Regulation of Src by α1 Na/K-ATPase

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A Dissertation

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Regulation of Src by α1 Na/K-ATPase

By

Qiqi Ye

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Sciences

Zi-jian Xie, Ph.D., Committee Chair

Patricia Komuniecki, Ph.D., Dean

College of Graduate Studies

The University of Toledo

August 2012
An Abstract of

Regulation of Src by α1 Na/K-ATPase

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Qiqi Ye

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Science

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The Na/K-ATPase is a membrane protein which undergoes E1/E2 conformational transition during an ion pumping cycle to translocate Na\(^+\) and K\(^+\) across the plasma membrane. It is found that α1 Na/K-ATPase associates with Src to form a receptor complex. Binding of ouabain to α1 Na/K-ATPase stimulates Src and Src related signaling cascades.

To understand the mechanism of Src regulation by α1 Na/K-ATPase, we hypothesized that Na/K-ATPase regulated Src through conformational transition. Specifically, E1 Na/K-ATPase inhibited Src while E2 Na/K-ATPase activated it. To test this hypothesis, purified Na/K-ATPase was stabilized in E1 and E2 state with N-ethylmaleimide (NEM)/oligomycin and fluoride compounds/ouabain, respectively. E1 enzyme was found to keep Src inactive while E2 enzyme failed to inhibit Src. Moreover,
when α1 Na/K-ATPase was trypsinated or chymotryptsinized to disrupt the coordinated domain movements, the capability of α1 Na/K-ATPase to control Src activity in response to ouabain was abolished.

To further test this potential regulation of Src by α1 Na/K-ATPase, E1/E2 α1 mutant cell lines were generated by rescuing the α1-knockdown PY-17 cells with α1 mutants (I279A and F286A) that are known to be defective in conformational transition. I279A (E1 mutant) was more effective in inhibiting basal Src activity than either wild-type or F286A (E2 mutant). While much higher ouabain concentration was required to stimulate Src in I279A-rescued cells, ouabain failed to produce detectable changes in Src activity in F286A-rescued cells. Furthermore, expression of both mutants inhibited integrin-induced activation of Src/FAK pathways, slowed cell spreading processes as well as cell growth.

Other Na/K-ATPase ligands such as Na⁺, K⁺ and cholesterol are known to alter the distribution of E1/E2. Consistently, test tube and cell experiments showed that redistribution of E1/E2 Na/K-ATPase by these ligands were capable of regulating Src and its downstream effectors.

Taken together, these findings suggested that this α1 Na/K-ATPase conformational transition-dependent regulation of Src renders an important mechanism to dynamically regulate cellular Src activity. Moreover, this receptor complex could sense multiple cellular ligands such as ouabain, intracellular Na⁺ and cholesterol, as well as extracellular K⁺, to maintain cellular homeostasis.
I dedicate this work to my dear mother Jing Wang for her love, support and encouragement.
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Introduction

Na/K-ATPase is a ubiquitously expressed integral membrane protein, transporting three \( \text{Na}^+ \) out of and two \( \text{K}^+ \) into the cells against their electrochemical gradients at the expense of hydrolyzing one molecule of ATP. Besides this classic pumping function, in recent decades extensive studies from this lab and others have explored non-pumping functions of \( \alpha_1 \) Na/K-ATPase (Arnon et al. 2000; Aizman et al. 2001; Liu et al. 2007b; Nguyen et al. 2007; Liu and Xie 2010).

One important constituent of this non-canonic signaling function of \( \alpha_1 \) Na/K-ATPase is Src kinase. Researchers from this lab and many other labs have reported the activation of Src by ouabain in different types of cells (Haas et al. 2000; Liu et al. 2004; Kometiani et al. 2005; Liu et al. 2007b; Nguyen et al. 2007; Karpova et al. 2010; Larre et al. 2010; Giannatselis et al. 2011). Moreover, GST pull-down assay indentify two interacting pairs between \( \alpha_1 \) Na/K-ATPase and Src: the second cytoplasmic domain (CD2) and nucleotide binding (N) domain of \( \alpha_1 \) subunit seem to associate with Src SH2 domain and kinase domain, respectively (Tian et al. 2006). Ouabain releases Src kinase domain from the receptor complex and activates the Na/K-ATPase-associated Src (Tian et al. 2006). The activation of Src results in transactivation of multiple downstream effectors, and regulates cellular activities such as clathrin-mediated endocytosis of \( \alpha_1 \) Na/K-ATPase (Liu et al. 2005). Studies from this lab have further mapped a 20 amino acid peptide (NaKtide) at the N domain of \( \alpha_1 \) subunit which has been identified to interact and inhibit Src (Li et al. 2009). Moreover, in vitro mutagenesis assay suggests an essential role of the \( \alpha \)-helix in NaKtide in mediating the inhibition of Src. Taken together, these data indicate that \( \alpha_1 \)
Na/K-ATPase may interact with Src. Moreover, this interaction may not only keep Src in an inactive state, it may also constitute a receptor complex for CTS (cardiotonic steroids) to activate protein kinases in live cells.

However, the underlying mechanism of α1 Na/K-ATPase-mediated Src regulation is still not clear. So herein, the first aim of this dissertation is to explore how α1 Na/K-ATPase regulates its associated Src activity. It is known that Na/K-ATPase undergoes E1/E2 conformational transition in a pumping cycle, and ouabain stabilizes Na/K-ATPase in E2P conformation. Therefore we hypothesized that the regulation of Src is related to the conformational transition of α1 Na/K-ATPase. To test this, in cell-free system purified Na/K-ATPase was stabilized in E1 or E2 state with different compounds, as well as trypsinized or chymotrypsinized to disrupt the coordinated domain movements. And the data indicated that E1 Na/K-ATPase inhibited Src, whereas the conversion from E1 to E2 might lead to Src activation. To further test this potential regulation of Src by α1 Na/K-ATPase, E1/E2 α1 mutant cell lines were generated, and the data presented here suggested that disturbing the balance of E1/E2 would affect the signaling function of α1 Na/K-ATPase, and consequently the cellular functions such as attachment/spreading, and proliferation.

Other Na/K-ATPase ligands such as Na\(^+\), K\(^+\) and cholesterol are known to alter the equilibrium of E1/E2. To study the physiological/pathological relevance of this E1/E2 Na/K-ATPase-dependent Src regulatory mechanism, we also tested whether these ligands were capable of regulating Src and its downstream effectors.


**Literature**

**Na/K-ATPase**

Na/K-ATPase, or Na pump, is a polytopical membrane protein expressed in most of the animals cells. It belongs to Type II P type ATPase, translocating three Na$^+$ out and two K$^+$ in the cells at the expense of hydrolyzing one ATP. The Na pump is well recognized to be responsible for the significantly different ionic concentration across the cell membrane. The concentration gradient it generates not only maintains the cell volume, it also provides driving force for the transport of ions, nutrients as well as neurotransmitters; moreover, it is fundamental to maintain the electrical excitability in nervous system.

However, it took around a century for the scientists to debate the existence of an active pump before Skou finally identified the real protein. The phenomenon of differed ionic distribution across the membrane was first recognized by physiological chemist C. Schimidt in mid 18th century (Clarke and Fan 2011). Later at the end of 19th century, Overton proposed that there was an active exchange of Na$^+$ and K$^+$ across the plasma membrane on the expenditure of energy when he studied the muscle contraction. However, his notion was ignored at that time. The concept of physical electro diffusion and the inherent impermeance of Na$^+$ was dominated in the scientific society until the active transport of Na$^+$ in both isolated muscle fibers and erythrocytes were demonstrated using radioisotopes and R. Dean proposed the existence of a membrane-bound pump (Schultz 1998; Clarke and Fan 2011). Moreover, the process of active transport of Na$^+$ and K$^+$ was linked to the hydrolysis of ATP, suggesting that the pump which Dean
proposed was an ATPase. In 1957 Skou indentified the real enzyme in the crab nerve whose activity was stimulated by the addition of Na\(^+\) and K\(^+\) (Skou 1957), and later on he confirmed that this newly identified ATPase could be specifically inhibited by heart glycosides (Skou 1960).

**Subunits of Na/K-ATPase**

Unlike most of the other members of the P type ATPase, Na/K-ATPase is an oligomeric protein which requires assembly of both \(\alpha\) and \(\beta\) subunit to be functional. The \(\alpha\) subunit is a 110 kDa membrane protein which spans the membrane 10 times, with both N-terminus and C-terminus in the cytosol. It contains the binding sites of Na\(^+\), K\(^+\) as well as ATP, and therefore it functions as a catalytic subunit. The Na/K-ATPase \(\alpha\) subunit shares highly structural similarity with other type II P type ATPases such as Ca\(^{2+}\)-ATPase and H\(^+\)/K\(^+\)-ATPase (Sweedner and Donnet 2001). It has 10 transmembrane helices, and three well defined cytosolic domains. The two K\(^+\) binding sites are located between \(\alpha\)M4, \(\alpha\)M5 and \(\alpha\)M6 transmembrane segments. Two Na\(^+\) binding sites are homologues to the K\(^+\) binding cavity based on the similarity between SERCA (sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase) and Na/K-ATPase (Morth et al. 2007). The third Na\(^+\) binding site is suggested to be located in the cavity among \(\alpha\)M5/\(\alpha\)M7/\(\alpha\)M8 or \(\alpha\)M6/\(\alpha\)M8/\(\alpha\)M9 (Morth et al. 2011). The three cytosolic domains employ distinct functions: the actuator (A) domain, consisting of the flexible N-terminus and the second cytoplasmic loop, is in control of the conformational transition of the Na/K-ATPase; the nucleotide-binding (N) domain, localized at the distal part of the cytoplasmic loop connecting the fourth and fifth transmembrane segments, contains the ATP binding pocket centered around the key
binding residue Arg 551; the proximal part of the large αM4/αM5 loop constitutes the phosphorylation (P) domain, and contains an Asp which accepts the γ-phosphate of the ATP from the N domain, and forms a covalent Asp-P bond.

There are four isoforms of α subunit in mammals with distinct tissue distribution (Sweadner 1989; Lingrel et al. 1990; Woo et al. 1999). α1 is prevalently expressed in many tissues; α2 is found in skeletal muscle, brain and heart tissue; α3 is mainly present in neuronal tissues; while the expression of α4 is restricted to spermatozoa. The sequence identity is around 85% among different isoforms, but the overall tertiary structural appears to be identical (Jorgensen and Andersen 1988). In addition, the rodent isoforms confer different affinities for digitalis and Na⁺ (Jewell and Lingrel 1991; Horisberger and Kharoubi-Hess 2002; Blanco 2005b), which suggests the regulatory role of isoforms adapting to different physiological demands.

The β subunit is a type II membrane protein composed of a single transmembrane segment, a short cytoplasmic N-terminus and a bulky extracellular C-terminus. The ectodomain contains two to eight glycosylation sites and three consecutive cystein pairs, which are known to be critical for the proper folding of the β subunit. The β subunit mainly associates with the α subunit at the αM7/M8 loop (Sarvazyan et al. 1995). The molecular mass of β subunit is close to 37 kDa, and its overall molecular mass depends on the level of glycosylation. Although the β subunit does not have an intrinsic catalytic function, it is an indispensable chaperon of the α subunit as suggested by the early work of Noguchi who showed that α subunit alone did not have ATPase activity (Noguchi et al. 1987). Further studies indicate that the β subunit helps the α subunit in two ways:
structurally, it assists the de novo synthesized α subunit in adopting the correct topology in the endoplasmic reticulum membrane, and then directs the αβ dimer to the plasma membrane (McDonough et al. 1990; Geering 1991; Geering 2001); functionally, it modifies the kinetic properties of the α subunit by affecting K⁺/Na⁺ affinity as well as the conformational transition (Eakle et al. 1992; Eakle et al. 1994; Eakle et al. 1995; Hasler et al. 1998). Like the α subunit, the β subunit also has isoforms with distinct tissue specific distribution. β1 is expressed ubiquitously in most cells, while β2 and β3 are selectively found in neurons, or muscle/lung/brain, respectively (Blanco 2005a).

