybrid analysis or immunoprecipitation in combination with mass spectrometry analysis may further our knowledge of the possible roles CHP3 plays within the myocardium.

The juxtamembrane region of NHE1 seems to be crucial for the physiological exchange activity of NHE1 (Ikeda *et al,* 1997; Wakabayashi *et al*, 1997b) since it interacts with several accessory interacting partners, working

either independently or in conjunction. Apart from interacting with the CHP proteins, this region also interacts with PIP_2 at two acidic clusters at residues 513-520 and 556-564 or rat NHE1 (Aharonovitz *et al*, 2000), with the second PIP_2 interaction site also binding ezrin independently of exchange activity (Denker *et al*, 2000; Baumgartner *et al*, 2004). Furthermore, Hisamitsu et al, reported that this region (residues 560-580) may be involved in dimeric interaction (Hisamitsu *et al*, 2004) (***Fig. 2)***. The importance of concerted interactions between NHE-binding proteins within the juxtamembrane region of the exchanger was recently demonstrated by the increase in the trafficking and transport activity of NHE3 via a cascade involving both CHP1 and Ezrin. Di Sole et al, showed that CHP1 expression is crucial for the plasmalemmal abundance and transport activity of NHE3 which is dependent on ezrin phosphorylation. Furthermore, ezrin was shown to be downstream of CHP1 expression since knockdown of CHP1 by siRNA abrogates NHE1 activation and surface expression in cells expressing wild-type ezrin, but not in cells overexpressing a pseudophosphorylated from of ezrin (Di Sole *et al*, 2009).

This region has also been demonstrated to be important in maintaining the structure and stabilization of the exchanger. Ammar et al, reported that a peptide corresponding to the juxtamembrane region of NHE1 that includes the CHP-binding site, but CHP-free, had no secondary structure in aqueous solution, and when expressed in *E. Coli* or mammalian cells as a single polypeptide, underwent rapid degradation (Ammar *et al*, 2006; *Unpublished data*). Furthermore, previous studies utilizing mutation of NHE1 in which the C-terminal tail was subsequently truncated indicated that the juxtamembrane region is crucial for the maintenance of the high intracellular pH sensitivity and function of the exchanger (Ikeda *et al*, 1997).

These data offer some interesting avenues in the pursuit of our understanding of the regulation of NHE1 within the myocardium, either by CHP3 or other interacting proteins. Although both chapter 3 as well as the unpublished results demonstrate the loss of NHE1 at cell surface of CHO/AP-1 fibroblasts and at the intercalated disks and transverse tubules of isolated rat cardiomyocytes

Fig. 2.

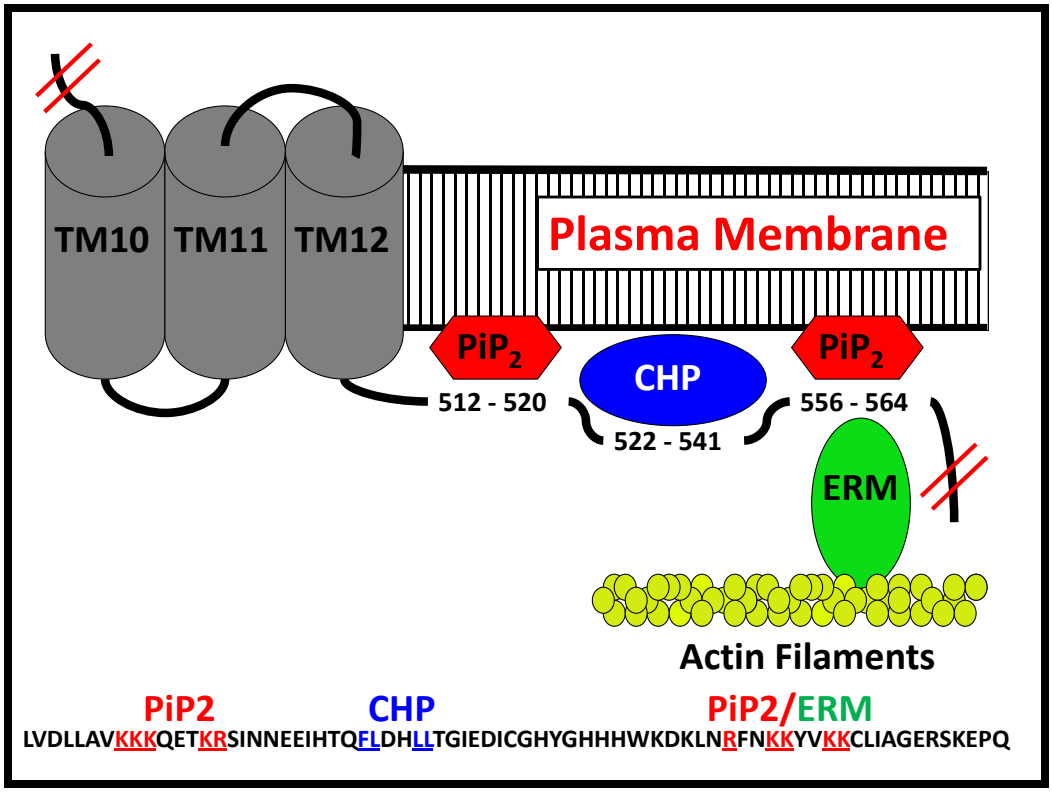


Figure 2. **Schematic representation of the juxtamembrane region of the cytosolic C-terminal tail of NHE1 and its interacting partners.** The importance of the juxtamembrane region of the C-terminal tail of NHE1 may be implied by both the numerous crucial NHE1 binding partners the interact with the exchanger within this domain. These include the inner membrane phosphoinositides, phosphotidylinositol-4,5-bisphosphate (**PIP_2**), the calcineurin B homologous proteins (**CHP**) and the ezrin/radixin/moesin family of actin bindings proteins (**ERM**). Residues crucial for the interaction of NHE1 with PiP_2 are indicated in red (Aharnonovitz *et al*, 2000) and for the interaction of the CHP proteins in blue (Pang *et al*, 2001, 2001; Zaun *et al*, 2008).

during ATP depletion, the molecular mechanisms that are responsible have not yet been determined. However, the corresponding inhibition of NHE1 by ATP depletion suggests a role for the interaction between NHE1 and PIP2 in maintaining the exchanger at the cell surface and further studies to this regard are definitely warranted.

In summary, the calcineurin B homologous protein 3 (CHP3) represents an NHE1 regulatory interacting protein whose expression is predominantly restricted to the heart in adult human tissue. Unlike CHP1, which has been shown to also be involved in vesicular traffic, other possible functions of CHP3 within the myocardium have yet to be elucidated. However, its ability to increase NHE1 activity through maturation and stabilization of the exchanger, ability to regulate calcineurin phosphatase activity, and its restricted tissue distribution make it an attractive candidate for study within the myocardium, particularly during pathophysiological states such as cardiac hypertrophy.

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