In some tissues, a single-span transmembrane protein from the FXYD family is found to interact with Na/K-ATPase α and β subunit. The FXYD protein family is defined according to the conserved extracellular FXYD (Phe, Tyr and Asp) motif, two invariant glycine residues and one serine residue (Sweadner and Rael 2000). So far, seven members (FXYD 1-7) have been identified according to different tissue and cell distribution pattern, as well as their specific association with different α/β isoenzymes. For example, FXYD 2, or γ subunit, is exclusively expressed in the kidney; specifically it is mainly found in the segments such as TAL (thick ascending limb of the loop of Henle) and the distal convoluted tubule (Pu et al. 2001; Wetzel and Sweadner 2001; Pihakaski-Maunsbach et al. 2003). FXYD 4, or CHIF (corticosteroid hormone-induced factor), is expressed exclusively in kidney medullary CCD (collecting duct) and distal colon (Shi et al. 2001). FXYD1, or PLM (phospholemman), is widely distributed in heart, brain and kidney (Feschenko et al. 2003; Wetzel and Sweadner 2003). FXYD proteins are short polypeptides with around 100 amino acids (Geering 2006). Most of FXYD family members (PLM, γ subunit, CHIF and FXYD7) are type I single-span transmembrane
proteins with an extracellular N-terminus and a cytoplasmic C-terminus (Garty and Karlish 2006). Multiple interacting sites have been identified between FXYD protein and α/β complex. For instance, FXYD motif is necessary for the interaction between Na/K-ATPase and FXYD2 or FXYD4 (Beguin et al. 2001). Chimeric and cross-linking studies suggest that the residues in the transmembrane segment and cytoplasmic tail also play a role in determining the structural stability with α/β complex (Lindzen et al. 2003; Fuzesi et al. 2005). On the other hand, the role of αM9 in the association with FXYD proteins has been confirmed by the mutagenesis studies as well as the crystal structure (Li et al. 2004; Morth et al. 2007). The interaction between FXYD protein and Na/K-ATPase could slightly modulate the kinetic properties of Na/K-ATPase. For example, the γ subunit is found to reduce the apparent affinity of intracellular Na⁺ and extracellular K⁺ in NRK-52E rat renal cells (Arystarkhova et al. 1999), and it also shifts the E1/E2 equilibrium toward E1 in Hela cells (Pu et al. 2001). CHIF, on the other hand, decreases the K₀.₅ value for cytoplasmic Na⁺ in both oocytes and Hela cells (Beguin et al. 2001; Garty et al. 2002). The differed tissue distribution and kinetic modulatory property imply different physiological roles among these isoforms. For instance, the reduced intracellular Na⁺ affinity by the γ subunit in TAL facilitates the secretion of Na⁺, whereas the increased cytoplasmic Na⁺ affinity by CHIF in CCD promotes the reabsorption of Na⁺.

Kinetics and Structure of the Na/K-ATPase

Na pump could operate under four distinct modes depending on the availability of substrates, and catalyze different reactions (Glynn and Karlish 1975; Robinson and
1) Na⁺/K⁺ exchange: Under physiological condition, Na pump actively exchanges internal Na⁺ with external K⁺ on the expenditure of intracellular ATP at a stoichiometry of 3 Na⁺/2 K⁺/1 ATP in red blood cells, dog brain and kidney (Garrahan and Glynn 1967; Sweadner and Goldin 1975; Goldin 1977). This net transport of one positive charge suggests that Na pump is electrogenic, contributing to the resting membrane potential in excitable cells (Thomas 1972). Under this working mode, the Na pump catalyzes Na⁺/K⁺-ATPase activity, which is stimulated by extracellular K⁺ and intracellular Na⁺, Mg²⁺ and ATP (Sen and Post 1964).

2) uncoupled Na⁺ efflux: In the absence of both extracellular Na⁺ and K⁺, the Na pump extrudes intracellular Na⁺ at a ratio of 3 Na⁺/1 ATP (Glynn and Karlish 1976). Under this operating mode, the Na pump catalyzes Na⁺-ATPase. Na⁺-ATPase only requires high affinity ATP binding (around 1μM), and is repressed by extracellular Na⁺.

3) Na⁺/Na⁺ exchange: In the absence of extracellular K⁺, but with intracellular and extracellular Na⁺ as well as intracellular ATP and ADP, the Na pump exchanges Na⁺ across the cell membrane at 1:1 ratio, resulting in no net movement of Na⁺ and no net hydrolysis of ATP (Cavieres and Glynn 1976). Under this mode, the Na pump catalyzes Na⁺ dependent ATP/ADP exchange, which is inhibited by extracellular K⁺.

4) K⁺/K⁺ exchange: In the absence of intracellular Na⁺, but with intracellular and extracellular K⁺ as well as intracellular ATP and Pi, the Na pump exchanges K⁺ across the cell membrane at 1:1 ratio, with no net movement of K⁺ (Post et al. 1975). Under this mode, the Na pump catalyzes K⁺-dependent phosphorylation by Pi (Post et al. 1975), which is inhibited by intracellular Na⁺. Besides the above mentioned reactions, the Na pump also contains intrinsic phosphatase activity, and can hydrolyze
exogenous phosphoric acid anhydrides (e.g., p-nitrophenyl phosphate) in the presence of K\(^+\) and Mg\(^{2+}\) (Robinson 1969; Robinson 1970).

Apart from the kinetic studies, work on the structure of Na/K-ATPase also sheds light on the understanding of this protein. Albers and Post first proposed that there were four major conformational states of the Na/K-ATPase \(\alpha\) subunit: two unphosphorylated state, E1 and E2, and their corresponding phosphorylated form, E1P and E2P (Siegel and Albers 1967; Post et al. 1969). These conformations are defined according to their different reactivities to substrates. For example, E1 has higher affinity for ATP (\(\mu\)M range) and Na\(^+\) (Neufeld and Levy 1969; Glynn and Karlish 1976), whereas E2 has lower affinity for ATP (mM range) and higher affinity for Pi and K\(^+\) (Glynn and Karlish 1976; Nagamune et al. 1986). While E1P is sensitive to ADP, E2P is dephosphorylated by K\(^+\). Proteolytic studies also suggest the existence of two distinct conformations of the Na/K-ATPase according to the different cleavage patterns. For example, in the presence of either 150mM KCl or ATP/MgCl\(_2\)/NaCl, conditions which promote E2 or E2P state, trypsin cleaves the \(\alpha1\) Na/K-ATPase and yields two polypeptides with molecular weight of 58 kDa and 48 kDa, respectively; while in the presence of 150 mM NaCl, a condition under which E1 state is predominated, a 78 kDa segment is produced (Jorgensen 1975; Jorgensen 1977; Jorgensen and Klodos 1978). Studies with endogenous protein fluorescence and exogenous fluorescent probes also support the distinct conformations of Na/K-ATPase. For example, an increase of intrinsic tryptophan fluorescence is detected by addition of KCl to the enzyme preparation, which corresponds to the conversion from E1 to E2 (Karlish and Yates 1978). Similar fluorescence changes are obtained by labeling the enzyme with exogenous fluorescein isothiocyanate.
Incorporating the results from the kinetic and structural studies, a ping-pong, or sequential model is proposed for Na/K-ATPase by Albers and Post as illustrated in Fig. 1. 1) E1-ATP→E1P(Na₃): high affinity ATP associates with E1 and induces the cytoplasmic Na⁺ binding to the pump, which leads to the phosphorylation of the pump and the occlusion of Na⁺; 2) E1P(Na₃)→E2P: Na⁺ is released during the subsequent transition from an ADP-sensitive phosphoenzyme to a K⁺ sensitive phosphoenzyme; 3)E2P→E2(K₂): extracellular K⁺ dephosphorylates the pump, and K⁺ is occluded in the pump; 4) E2(K₂) →E1: ATP with low binding affinity accelerates K⁺ release into the cell (Skou 1990).

Figure 1 Albers-Post Kinetic Scheme of Na/K-ATPase
In accordance with the early kinetics and structural studies, the recently revealed crystal structures of Na/K-ATPase and SERCA confirm the structural changes during the pumping cycle of these two enzymes (Toyoshima et al. 2000; Toyoshima et al. 2004; Yatime et al. 2011). According to the crystal structure of SERCA, large scale coordinated domain rearrangements occur during the conformational transition (Toyoshima et al. 2004). Although the conformation of Na/K-ATPase has not been fully resolved yet, based on the known high similarity between α1 Na/K-ATPase and SERCA (Sweadner and Donnet 2001), as well as the results from the proteolytic studies, similar domain movements can also be applied to α1 Na/K-ATPase. During E2-E1 transition, with the help of low affinity ATP binding, release of two K\(^+\) exchanges with three Na\(^+\), and three cytoplasmic domains adopt a relatively relaxed configuration. The binding of ATP with high affinity cross-links the N domain and P domain. Consequently, the formation of aspartyl-phosphoanhydride triggers the separation of the N domain with the P domain. A conserved TGES motif in A domain is critical in stimulating the dephosphorylation of the aspartic acid. In order for this motif to access the phosphorylation site, the A domain rotates about 120° around the P domain during E1~P to E2~P transition, and TGES loop wedges into the gap between the N domain and P domain. Therefore these three cytoplasmic domains form a compact headpiece. Once the Na/K-ATPase is dephosphorylated, the cycle is completed by liberating two K\(^+\).
Oligomerization of Na/K-ATPase

It is generally accepted that the α and the β subunits form αβ heterodimer in equal molar ratio, while the γ subunit is associated with αβ heterodimer in a tissue-specific manner. However, some kinetic data cannot be explained by a single form of αβ heterodimer, i.e., the discrepancy of the kinetics of E1P to E2P conversion between pre-steady state and steady state (Froehlich et al. 1997). On the other hand, extensive studies have suggested that Na/K-ATPase may form oligomers in the plasma membrane. For example, chemical cross-linking studies show the non-covalent association of α-subunits in the native state (Askari and Huang 1980; Askari et al. 1980). A series of fluorescence studies further suggest a tetramer model of Na/K-ATPase (Tsuda et al. 1998; Taniguchi et al. 2001). Fractionation of the solubilized enzyme by high performance gel chromatography and the analysis of the preparations by low angle laser light scattering photometry indicates that Na/K-ATPase contains two major protein contents (αβ-protomer and (αβ)2-diprotomer), as well as small amounts of higher oligomer forms (Kobayashi et al. 2007). Observation from electron microscopy also reveals the existence of (αβ)2 and (αβ)4 in solubilized pig kidney enzyme and dog kidney enzyme (Yokoyama et al. 1999). Interestingly, some studies further suggest that part of CD3 of Na/K-ATPase is involved in the specific α/α association (Koster et al. 1995; Koster et al. 1997; Costa et al. 2003; Laughery et al. 2004). It is still not clear whether this interaction is involved in Na/K-ATPase oligomerization. If it does, will the α/α oligomerization affect Src binding and its kinase activity? Some studies also suggest that the formation of oligomer is affected by ions. For example, K+ strengthens the protein-protein interaction while Na+ antagonizes its effect (Kobayashi et al. 2007). Moreover, E2/E2(P) is likely to form a
compact oligomer (Hayashi et al. 1997). Therefore, the ion-induced conformational changes and oligomer formation of Na/K-ATPase will add another layer of complexity to the Na/K-ATPase/Src interaction.

**Src**

Since the discovery of the tyrosine phosphorylation as an important post-translational modification of the protein activity, extensive studies have revealed the essential role of protein kinases in many signaling pathways including growth factors, hormones, neurotransmitters and toxins. On the other hand, malfunction of some kinases are involved in the development of many diseases.

SFKs (Src family kinases) are non-receptor tyrosine kinases. The v-Src is the first identified oncogene which encodes the transforming v-Src protein with a constitutive kinase activity, and is responsible for the cell-transforming ability of Rous sarcoma virus (Brugge and Erikson 1977). As the cellular homologue of the v-Src gene, the c-Src gene is the first identified proto-oncogene, and its product, c-Src protein, is ubiquitously expressed and possesses tyrosine kinase activity (Stehelin et al. 1976; Shalloway et al. 1981). Later on, other eight proteins are found in SFKs based on the similarity in sequence and structure. These nine Src family members are divided into two groups according to their distinct cellular distribution: ubiquitously expressed tyrosine kinases such as Src, Fyn, Yes, and limited expressed members such as Fgr, Lyn, Hck, Lck, Blk, Yrc and Yrk (Martins-Green et al. 2000; Martin 2001).
SFKs are important regulators in various signal transduction pathways including cytokines, hormones, growth factors as well as stress responses. Moreover, SFKs are involved in controlling cell proliferation, replication, growth, adhesion and motility (Thomas and Brugge 1997). SFKs also play a role in other physiological processes as suggested by certain SFKs knock-out mouse models. For example, deficiency of Src affects the bone formation and causes osteopetrosis (Soriano et al. 1991). Fyn is involved in the fatty acid metabolism, and Fyn null mice have been shown to have increased peripheral tissue insulin sensitivity and improved glucose tolerance (Bastie et al. 2007).

**Structure of Src**

c-Src tyrosine kinase contains 535 amino acids which constitute several well characterized domains (Boggon and Eck 2004): 1) N terminal segment includes SH4 (Src homology domain 4) and a unique domain with 50-70 residues which has the least conservation among SFKs. The SH4 domain is important for fatty acid modification after translation. It contains a consensus sequence as Met-Gly-XXX-Ser for the irreversible attachment of the 14-carbon saturated fatty acid myristate, as well as a Met-Gly-Cys sequence for the reversible acylation by the 16-carbon saturated palmitic acid. In addition, conserved positive charged lysine residues and cysteine residues have also been found in N-terminus. All of these signals may contribute to the membrane targeting and the subcellular compartmentalization of Src (Resh 1994). On the other hand, the unique region is important for binding with specific signaling proteins among SFKs. 2) Two homologous regulatory modules include SH3 domain (residues 81-142) and SH2 domain (residues 148-245). Both are common motifs in mediating the protein-protein interaction in signaling cascades. SH3 preferentially associates with polypeptides containing proline-
rich sequences which adopt a characteristic polyproline II conformation; while SH2 preferentially binds to a phosphotyrosine in a specific context (for example, with leucine or isoleucine at pTyr+3 position). 3) A highly conserved kinase or SH1 domain (residues 267-520) consists of a small N-terminal lobe and a large C-terminal lobe. The N lobe contains a Gly-rich loop and the catalytically important helix C; the C lobe contains the activation loop (residues 404-432) and the catalytic loop. In the kinase domain, several important structural elements are critical in determining the overall kinase activity. For example, the intramolecular ionic interaction between Glu 319 in helix C and Lys 295 determines whether the activation loop can accommodate the phosphate of ATP. Hence the kinase activity of SFKs is dependent on helix C configuration. Another important determinant is the phosphorylation state of Tyr 416 in the activation loop. The unphosphorylated Tyr 416 prevents the substrate binding, and stabilizes a closed and restrained conformation of the kinase domain. 4) A short C-terminal regulatory tail includes another tyrosine residue (Tyr 527) functioning as a negative regulation point. Mutations or truncations of C-terminus have been found to elevate the Src activity (Brown and Cooper 1996; Xu et al. 1999; Young et al. 2001; Cowan-Jacob et al. 2005).

Depending on the phosphorylation level of this C-terminal tyrosine, Src adopts two main conformations as revealed by the crystal structure (Fig. 2) (Young et al. 2001). 1) An “assembled state” with Tyr 527 phosphorylated (Xu et al. 1999; Young et al. 2001): in this inactive conformation with a low basal level of kinase activity, two intramolecular associations are revealed. One is between the SH2 domain and the phosphorylated Tyr 527, the other is between the SH3 domain and the 14-amino acid linker region (residues 246-259) connecting the SH2 domain and the kinase domain. These two interacting pairs
pack SH2 domain and SH3 domain against the C-terminal lobe and N-terminal lobe of the kinase domain, respectively, resulting in a highly compacted, auto-inhibited conformation. In this inactive form, the activation loop adopts an $\alpha$ helix configuration which wedges in the cleft between the two lobes of the kinase domain, therefore making the catalytic site inaccessible to the substrate. 2) An unrestrained form with Tyr 527 unphosphorylated (Cowan-Jacob et al. 2005): with the release of Tyr 527 from the SH2 domain, the overall orientation of the SH2 and SH3 domains to the kinase domain has changed: SH2 and SH3 domains rotate $130^\circ$ perpendicular to the kinase domain. In this relatively less restrained form, helix C occupies an active position. The activation loop is extended instead of forming an ordered helix, allowing the access of substrates to the catalytic site (Cowan-Jacob et al. 2005). It is important to mention that Tyr 416 needs to be phosphorylated in order to form a more stable and further active form (Williams et al. 1997).

Figure 2 Schematic Presentation of Src Structure and Regulation

Cell. 2001 Apr 6;105(1):115-26
Regulation of Src

As mentioned before, since SFKs control many cellular functions, various mechanisms are involved in tightly controlling the catalytic activity. Any factors modulating the intramolecular interactions will affect its assembled conformation and consequently alter its activity. One way is to modulate the interaction between the SH2 domain and the C-terminal tail by regulating the phosphorylation extent of Tyr 527. Activation of phosphatase SHP1 by thrombin treatment, phosphatase SHP2 by platelet-derived growth factor (PDGF) (Peng and Cartwright 1995), CD45 through T cell receptor signaling (Cahir McFarland et al. 1993), all have been shown to dephosphorylate Tyr 527 and induce Src activation in different cell lines. A second mechanism is to directly disrupt the relatively low affinity intramolecular binding, which will lead to the consequent opening up of the assembled two lobes of kinase domain. Many high affinity binding competitors have been shown to work as Src activators. For example, PDGF receptor (Alonso et al. 1995) can compete for the SH2 domain binding; Sin displaces the SH3 domain/linker interaction (Alexandropoulos and Baltimore 1996); FAK and p130Cas interact with both SH2 and SH3 domains (Thomas et al. 1998; Burnham et al. 2000). All of these proteins cause the increment of Src activity. In addition, other allosteric mechanisms which destabilize the restrained conformation of Src will also induce the activation. For example, phosphorylation at the unique region of Src (like Thr 34, Thr 46, Ser 72) is known to increase Src activity even though the C-terminus is still phosphorylated, suggesting that the association between the SH2 domain and the C-terminus is released by this specific phosphorylation (Brown and Cooper 1996). Direct
interaction of Gαs with kinase domain also causes conformational changes and activates Src (Ma et al. 2000).

**Na/K-ATPase as Signal Transducer**

**CTS**

CTS include plant-derived digitalis such as digoxin and ouabain, and vertebrate-derived aglycones such as bufalin and marinobufagenin (MBG) (Akera and Brody 1976; Schoner and Scheiner-Bobis 2007). Unlike many other steroids, some CTS have one or several sugar molecules attached to its steroidal genin. Although digitalis has been used to manage congestive heart failure for over 200 years, its underlying mechanism is not recognized until the identification of Na/K-ATPase by Skou in 1950s (Skou 2004). Now it is well known that digitalis is a specific Na/K-ATPase ligand, which can inhibit the Na/K-ATPase activity in a dose-dependent manner (Skou 2004). Consequently, the intracellular Na\(^+\) concentration is increased. The Na\(^+\) elevation reverses the working mode of the membrane Na\(^+\)/Ca\(^{2+}\) exchanger, leads to the increase of intracellular Ca\(^{2+}\), which in turn enhances the contractility of cardiac muscle, a phenomenon termed “positive inotropy” (Blaustein and Hodgkin 1969; Iwamoto et al. 2004).

Ouabain, the most water-soluble CTS, has been shown to bind to the extracellular site of Na/K-ATPase by early mutagenesis studies (Lingrel et al. 1998). X-ray crystallography studies further pinpoint the binding pocket to be among H1-H2, H3-H4 and H5-H6 extracellular loops of α1 subunit (Yatime et al. 2011). Moreover, ouabain
preferentially binds to E2-P state as suggested by the facilitated association in the presence of Na\(^+\)/Mg\(^{2+}\)/ATP or Mg\(^{2+}\)/Pi, both of which promote the E2-P form of the enzyme (Albers et al. 1968; Schwartz et al. 1968); and its binding is antagonized by K\(^+\) (Akera and Ng 1991).

EO (endogenous ouabain) has been isolated in human plasma, bovine adrenal gland and hypothalamus (Hamlyn et al. 1991; Schneider et al. 1998). Moreover, de novo synthesis of ouabain has been found in cultured adrenal hypothalamic cells (Perrin et al. 1997). Studies have also shown that the production and release of ouabain are regulated by multiple physiological stimuli including ACTH (adrenocorticotropic hormone) and angiotensin II (Hamlyn et al. 1991; Laredo et al. 1997). The level of circulating EO is in the range of subnanomolar to nanomolar (Hansen 2003). Increase in EO has been observed in renal failure, congestive heart failure and adrenocortical adenomas (Pierdomenico et al. 2001; Wang et al. 2003). Moreover, the level of plasma EO is correlated with salt-handling and blood pressure (Manunta et al. 1999). For example, increase in plasma EO has been observed in human with chronic high-salt diet and in salt-sensitive hypertensive animal models (Fedorova et al. 2000; Manunta et al. 2006). About 50% of patients with essential hypertension have substantially elevated levels of EO (Manunta et al. 1999). These data implies that ouabain is actually an endogenous hormone.

**Function and Mechanism of CTS**

**Calcium Signaling**
Calcium is a highly versatile intracellular signal, occurring either transiently or in an oscillatory manner. It is involved in many vital cellular processes, such as proliferation and apoptosis. Therefore, the regulation of calcium is diversified in a cell-specific manner.

In cardiac and smooth muscle cells, partial inhibition of α2 and α3 Na/K-ATPase by low dose of ouabain induces the local increase of intracellular Na⁺, which in turn reverses the mode of NCX1 (sodium/calcium exchanger 1) and results in an elevation of intracellular Ca²⁺. This transient increase of local Ca²⁺ will activate SERCA and amplify the Ca²⁺ signaling from sarco/endoplasmic reticulum. The elevation of Ca²⁺ will lead to an augmented contraction of smooth muscle cells, an increase of myogenic tone and total peripheral vascular resistance, and finally the hypertension (Davis and Hill 1999). This regulation of Ca²⁺ signaling is enabled by a specific microdomain arrangement called “plasmaErosome”. Through the conserved N-terminus, α2 and α3 Na/K-ATPase are tethered to the plasma membrane where the NCX1 is co-localized. This plasma microdomain is situated adjacent to jS/ER (junctional sarcoplasmic/endoplasmic reticulum). Therefore, the local ion changes induced by ouabain can be transmitted into jS/ER (Juhaszova and Blaustein 1997; Arnon et al. 2000). This mechanism suggests the role of CTS as an important regulator of blood pressure. Gene-replacement animal studies further support this concept. For example, compared to the wild-type mice, mice in which ouabain-insensitive α1 is replaced with humanized ouabain-sensitive α1, develop more severe hypertension when ACTH is administered (Dostanic-Larson et al. 2005). Similarly, knock-down of 50% of α2 induces elevation of blood pressure but not knock-down of α1 (Zhang et al. 2005); while overexpression of α2 but not α1 could reduce the blood pressure (Pritchard et al. 2007).
On the other hand, Aperia’s lab first reported that in renal epithelial cells ouabain could trigger slow calcium oscillation at noninhibitory concentrations (Aizman et al. 2001). Further investigations suggest a direct interaction between α1 Na/K-ATPase and IP3R (inositol trisphosphate receptor), a calcium channel widely distributed in ER (Miyakawa-Naito et al. 2003). This interaction is mediated through the N-terminus of α1 Na/K-ATPase, and the conformational change of Na/K-ATPase upon ouabain binding facilitates this association (Zhang et al. 2006). This ouabain dependent calcium oscillation further activates calcium-dependent transcriptional factors such as NF-κB to regulate apoptosis and growth (Li et al. 2006). Consistent with the above findings, my lab mates also find a direct association between Na/K-ATPase and IP3R; moreover, Na/K-ATPase also binds with PLC-γ (phospholipase C) via CD3 domain, and stimulates this phospholipase through phosphorylation. Therefore, Na/K-ATPase tethers these two proteins together, facilitating the formation of a Ca$^{2+}$- regulatory complex (Yuan et al. 2005).

**Protein Phosphorylation**

Besides the generation of second messengers, Na/K-ATPase could directly convert ouabain binding to the activation of protein kinase cascades and phospholipase by phosphorylation. Ouabain stimulated phosphorylation has been observed in cardiac myocytes, LLC-PK1 and A7r5 cells (Haas et al. 2000). Functionally, ouabain binding to the Na/K-ATPase results in the activation of Src/EGFR/Ras/Raf/MEK/ERK pathway, the production of ROS in mitochondria, and the generation of second messengers (i.e., an increase in Ca$^{2+}$ in epithelial cells) (Haas et al. 2002; Chen et al. 2008). The ultimate
cellular effects are diversified depending on the cell and tissue type. For example, ouabain promotes hypertrophic growth in cardiac myocytes and proliferation in renal epithelial cells, but it inhibits growth in some cancer cells (Li et al. 2009). In LLC-PK1 cells, ouabain has been found to coordinate the membrane expression of NHE3 and Na/K-ATPase, suggesting a potential role of CTS in pump/leak coupling and Na$^+$ handling (Cai et al. 2008a; Liu et al. 2011).

Moreover, a recent study from Blanco’s laboratory suggests that endogenous CTS may play an important role in the pathogenesis of ADPKD (autosomal dominant polycystic kidney disease) (Nguyen et al. 2007). Application of sub-nanomolar concentrations of ouabain basolaterally is sufficient to stimulate the proliferation of ADPKD cells, but not the normal human kidney cells, via the activation of ERK pathway (Nguyen et al. 2007). Low concentration of CTS also induce cell-cell attachment by elevating the membrane expression of connexin 32 through protein phosphorylation cascades (Larre et al. 2006), and promote cell detachment by redistributing cell-cell attaching molecules such as occludin through Rho/Rac and MAPK signaling pathways (Contreras et al. 1999).

**Na/K-ATPase/Src Forms a Receptor Complex**

Like cytokine receptors and GPCRs, Na/K-ATPase does not have intrinsic kinase activity. Therefore it requires coupling with non-receptor kinase to fulfill the phosphorylation capability. Based on the previous studies from this lab, Src kinase turns out to be one of the most proximal kinase associating with Na/K-ATPase, and it trans-activates EGFR to propagate the signaling cascades. First, pretreatment with Src kinase
inhibitors (PP2 or herbimycin A) abrogates the ouabain-stimulated phosphorylation. Moreover, ouabain-induced transactivation of EGFR is only observed in SYF+c-Src cells, but not in SYF cells. Second, Src is found to be coimmunoprecipitated with α1 Na-K/ATPase in a ouabain-regulatory manner. This is consistent with the confocal imaging showing that α1 Na/K-ATPase co-localizes with Src in the plasma membrane in LLC-PK1 cells (Tian et al. 2006). The close proximity between α1 Na/K-ATPase and Src is further confirmed by FRET analysis (Tian et al. 2006). Third, GST pull-down assay indicates that the association between α1 Na/K-ATPase and Src is specific through two intermolecular interacting pairs: one is between Src SH2 domain/Na/K-ATPase CD2 domain, and the other is between Src kinase domain and Na/K-ATPase CD3 domain (Tian et al. 2006) (Fig. 3). The latter interaction keeps Src in an inactive state by inhibiting Tyr 418 phosphorylation without affecting the phosphorylation of Tyr 529 (Tian et al. 2006).

Figure 3 Schematic Presentation of Ouabain-Regulated Na/K-ATPase/Src Interaction

![Diagram of Ouabain-Regulated Na/K-ATPase/Src Interaction](image)
Moreover, it is reported that the number of $\alpha_1$ Na/K-ATPase in the plasma membrane is close to one million/cell in LLC-PK1 cells (Liang et al. 2007). To estimate the potential capacity of $\alpha_1$ Na/K-ATPase-mediated Src regulation in these cells, the number of Src in these cells was measured semi-quantitatively by Western blot analysis of cell lysates and the known amount of purified Src. As depicted in Fig. 4 (Li, Xie, unpublished data), it was estimated that there were about $2 \times 10^5$ Src per LLC-PK1 cell in average.

**Figure 4 Quantification of Src Molecule in Each LLC-PK1 Cell**

![Image](image)  

Fig. 4 Confluent LLC-PK1 cells were lysed in modified RIPA solution. Protein amount in the cell lysates was measured using Lowry assay. Cell numbers were counted with a hemocytometer. The calculated protein amount in each LLC-PK1 cell is 0.845 ng. Then, 0.8 ng of purified Src (Upstate, Lake Placid, NY) and 20 µg of LLC-PK1 cell lysate were subjected to Western blot analysis with an antibody against c-Src (Santa Cruz, CA). ImageJ was used to quantitate the image. The Src molecule per LLC-PK1 cell is around $2 \times 10^5$.

**The Interaction Between $\alpha_1$ Subunit N Domain and Src**

Further characterization of the Na/K-ATPase/Src interaction have led the identification of a 20 amino acid peptide located at the less structural N-terminus of the Na/K-ATPase N domain (NaKtide) (Li et al. 2009). NaKtide is found to bind to and inhibit Src with similar potency as purified $\alpha_1$ Na/K-ATPase (Li et al. 2009). According to the crystal structure, the N-terminus of NaKtide forms a helix (T417 to L427) followed by a loop tail (C428 to Q434) (Fig. 5).
Figure 5 The Helical N-terminus of NaKtide

Fig. 5 A, amino acid sequence of NaKtide. B, T417 to L427 forms a helical structure (labeled in red), which is followed by a loop tail (C428 to Q434) (labeled in green). The 3D structure was generated based on Na/K-ATPase crystal structure (PDB ID: 3b8e) using SPDBView V3.7 program.

Unpublished mutagenesis experiments from this lab indicate that this helical structure is important for NaKtide to inhibit Src. It is known that proline replacement will bend the backbone of a helix (Barlow and Thornton 1988; Yun et al. 1991). To further understand the molecular basis of α1 Na/K-ATPase mediated Src regulation and to reveal the importance of the helical structure, several alanine to proline mutants were constructed based on a rat α1 cDNA expressing vector that was described in the previous publications (Liang et al. 2006): A420P and A425P within the helix, as well as A416P at the N-terminus to the helical structure. To reduce the interference from endogenous α1 Na/K-ATPase, α1 knockdown PY-17 cells were used as parent cells. PY-17 cells are derived from pig LLC-PK1 cells, and the amount of endogenous α1 Na/K-ATPase is reduced more than 90% by introduction of the α1-specific siRNA (Liang et al. 2006). Note that these cells do not express other isoforms of Na/K-ATPase. Wild-type rat-α1 rescued PY-17 cells (AAC-19) were used as a control.
Like wild-type rat α1 (Liang et al. 2006), after ouabain selection of the transfected PY-17 cells, numerous clones for A416P, A420P and A425P with varied expression level of rat α1 mutant were generated. As depicted in Fig. 6 (Lai, Ye and Xie, unpublished data), the clone with the expression level of rat α1 mutant comparable to that of wild-type rat α1 in AAC-19 cells was picked for each mutation for further analysis. As reported, no detectible signal of rat α1 was observed in the PY-17 cell lysates (Fig. 6); however, when the blot was analyzed by a generic α1-specific monoclonal antibody, a weak signal was detected in samples from PY-17 cells as previously reported (Liang et al. 2007).

**Figure 6 Generation of Stable Cell Lines Expressing A to P Mutants**

Fig. 6 A416P, A420P and A425P cell lines were generated by transfection of rat α1 Na/K-ATPase mutant expression vectors into PY-17 cells. Total cell lysates from different cell lines were separated by SDS-PAGE and analyzed by Western blot with anti-NASE (Texas Tech University, Lubbock, TX) for the expression of specific rat α1 Na/K-ATPase and with anti-α1 (α-6F) (The University of Iowa, IA) for total α1. α-tubulin was used as loading control (Sigma-Aldrich). A representative Western blot is shown, and the quantitative data (mean±s.e.m.) were calculated from at least three separate experiments as relative ratio of total α1/tubulin. **, P<0.01.
The Western blot data were further verified by immunostaining of these cell lines. Strong and comparable signals were detected in the plasma membrane area in all of the rescued cell lines but not the parental PY-17 cells (Fig. 7A, Lai, Ye and Xie, unpublished data). Biotinylation analysis also indicated that the ratio of biotinylated surface α1 to total α1 in the cell lysates was similar among AAC-19, A416P, A420P and A425P cells (Fig. 7B, Lai, Ye and Xie, unpublished data).

**Figure 7 Expression of Na/K-ATPase α1 and β1 Subunit in Mutant Cells**

**A**

AAC-19  
PY-17  
A416P  
A420P  
A425P

**B**

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**C**

AAC-19  
PY-17  
A416P  
A420P  
A425P

**Fig. 7** A, cells were cultured on cover slips, and immunostained with anti-α1 antibody (Upstate, Lake Placid, NY). B, one 60 mm dish of each cell line was biotinylated as described under “Material and Methods”. Afterward, the cells were solubilized in 400 µl of lysis buffer, and then analyzed. The total cell lysate (T) 25 µg and biotinylated membrane protein (M) from 250 µg total protein were subjected to SDS-PAGE and probed with anti-α1 antibody (α-6F) (The University of Iowa, IA). Representative Western blots are shown, and quantitative data are calculated based on at least three independent experiments as relative ratio of membrane α1 to total α1. Values are mean±s.e.m.. C, total cell lysates from different cell lines were separated by SDS-PAGE and analyzed by Western blot for the expression the β1-Na/K-ATPase (Upstate, Lake Placid, NY). A representative Western blot is shown.
Knockdown of α1 subunit reduced the expression and glycosylation of β1 subunit (Liang et al. 2006). As shown in Fig. 7C (Lai, Ye and Xie, unpublished data), all three α1 mutants were able to rescue the expression and glycosylation of β1 as did wild-type α1. These findings indicate that the expressed mutant α1 is fully capable of assembling with β1 subunit into a functional Na/K-ATPase, which is consistent with the findings depicted in Fig. 7A and 7B.

Pumping function of mutant Na/K-ATPase was not altered as suggested by the same ouabain-sensitive $^{86}\text{Rb}^+$ uptake activity (Fig. 8A, Lai, Ye and Xie, unpublished data). Moreover, the expressed mutants were not predisposed to either E1 or E2 conformation as illustrated by the similar sensitivity to vanadate (Fig. 8B, Lai, Ye and Xie, unpublished data). The above findings indicate that expression of mutants restore the total cellular α1 Na/K-ATPase as well as the pumping capacity in A416P, A420P and A425P cells to the level comparable to that in AAC-19 cells.

**Figure 8 Pumping Activity in Mutant-Rescued Cells**

Fig. 8 A, ouabain-sensitive $^{86}\text{Rb}^+$ uptake was assayed as described under “Material and Methods”. Values are normalized to per mg protein, and then calculated as % of AAC-19 cells. Data are presented as the mean±s.e.m. (n=3). B, vanadate-sensitive Na/K-ATPase activities of wild-type α1 Na/K-ATPase and mutants. Crude membrane fraction were prepared and assayed for the ouabain-sensitive Na/K-ATPase activity as described under
“Material and Methods” in the presence of indicated concentrations of vanadate. The data points are shown as percentage of the Na/K-ATPase activity in the absence of vanadate.

It is previously reported that knockdown of Na/K-ATPase increases basal Src activity in PY-17 cells, and that rescuing the knockdown cells with rat α1 restores basal Src activity in AAC-19 cells (Liang et al. 2006), which suggests the Src regulatory role of Na/K-ATPase. This is consistent with the Western blot analysis of active Src as indicated by Tyr 418 phosphorylation (Fig. 9A, Lai, Ye and Xie, unpublished data). Interestingly, only the expression of A416P, but not A420P and A425P, showed the same effect as wild type α1. The basal Src activity in both A420P and A425P cells were as high as that in PY-17 cells.

**Figure 9 Regulation of Src by Mutant α1**

Fig. 9 A, total cell lysates from different cell lines were separated by SDS-PAGE and analyzed by Western blot with an antibody against c-Src (Santa Cruz Biotechnology, Santa Cruz, CA) and pY418 Src (Invitrogen, Carlsbad, CA), and activated Src was represented as a ratio of activated Src over total Src. Quantitative data are mean ± s.e.m. from at least three experiments. *, P<0.05. B, Cells were cultured on cover slips, and immunostained with anti-pTyr 418 Src antibody (Invitrogen, Carlsbad, CA).
This result was further confirmed by immunostaining of these cultured cells with an anti-Tyr 418 antibody. As shown in Fig. 9B (Lai, Ye and Xie, unpublished data), in contrast to A416P cells, both A240P and A425P showed much higher positive staining of active Src. These data suggest that bending the helical structure at NaKtide position (A420, A425) in intact α1 subunit may reduce the capability of α1 Na/K-ATPase to interact and regulate Src; while A416, which is at N-terminus to this helical structure in native Na/K-ATPase, does not affect the regulatory effect of α1 Na/K-ATPase on basal Src activity.

As mentioned above, binding of ouabain to α1 Na/K-ATPase has been shown to stimulate Src pathway within min and increase the expression of α1 Na/K-ATPase in hours (Liang et al. 2006; Li et al. 2009). These changes in response to ouabain (10 to 100 μM) were also observed in the control AAC-19 cells (Fig. 10A and 10B, Lai, Ye and Xie, unpublished data). If α1 Na/K-ATPase and Src indeed formed a receptor complex, ouabain would not stimulate Src in A420P mutant rescued cells. As expected, ouabain was able to stimulate Src in A416P, but not A420P cells (Fig. 10A). Similarly, ouabain failed to increase the expression of α1 in A420P cells (Fig. 10B).
Fig. 10 A, cells were treated with different concentration of ouabain for 10 min. Equal protein amount of cell lysates was analyzed for expression of pTyr 418 Src (Invitrogen, Carlsbad, CA) and c-Src (Santa Cruz Biotechnology, Santa Cruz, CA). A representative Western blot and the quantification data from three or four independent experiments are shown. *, P<0.05. B, cells were treated with different concentration of ouabain for 24 hr. Equal protein amount of cell lysates was analyzed for Na/K-ATPase α1 expression with anti-α1 antibody (α-6F) (The University of Iowa, IA) and anti-α-tubulin (Sigma-Aldrich). A representative Western blot and the quantification data from three or four independent experiments are shown. *, P<0.05.

To seek further evidence that the helical structure is important for the formation of a functional Na/K-ATPase/Src complex, these studies were repeated in A425P cells. As shown in Fig. 10A and 10B, A425P mutant, like A420P mutant, lost its ability to allow ouabain to stimulate Src pathway. It is important to note that AAC-19 cells express ouabain-insensitive rat α1 Na/K-ATPase. Therefore, µM instead of nM ouabain was used in these experiments (Liang et al. 2006).

The defect in ouabain signaling cannot be attributed to the reduced ouabain sensitivity of the mutants. In fact, ouabain produced a similar dose-dependent inhibition of $^{86}$Rb$^+$ uptake in A416P and A420P cells with IC$_{50}$ of 117.8 µM and 133.7 µM, respectively (Fig. 11, Lai, Ye and Xie, unpublished data), which are close to the value of wild type rat α1 (around 100 µM) (Liang et al. 2006).
Fig. 11 Cells were pretreated with different concentrations of ouabain as indicated for 10 min and assayed for $^{86}\text{Rb}^+$ uptake as described under “Material and Methods”. Data are shown as % of non-treated control value and are presented as the mean ± s.e.m. of three independent experiments. Curve fit analysis was performed by Graphpad software.

In addition to Src, α1 Na/K-ATPase also interacts with PI3K and regulates the PI3K/Akt pathway (Liu et al. 2007b). Specifically, it has been reported that ouabain could stimulate PI3K and Akt in LLC-PK1 cells. Interestingly, ouabain stimulated Akt in both A416P and A420P cells (Fig. 12, Lai, Ye and Xie, unpublished data), contrary to the effect of ouabain on Src, indicating that the inhibition of ouabain-induced signal transduction by A420P mutation is specific to the Src-related pathway.

Fig. 12 Cells were treated with indicated concentration of ouabain for 10 min. Equal protein amount of cell lysates was analyzed with anti-pAkt (Cell Signaling, Danvers, MA)
and anti-Akt (Cell Signaling, Danvers, MA). A representative Western blot and the quantification data from three or four independent experiments are shown. *, P<0.05.

What is the mechanism of ouabain induced Src activation?

The above experiments suggest a direct interaction between α1 Na/K-ATPase and Src. Moreover, association with the α helix in NaKtide keeps Src inactive. The next question is how ouabain, which binds to Na/K-ATPase extracellularly, activates Src which associates with α1 Na/K-ATPase at cytoplasmic site.

Binding of ouabain to the Na/K-ATPase has been found to free the kinase domain from the N domain without interfering the interaction between SH2 and CD2 domains (Tian et al. 2006). However, the overall binding affinity between α1-Na/K-ATPase and Src is not altered (Tian et al. 2006). This suggests that the binding affinity of N domain/kinase domain interaction might be lower than that of CD2/SH2 domain interaction. Indeed, GST pull-down assay showed that the binding of CD2 to Src occurred at a lower concentration than that of the N domain (ND1) (Fig. 13A) (Ye et al. 2011).

Figure 13 Interaction Between the α1 Na/K-ATPase and Src
Fig. 13 A, GST pull-down analysis showing the concentration-dependent interaction between Src and two domains of the α1 subunit. Indicated amount of GST-fused proteins were incubated with 100 ng purified His-Src, and the bound His-Src in the pull down pellets was detected by Western blot with anti-His antibody (GE, Buckinghamshire, England) shown in the upper panel. Lower panel shows the Coomassie blue staining of GST, GST-CD2 (amino acid residues 152-288), and GST-ND1 (amino acid residues 379-435, first 57 amino acids of the N domain). n=3. B, purified Na/K-ATPase was incubated with either His-Src buffer or His-Src (at molar ratio of 1:1). Ouabain-sensitive Na/K-ATPase activities were measured as described under “Material and Methods”. Activity was calculated and expressed as % of control. Quantitative data from three independent experiments are presented as mean ± s.e.m.

Since the above GST pull-down assay suggests that Src directly interacts with Na/K-ATPase, it is logical to test whether binding of Src interferes with the pumping activity of Na/K-ATPase. To answer this question, the effect of Src on Na/K-ATPase activity was determined (Fig. 13B) (Ye et al. 2011). The same molar amount of purified Na/K-ATPase and His-Src was incubated together. No change of ouabain-sensitive Na/K-ATPase activity was observed under this condition. Higher molar ratio (up to 5 Src: 1 Na/K-ATPase) was also tested, and no effect of Src on Na/K-ATPase activity was detected.

During the pumping cycle, the α1 Na/K-ATPase undergoes E1 to E2 conformational transition, and ouabain is known to stabilize the Na/K-ATPase at E2 like form (Skou and Esmann 1992). Therefore, we speculated that the α1 Na/K-ATPase might regulate Src in a conformation-dependent manner. To seek further evidence, my co-worker Dr. Zhichuan Li and I compared the crystal structure of E1 and E2 conformation of Na/K-ATPase and SERCA. During E1P to E2P transition, Na/K-ATPase A domain rotates about 120º, and wedges into the gap between the N domain and P domain (Toyoshima et al. 2004). This movement of A domain, on the one hand, may work with SH2 domain as a hinge and
affect the interaction between the kinase domain and N domain; on the other hand, it makes the three cytosolic domains form a more compact configuration, which is less likely to accommodate two intermolecular associations simultaneously. Moreover, the Na/K-ATPase activity was not affected when incubated with purified Src at a 1:1 molar ratio (Fig. 13B), indicating that Src did not continuously bind to Na/K-ATPase through two intermolecular interactions to impede E1/E2 transition. Combined with the relative unstable interaction between the N domain/kinase domain, we hypothesized that during the conformational transition from E1 to E2, the kinase domain would be released from the N domain by the coordinated cytosolic domain movements, expose its active site and gain autophosphorylation (Fig. 14) (Ye et al. 2011).

**Figure 14 Cartoon of α1 Na/K-ATPase/Src Interaction**

![Figure 14 Cartoon of α1 Na/K-ATPase/Src Interaction](image)

Fig. 14 Crystal structure of E1, E2 Na/K-ATPase, and Src is based on SERCA1a (PDB ID: 1SU4), Na/K-ATPase (PDB ID: 2ZXE), and Src (PDB ID: 1Y57). The 3D structure was generated using SPDBView V3.7 program. The A domain in α1 subunit is in blue, the N domain in black. The SH2 domain of Src is in orange, kinase domain in cyan, activation loop and Tyr 418 in red. In E1-like conformation, the kinase domain (in cyan) is tightly bound with the N domain (in black) and Tyr 418 is buried (boxed area),
therefore Src activity is suppressed; while in E2-like conformation, the kinase domain is moved away from the N domain and Tyr 418 is exposed (boxed area), resulting in Src activation.
Materials and Methods

Materials

The monoclonal anti-His antibody was from GE (Buckinghamshire, England). Polyclonal anti-Na/K-ATPase β1, anti-Na/K-ATPase α1 polyclonal was from Upstate (Lake Placid, NY); monoclonal anti-α-tubulin was from Sigma-Aldrich. Monoclonal anti-α1 antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Polyclonal rat α1-specific antibody (anti-NASE) was provided by Dr. Thomas Pressley (Texas Tech University, Lubbock, TX). Polyclonal anti-pFAK, polyclonal anti-pAKT, polyclonal anti-AKT were purchased from Cell Signaling (Danvers, MA). Monoclonal anti-caveolin-1, monoclonal anti-FAK, monoclonal anti-Src antibody, polyclonal anti-ERK1/2 antibody, monoclonal anti-pERK1/2 antibody, goat anti-rabbit and goat anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-pY418Src and Image-iT FX signal enhancer was purchased from Invitrogen (Carlsbad, CA). Antifade kit, Alexa Fluor 488-conjugated anti-rabbit antibody, Alexa Fluor 546-conjugated anti-rabbit antibody were from Molecular Probes (Eugene, OR). Recombinant human Src was purchased from Upstate (Lake Placid, NY). AG1478 was obtained from Cayman Chemical (Ann Arbor, MI). Radioactive $^{86}$Rb$^+$ was from PerkinElmer Life Science Products (Boston, MA). QuickChange mutagenesis kit was purchased from Stratagene (La Jolla, CA). EZ-link NHS-SS-biotin and streptavidin-agarose beads were from Thermo Scientific. The CMV promoter-driven pEYFP-C1 vector was from Clontech (Palo Alto, CA). The pGEX-4T-1 and pTrc-His A vectors were from GE Healthcare and Invitrogen, respectively. Escherichia coli BL21 was obtained from Invitrogen. AG1478 was obtained from
Cayman Chemical (Ann Arbor, MI). Other chemicals of the highest purity were all obtained from Sigma-Aldrich.

**Cell Culture and Treatment**

Cells used in this work were all derived from LLC-PK1 cells that were originally from ATCC. The generation of \( \alpha_1 \) knockdown PY-17 cells and the rat \( \alpha_1 \)-rescued PY-17 cells (AAC-19) were done as previously described (Liang et al. 2006). The generation of mutant \( \alpha_1 \)-rescued stable cell lines was described below. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS. After cells reached 95-100% confluence, they were serum-starved overnight and used for experiments unless indicated otherwise. The modified \( K^+ \) or \( Na^+ \) medium was prepared as DMEM except that the amount of \( K^+ \) or \( Na^+ \) was reduced according to the treatment protocol (Bowen and McDonough 1987). Choline chloride was added to maintain the ionic strength of the medium.

**Site-Directed Mutagenesis and the Generation of Mutant-Rescued Stable Cell Lines**

Site-directed mutagenesis of rat \( \alpha_1 \) subunit (I279A or F286A, numbered according to the GenBank™ accession number NM_01254) was performed by Quick-change mutagenesis kit using pRc/CMV-\( \alpha_1 \) AACm1 vector as a template (Liang et al. 2006). The pRc/CMV-\( \alpha_1 \) AACm1 carries silent mutations that prevent the binding of \( \alpha_1 \)-specific siRNA to the transcript of rat \( \alpha_1 \) mRNA (Liang et al. 2006). The mutants were verified by DNA sequencing. To generate stable cell lines, the \( \alpha_1 \) knockdown PY-17 cells were transfected with different vectors expressing either I279A or F286A \( \alpha_1 \) mutant using Lipofectamine 2000 as previously described (Liang et al. 2006). The cells were
then selected with ouabain (3μM) 24 h after transfection. The ouabain resistant colonies were isolated and expanded into stable cell lines. Cells were then cultured in the absence of ouabain for three generations before used for the

**Preparation of Purified Na/K-ATPase**

Purified Na/K-ATPase preparations from pig kidney outer medulla, with specific activities between 1000-1400 μmol Pi/mg/h, were obtained using modified Jorgensen method as previously described (Liu et al. 2007a). According to previous report from this lab, more than 90% of protein content in the preparation is Na/K-ATPase (Tian et al. 2006). Briefly, the outer part of medulla was dissected from the pig kidney and kept in cold buffer A solution (30 mM histidine, 250 mM sucrose, 1 mM EDTA Na₂H₂O, pH 7.4) containing 20 μM PMSF. The mixture was stirred, homogenized, and filtered through cheesecloth. In order to get microsomes, the obtained homogenate was centrifuged at 8,000 rpm X 30 min, and the supernatant was further centrifuged at 18,000 rpm X 30 min. The pellet was re-suspended in buffer A. The microsome activation buffer (5 M imidazole, 20 mM ATP, 20 mM EDTA Na₂H₂O, pH 7.5), water and microsomes were added in order into a beaker. The mixture was stirred at 25 °C, and during stirring 5 ml of SDS (56 mg SDS in 5 ml / H₂O) was added drop by drop. After 30 minutes, 12 g of sucrose was added into the mixture. After the sucrose was dissolved, put the beaker on ice. 32 ml light gradient buffer (25 mM imidazole, 1 mM EDTA Na₂H₂O, pH 7.5), 26 ml SDS treated microsomes, 26 ml medium gradient buffer (44% glycerol, 25 mM imidazole, 1 mM EDTA Na₂H₂O, pH 7.5), 11 ml heavy buffer (64% glycerol, 25 mM imidazole, 1 mM EDTA Na₂H₂O, pH 7.5) were layered in order under previous fraction into a centrifuge tube for 45 Ti rotor. The tubes were spun at 45,000 rpm X 165 min. 5 ml of
the solution from the bottom of tubes was carefully removed and discarded. Then 10 ml
(or visible lower white ring fraction, the Na/K-ATPase containing fraction) was removed
from the bottom, and transferred to tubes for 70Ti rotor, spinned at 49,000 rpm X 100
min. The pellet was re-suspended in buffer A, homogenized by hand, and freeze at – 80
°C for future use.

**Chemical Modifier Treatment of the α1 Na/K-ATPase**

To examine the effect of the α1 Na/K-ATPase conformation on Src activity, the
enzyme was stabilized in distinct conformation with fluoride compounds (E2),
NEM/AMPPNP (E1) and oligomycin (E1). Treatment of the α1 Na/K-ATPase with
fluoride compounds was performed according to Danko et al. (Danko et al. 2004).
Purified Na/K-ATPase (18 μg) was exposed to aluminum fluoride in the presence of 2
mM EGTA, 100 mM KCl, 50 mM MES/Tris (pH 6.0), 0.1 mM MgCl₂, 3 mM KF, and 50
μM AlCl₃ for 1.5 h at 25°C. The same amount of enzyme was exposed to beryllium
fluoride in the presence of 2 mM EGTA, 50 mM LiCl, 50 mM MOPS/Tris (pH 7.0), 5
mM MgCl₂, 0.5 mM KF, and 20 μM BeSO₄ for 4 h at 25°C. After treatment, the Na/K-
ATPase was centrifuged at 100,000 g for 45 min in order to remove unbound chemicals,
and the preparations were further subjected to Src autophosphorylation assay (see below)
to detect their effect on Src. The α1 Na/K-ATPase was treated with NEM/AMPPNP
according to Hegyvary (Hegyvary 1976) with modification. In brief, 8 μg purified Na/K-
ATPase was incubated in the reaction buffer containing 40 mM TES (pH 7.0), 100 mM
KCl, 5 mM MgCl₂, 20 mM NaCl, 5 mM NEM and 1 mM AMPPNP. The reaction
continued for 10 min at 30°C and stopped by 1/10 volume of 1M β-mercaptoethanol.
Similarly, after treatment the Na/K-ATPase was centrifuged at 100,000 g for 45 min in
order to remove unbound chemicals, and the preparations were further subjected to Src autophosphorylation assay (see below) to detect their effect on Src. Treating the α1 Na/K-ATPase by oligomycin was according to Hegyvary (Hegyvary 1976) with modification. Indicated amount of oligomycin was incubated with 1 μg Na/K-ATPase in 50 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl (choline chloride was added to maintain the ionic strength at 155mM). The preparations were subjected to Src autophosphorylation assay as mentioned below.

**Trypsin/Chymotrypsin Cleavage of the α1 Na/K-ATPase**

Cleavages of the Na/K-ATPase were obtained by addition of trypsin and chymotrypsin with established procedures according to Zolotarjova et al. (Zolotarjova et al. 1995) and Huang et al. (Huang et al. 1990), respectively. Purified Na/K-ATPase (0.5 mg/ml) was exposed to 0.7μg/ml trypsin in a solution containing 12 mM KCl, 15 mM Tris-HCl (pH 7.4). Similarly, purified enzyme (0.5 mg/ml) was digested by 25μg/ml chymotrypsin in a solution containing 10mM NaCl and 15 mM Tris-HCl (pH 7.4) until more than 75% enzyme activities were lost. The reaction was stopped by addition of trypsin-chymotrypsin inhibitor (Sigma, at the dose of 0.7 mg/μg trypsin or 10 μg/μg chymotrypsin).

**Assays of Src autophosphorylation**

Purified Na/K-ATPase after treatment was incubated with 1 unit purified Src for 30 min at 37°C in a solution containing 50 mM Tris-HCl (pH 7.4), different ion compositions indicated in the figure legends (with addition of choline chloride to maintain the ionic strength), and in some cases followed by ouabain exposure (10 μM, 5 min) as indicated in the figure legends for positive control. Reaction was started by the
addition of 2 mM Mg\textsuperscript{2+}/ATP. The reaction continued for 10 minutes, and was stopped by adding SDS sample buffer. The Src activity was determined by phosphorylation of Src Tyr 418 using immunoblot analysis.

**Immunoblot Analysis**

The immunoblot analysis was performed according to the previous protocol (Li et al. 2009). Briefly, after indicated treatment, cells were solubilized in modified ice-cold radioimmunoprecipitation (RIPA) buffer containing 0.25% sodium deoxycholate, 1% nonidet P-40, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate, 1mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.4). Cell lysates were then centrifuged at 16,000 x g for 15 minutes. Supernatants were collected and protein content was measured. Proteins were separated by SDS-PAGE, transferred to an Optitran membrane and blotted by specific antibodies.

**Ouabain Sensitive Na/K-ATPase Activity**

The Na/K-Activity was assayed according to the protocol previously described (Liang et al. 2006) with modification. Briefly, cells were harvested in Skou C buffer (30 mM histidine, 250 mM sucrose, 1mM EDTA, pH 7.4) and briefly sonicated. After centrifugation (800 x g for 10 min), the post nuclear fraction were further centrifuged (45,000 x g for 45 min) in order to get crude membrane. The crude membrane pellet was resuspended in Skou C buffer and treated with alamethicin (0.1mg/mg of protein) for 10 min at room temperature. The preparation was then incubated in the buffer containing 50 mM Tris (pH 7.4), 1 mM EGTA, 3 mM MgCl\textsubscript{2}, 25 mM KCl, 100 mM NaCl, 5 mM NaN\textsubscript{3} and 2 mM ATP. Phosphate generated during the ATP hydrolysis was measured by
BIOMOL Green Reagent (Enzo Life Science). Ouabain-sensitive Na/K-ATPase activities were calculated as the difference between the presence and absence of 5 mM ouabain. In certain experiments, indicated vanadate amount was added in the reaction mixtures.

**Ouabain Sensitive $^{86}\text{Rb}^+$ Uptake Activity**

The transport function of Na/K-ATPase was assessed by measuring the ouabain-sensitive uptake of the K$^+$ congener, $^{86}\text{Rb}^+$, as described with minor modification (Liang et al. 2007). Briefly, cells were cultured in 12-well plates over 90% confluence and serum starved overnight before experiment. The cells were washed and incubated in culture medium with or without 5 mM ouabain over 10 minutes at 37 °C. $^{86}\text{Rb}^+$ (1µ Ci/well) was added for 10 minutes at 37 °C, and the reaction was stopped by washing with ice-cold 0.1M MgCl$_2$. The cells were incubated in 10% trichloroacetic acid (TCA) for 45 minutes and TCA soluble $^{86}\text{Rb}^+$ was counted in a Beckman scintillation counter. TCA-precipitated proteins were dissolved by 0.1N NaOH, 0.2% SDS solution and the concentration was determined using the BioRad Protein Assay Kit (BioRad Laboratories, Hercules. CA). All counts were normalized to protein amount.

**Cell Surface Biotinylation and Streptavidin Precipitation**

Biotinylation assay was conducted according to Liang *et al.* (Liang et al. 2007). Briefly, cells were cultured on 60-mm Petri dishes until they reach 90 % confluence. The cells were then rinsed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4), and incubated with 2 ml of NHS-SS-biotin (1.5mg/ml) freshly dissolved into biotinylation buffer (10 mM triethanolamine, pH 9.0, 150 mM NaCl) for 25 min at 4 °C on a gently shaking rocker. Cells were then rinsed
twice with PBS containing 100 mM glycine, and incubated in the same buffer for 20 min at 4 °C to quench the un-reacted biotin. Cells were then rinsed twice with PBS, and solubilized in 400µl lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5) for 60 min on rocker with gentle motion. Cell lysates were collected by centrifugation at 16,000 x g for 10 min. Cell lysates (250 µg) was incubated overnight with 150µl of streptavidin-agarose beads in lysis buffer with a total volume of 800 µl at 4 °C with end over-end rotation. The pellet was collected, rinsed, resuspended in 100 µl of Laemmli loading buffer, and subject to Western blot analysis.

**Cell Counting Assay**

Cell growth was measured as previously described (Li et al. 2009). Briefly, cells were seeded at density of 20,000 / well in 12-well plates in DMEM containing 10% FBS. At indicated time point, cells were trypsinized, stained by trypan blue and counted with hemocytometer.

**Cell Spreading Assay**

Cell spreading was assayed according to Richardson et.al (Richardson et al. 1997) with slight modification. After cells were trypsinized, 200,000 cells were seeded to a 60-mm-diameter dish which contained 4ml pre-warmed DMEM containing 10% FBS. The cells were then allowed to spread for indicated time period at 37 °C. For each experiment, five random fields were photographed. A total of at least 300 cells were counted for each experiment condition. Spreading cells were defined as those that had extended processes, lacked a rounded morphology, and were not phase bright.

**Cell Spreading Associated Kinase Activity Assay**
The assay was performed according to Schlaepfer et al (Schlaepfer and Hunter 1997). Cells were grown up to 90% confluence and serum starved with DMEM +0.5% FBS for 24 hours. Dishes (10 cm) were coated overnight with 10ug/ml fibronectin (in PBS) at 4 °C on a shaker. On the day of the experiment the dishes were incubated in serum free media at 37 °C incubator for 1 hour. Cells were harvested using 0.05% trypsin / 0.53 mM EDTA, and trypsin was neutralized by adding 0.5mg/ml of Soybean Trypsin inhibitor in PBS. The cells were then washed, suspended in serum free medium and incubated for 1 hour at 37 °C. Cells (6 millions) were plated in the pre-warmed dishes and allowed to attach/spread for 0, 30 or 60 minutes. At the indicated time points, the dishes were removed from incubator, and cell lysates were collected and subjected to Western blot.

**Confocal Fluorescence Microscope**

The imaging studies were conducted as previously described (Liang et al. 2006). Cells were seeded on cover-slips until they reached 90% confluence. The cells were then fixed with pre-chilled (-20 °C) methanol for 15 min. The fixed cells were blocked with either PBS containing 1% FBS for 30 minutes (for analyzing total α1 Na/K-ATPase) or Image-iT Max Signal Enhancer (for Src pY418) on ice, and incubated with primary antibody overnight at 4°C, followed by washing and incubation with Alexa Fluor conjugated secondary antibody. The stained cells on cover-slips were washed, mounted, and then visualized using a Leica DMIRE2 microscope (Wetzlar, Germany).

**Analysis of Data**

Data are given as the mean ± s.e.m.. Statistical analysis was performed using the Student t test, and significance was accepted at p < 0.05.
Results

Part I α1 Na/K-ATPase Regulates Src Activity in an E1/E2 Conformation Dependent Manner

Identification of E1/E2 Dependent Regulation of Src Activity

To test this E1/E2 regulation model, the purified Na/K-ATPase was first treated with 10 µM ouabain (dissolved in H2O). Because previous study from this lab shows that the level of phosphorylated Tyr 418 Src correlates with Src kinase activity using a commercially available kinase assay kit (Li et al. 2009), Src activity was indicated as pY418 Src in this study. As shown in Fig. 15A, ouabain treatment increased phosphorylation of Src Tyr 418; while it has no effect on Tyr 529 as previously reported (Tian et al. 2006). Therefore, in most of the following in vitro cell-free experiments ouabain was used as the positive control as indicated. Although small traces of endogenous Src (or SFKs) could be detected with anti-Src antibody in some of the purified Na/K-ATPase preparations, the stimulation of endogenous Src by ouabain was not observed (Fig. 15A).

Figure 15 E2-Na/K-ATPase Activates Associated Src
Fig. 15 A, purified Na/K-ATPase (NKA) was incubated with purified Src in 50 mM Tris-HCl (pH 7.4) containing NaCl 150 mM and KCl 5 mM for 30 minutes. 10 µM ouabain (dissolved in H₂O) was added for 5 minutes reaction, followed by addition of 2 mM Mg²⁺/ATP. The reaction continued for 10 minutes, and the preparation was subjected to Western blot analysis. Src Tyr418 phosphorylation and total Src were probed with antibody against pY418 Src (Invitrogen, Carlsbad, CA), and c-Src (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. A representative Western blot is shown. B, to stabilize α1 Na/K-ATPase in the E2P conformation, purified Na/K-ATPase was treated with beryllium fluoride or aluminum fluoride as described under “Methods and Materials”. The ouabain-treated and non-treated α1 Na/K-ATPase/Src complex was used as the positive and negative control for the experiments, respectively. The enzyme preparations were then incubated with purified Src in 50 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl and 5 mM KCl for 30 minutes. 10 µM ouabain (dissolved in H₂O) was added as indicated for 5 minutes reaction, followed by addition of 2 mM Mg²⁺/ATP. The reaction continued for 10 minutes, and preparation was subjected to Western blot analysis. Src Tyr 418 phosphorylation was probed with antibody against pY418 Src (Invitrogen, Carlsbad, CA), and activated Src was represented as a ratio of Tyr 418-phosphorylated Src over total Src. A representative Western blot is shown, and quantitative data are presented as mean ± s.e.m. of at least three independent experiments. **, p<0.01.

The purified Na/K-ATPase was first pretreated with inhibitors which could also stabilize the E2 conformation like ouabain. The pre-bound Na/K-ATPase was then incubated with purified Src to assess its activity. Fluoride compounds (BeF₃⁻ and AlF₄⁻) have been shown to stabilize the Na/K-ATPase in E2 form (Missiaen et al. 1988; Danko et al. 2004). Specifically, the BeF₃⁻ forms a covalent bond with Asp, which represents the ground state, Na/K-ATPase E2 ~ P; while Na/K-ATPase with AlF₄⁻ mimics the transition state, Na/K-ATPase E2-P. The Na/K-ATPase fixed by fluoride compounds indeed elevated Src activity to the same extent as ouabain, reflected by an increase in Src Tyr 418 phosphorylation (Fig. 15B) (Ye et al. 2011).

On the other hand, Na/K-ATPase inhibitors which stabilize the pump at E1 form were also tested. For example, NEM is known to react with the sulfhydryl groups of Na/K-
ATPase and promote occlusion of Na\(^+\), therefore it inhibits E1P-E2P conversion (Hegyvary 1976). Na/K-ATPase was reacted with NEM (dissolved in H\(_2\)O) as described under “Material and Methods”. In contrast to ouabain, phosphorylation of Src Tyr 418 was completely inhibited (Fig. 16A) when the purified Na/K-ATPase was incubated with NEM (Ye et al. 2011).

The effect of oligomycin, another E1 stabilizer but with less potency compared to NEM (Hegyvary 1976; Taylor 1981), on Src activity was also determined. As illustrated in Fig. 16B, indicated amount of oligomycin (dissolved in same volume of ethanol) was added to the bottom of the eppendorf tube (same volume of ethanol was used for control sample) (Ye et al. 2011). The eppendorf tube was then air-dried, and purified Na/K-ATPase as well as the reaction buffer was added for further incubation. Na/K-ATPase/Src incubated in the absence of Na\(^+\) and K\(^+\) was used as positive control in this case. Oligomycin exhibited a dose-dependent inhibition of Src activity. It is important to mention that these compounds do not interfere with Src activity by themselves under the same experimental conditions (Fig. 16C) (Ye et al. 2011).
Disruption of Coordinated Domain Movements Alters Na/K-ATPase-Mediated Src Regulation

The above findings suggest the α1 Na/K-ATPase conformation-transitional effect on Src. As illustrated in Fig 14, the coordinated domain movements occurring during the E1/E2 conformation transition seems important in regulating Src activity, therefore this hypothesis is further tested by disturbing the domain movements through proteolysis. In the presence of Na⁺, chymotrypsin cleaves E1 Na/K-ATPase at Leu 266 in the A domain, producing an 83 kDa fragment (Fig. 17A) (Ye et al. 2011). While the 83 kDa peptide retains the ability to form a ouabain-sensitive phosphoenzyme intermediate (EP), the chymotrypsin cleavage disrupts the coordinated movements of A and N domains, inhibits
the transition from E1 to E2, and results in complete inhibition of ATPase activity (Jorgensen and Andersen 1988; Jorgensen 1992; Liu et al. 1996). As expected, while Na\(^+\) (lane 1) and ouabain (lane 2) were able to stimulate the intact \(\alpha_1\) Na/K-ATPase-associated Src, they failed to do so after more than 75% of \(\alpha_1\) Na/K-ATPase was digested by chymotrypsin (Fig. 17B) (Ye et al. 2011). Moreover, less digestion of \(\alpha_1\) Na/K-ATPase by chymotrypsin resulted in a partial inhibition of ouabain-induced Src activation (Fig. 17C) (Ye et al. 2011).

**Figure 17 Effects of Chymotrypsin and Trypsin Digestion on \(\alpha_1\) Na/K-ATPase-Mediated Src Regulation**

Fig. 17 A, generation of 83 kDa fragment by chymotrypsin. Purified Na/K-ATPase was digested by chymotrypsin in the presence of 10 mM Na\(^+\) as described under “Materials
A representative preparation was Coomassie blue-stained, showing that a majority of full length α1 is converted to 83 kDa fragment. The similar preparations were used for the experiments presented in B. B, α1 Na/K-ATPase was subjected to chymotrypsin or trypsin digestion. The enzyme preparation was incubated with purified Src in 50 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl in the presence or absence of 10 μM ouabain, and subjected to Src autophosphorylation assay. The non-digested enzyme was used as control. Src Tyr 418 phosphorylation was probed using Western blot with antibody against pY418 Src (Invitrogen, Carlsbad, CA), and activated Src was represented as a ratio of Tyr 418-phosphorylated Src over total Src. C, time-dependent effect of chymotrypsin on ouabain-induced stimulation of Na/K-ATPase-associated Src. Na/K-ATPase was subjected to chymotrypsin digestion as in A for different time periods as indicated. Afterwards, the control and digested preparations were incubated with purified Src for Src autophosphorylation assay as in B. D, effects of chymotrypsin and trypsin-digested Na/K-ATPase on total Src. Chymotrypsinized or trypsinized α1 Na/K-ATPase was incubated with purified Src in Tris-HCl buffer as in B for 30 minutes. Total Src is probed by Western blot with antibody against c-Src (Santa Cruz Biotechnology, Santa Cruz, CA). A representative Western blot is shown, and quantitative data are presented as mean ± s.e.m. of at least three independent experiments. *, p<0.05; **, p<0.01.

Employing the same idea, the E2 Na/K-ATPase was digested by trypsin in the presence of K⁺, and subjected to the same assay. Unlike chymotrypsin, trypsin cleaves E2 Na/K-ATPase at Arg 438 in the N domain, producing a 48 kDa fragment that retains the capability of forming EP (Carilli et al. 1982; Jorgensen and Andersen 1988). However, disruption of the coordinated movements of the A and N domains by trypsin was equally effective in inhibiting Na⁺ and ouabain-induced activation of Src as chymotrypsin digestion (Fig. 17B). It is important to mention that the protease-digested α1 Na/K-ATPase was washed and then incubated with Src in the presence of trypsin-chymotrypsin inhibitor. Under these conditions, no detectable digestion of Src by proteases was observed (Fig. 17D) (Ye et al. 2011).

**Construction of Cell Lines Stably Expressing I279A and F286A α1 Na/K-ATPase Mutants**
The above *in vitro* cell-free studies suggest that α1 Na/K-ATPase may regulate Src in a conformation-dependent manner. To seek further evidence, several stable cell lines expressing either E1 or E2 α1 Na/K-ATPase mutant were generated. Based on studies from Vilsen’s lab, I279A has decreased $K_{\text{0.5}}(\text{ATP})$ and $E_2(K_2)$ percentage, and increased $K_{\text{0.5}}(\text{vanadate})$ and $K^+$ deocclusion rate; while F286A has decreased $K_{\text{0.5}}(K^+)$, $K_{\text{0.5}}(\text{vanadate})$, $K^+$ deocclusion rate and E1P/E2P ratio, as well as increased $E_2(K_2)$ percentage, E1P→E2P conversion rate, E2P dephosphorylation rate and $K_{\text{0.5}}(\text{ATP})$. Therefore, according to the Albers-Post scheme, I279A affects the conformational change of the dephosphoenzyme, specifically, it stimulates the E2→E1 step, and accumulates a higher extent of E1-like conformation (E1) compared to the wild type; while F286A affects both phosphoenzyme and dephosphoenzyme conversion, and accumulates more E2-like conformation (E2/E2P, mainly E2 considering the increased E2P dephosphorylation rate) (Toustrup-Jensen and Vilsen 2003a). Moreover, these studies have also shown that these two mutants produce more pronounced kinetic changes than most of the other mutants, and both mutations are in the third transmembrane domain (Toustrup-Jensen and Vilsen 2002; Toustrup-Jensen and Vilsen 2003a; Toustrup-Jensen and Vilsen 2003b; Toustrup-Jensen and Vilsen 2005).

Similar to the creation of A to P mutants as mentioned in “Literature”, the two mutants were generated and transfected into α1 knock-down PY-17 cells. While transfection of PY-17 cells with F286A mutant, like wild-type α1 (Liang et al. 2006), resulted in numerous clones, I279A mutant only produced three clones in the presence of ouabain. In general, the level of F286A mutant expression appeared to be higher than that of I279A mutant in the rescued cells as revealed by Western blot (Fig. 18A). Among the three I279A clones, clone number 3 (I279A-3) expressed the highest amount of
mutant α1 (Fig. 18A). Further analysis of cell lysates from different clones revealed that the expression of mutant α1 in I279A-3 cells was similar to that in clone number 19 (F286A-19) from F286A mutant transfected cells (Fig. 18B). Moreover, the total α1 amount in these cells was comparable to that in control AAC-19 cells (Fig. 18C).

**Figure 18** Generation of I279A and F286A Mutant α1 Cell Lines

Fig. 18 Total cell lysates were harvested, separated by SDS-PAGE and analyzed by Western blot with specific rat α1 Na/K-ATPase antibody anti-NASE (Texas Tech University, Lubbock, TX) (A and B), or total α1 antibody α6F (the University of Iowa, IA) and α-tubulin antibody (Sigma-Aldrich) (C). A representative Western blot is shown, and the quantitative data are mean ± s.e.m. from at least three experiments. *, p<0.05;**, p<0.01.

Consistently, confocal imaging analysis showed comparable expression of membrane α1 in all three rescued cell lines (Fig. 19A). Surface biotinylation analysis done by my co-worker Fangfang Lai confirmed that the ratio of surface biotinylable α1 to total α1 was similar among these cell lines (Fig. 19B).
Figure 19 Expression of Na/K-ATPase α1 and β1 in Mutant Cells

Fig. 19 A, cells were cultured on coverslips, and immunostained with polyclonal anti-α1 antibody (Upstate, Lake Placid, NY), showing that the majority of expressed α1 (both α1 mutant and endogenous) are in/close to the plasma membrane. B, ratio of biotinylable α1 Na/K-ATPase over total α1 Na/K-ATPase in indicated cell lines. Cells were biotinylated as described under “Materials and Methods”. The same volume of bound fraction and total cell lysates was analyzed by Western blot with anti-α1 antibody α6F (the University of Iowa, IA), and the quantitative data are mean ± s.e.m. from at least three experiments. C, the expression of β1 in different cell lines was detected by Western blot with anti-Na/K-ATPase β1 (Upstate, Lake Placid, NY). A representative Western blot is shown.

Na/K-ATPase Activity in I279A and F286A Mutant-Rescued Cells

To characterize the function of mutant Na/K-ATPase, the expression of β1 subunit was checked. As shown in Fig. 19C, expression of either mutant was able to rescue the expression and glycosylation of β1 as did wild-type α1. These findings indicate that the expressed mutant α1 is fully capable of assembling with the β1 subunit into a functional Na/K-ATPase, and rescuing the membrane expression of α1 Na/K-ATPase.
To assess the pumping capability of these mutants, ouabain-sensitive ATPase activity in crude membrane preparations made from different cell lines was measured. As depicted in Table 1, Na/K-ATPase activity in I279A-3 and F286A-19 cells was about half of that in AAC-19 cells, respectively. It is important to note that both mutant-rescued cell lines not only expressed more α1 Na/K-ATPase, but also exhibited much higher Na/K-ATPase activity than that of PY-17 cells (about 24% of the AAC-19 cells). To further test the pumping capacity of these rescued cell lines, ouabain-sensitive $^{86}\text{Rb}^+$ uptake activity was assessed. As shown in Table 1, I279A-3 and F286A-19 cells exhibited 49% and 53% ouabain-sensitive $^{86}\text{Rb}^+$ uptake activity in comparison to that of AAC-19 cells, respectively.

| Table 1 Ouabain-Sensitive Na/K-ATPase Activity and $^{86}\text{Rb}^+$ uptake Activity in Cells |
|-----------------------------------------------|-----------------------------------------------|
| Cell lines                                   | AAC-19 | I279A-3 | F286A-19 |
| Pump Activity / α1 (%) , n≥3                  | 100    | 42±15   | 52±6     |
| Rb$^+$ Uptake Activity / ng protein (%) , n≥3 | 100    | 49±4    | 53±3     |

To verify that the E1/E2 transition was indeed altered in the mutant-rescued cells, vanadate-dependent Na/K-ATPase activity was measured. Vanadate preferentially binds to E2($K_2$) and blocks the $K^+$ deocclusion. Therefore, the sensitivity to vanadate-induced inhibition of ATPase activity will indicate the relative amount of E2 accumulated in the whole preparation. Compared to AAC-19 cell, vanadate titration curve in Fig. 20 exhibited a reduced vanadate affinity in I279A-3 cell, indicating that I279A indeed poised E1/E2 balance to E1 as reported (Toustrup-Jensen and Vilsen 2003a). By contrast,
the vanadate affinity in F286A-19 cells increased, suggesting that F286A accumulating more E2.

**Figure 20 Vanadate-Sensitive Na/K-ATPase Activities of the Wild-Type and the E1/E2 α1 Na/K-ATPase**

![Graph showing vanadate-sensitive Na/K-ATPase activities](image)

Fig. 20 Crude membrane fraction was prepared and assayed for ouabain-sensitive Na/K-ATPase activity as described under “Material and Methods” in the presence of indicated concentrations of vanadate. The data points are shown as percentage of Na/K-ATPase activity in the absence of vanadate. The values are mean from at least three independent experiments.

Taken together, the above findings indicate that the expression of mutants restore total cellular α1 Na/K-ATPase in I279A-3 and F286A-19 cells to the level comparable to that in AAC-19 cells, and that the expressed α1 Na/K-ATPase mutants display a defect in E1/E2 conformational transition, resulting in an inhibition of pumping activity in these cells in comparison to that in AAC-19 cells. However, the pumping activities in the I279A-3 and F286A-19 cells were much higher than that of parental PY-17 cells.

**Regulation of Src and Src Effectors by Mutant α1**

The *in vitro* data mentioned above suggests that α1 Na/K-ATPase regulates Src in a conformation-dependent manner. If this is true and if α1 Na/K-ATPase is a major
regulator of Src in LLC-PK1 cells, Src activity would be expected to be different in I279A-3 and F286A-19 cells although they express the similar number of Na/K-ATPase, and possess similar pumping activity. Indeed, when the activity of Src was measured by Western blot analysis, basal Src activity in I279A-3 cells was about half of that in F286A-19 cells (Fig. 21A). There was no detectable difference in total Src in these two cell lines. To verify the immunoblot findings and to visualize the distribution of active Src in these cells, I279A-3 and F286A-19 cells were stained with anti-pY418 antibody. As depicted in Fig. 21B, more active Src resided in the plasma membrane area in F286A-19 cells than that in I279A-3 cells.

To further assess whether the expression of I279A mutant could indeed inhibit cellular Src, Src activity was compared among Na/K-ATPase knockdown PY-17, rat α1-rescued AAC-19, and I279A mutant-rescued I279A-2 and I279A-3 cells. Consistent with the previous report (Liang et al. 2006), knockdown of α1 Na/K-ATPase increased basal Src activity in PY-17 cells and expression of wild-type rat α1 reduced basal Src activity in AAC-19 cells (Fig. 21C). Similarly, expression of I279A mutant α1 could also reduce cellular Src activity in a mutant α1 amount-dependent manner. Moreover, while similar amount of α1 was expressed in I279A-3 and AAC-19 cells, cellular Src activity was significantly lowered in I279A-3 cells, suggesting that the accumulation of I279A mutant in the E1-like state may be more effective in keeping cellular Src in an inactive state. This notion was further supported by the fact that while I279A-2 cells expressed less α1 (about 60% of I279A-3 cells, Fig. 19B) than that of AAC-19 cell; it had the same basal Src activity as that in AAC-19 cells (Fig. 21C).
Figure 21 Regulation of Basal Src Activity by the Expressed Mutants

Fig. 21 A, total cell lysates collected from F286A-19 and I279A-3 cells were separated by SDS-PAGE and analyzed by Western blot with antibody against Src pY418 (Invitrogen, Carlsbad, CA) and c-Src (Santa Cruz Biotechnology, Santa Cruz, CA). A representative Western blot is shown, and the quantitative data are mean ± s.e.m. from at least three independent experiments. *, p<0.05. B, cells were cultured on coverslips, and immunostained with anti-Src pY418 antibody (Invitrogen, Carlsbad, CA). Representative images from three separate experiments are shown. C and D, total cell lysates collected from different cell lines were separated by SDS-PAGE and analyzed by Western blot with antibody against Src pY418 (Invitrogen, Carlsbad, CA) and c-Src (Santa Cruz Biotechnology, Santa Cruz, CA). The quantitative data are mean ± s.e.m. from at least three independent experiments. **, p<0.01 vs PY-17; #, p<0.01 vs AAC-19.

When the same set of experiments were conducted to assess F286A mutant-mediated regulation of Src activity, the basal Src activity in F286A-19 cells was significantly reduced compared to that in PY-17 cells (Fig. 21D). Moreover, there was no statistical difference in Src activity between F286A-19 and AAC-19 cells although on average it was a bit higher in F286A-19 cells. To be sure, the same experiment was repeated in
F286A-11 cells that express less F286A mutant α1. As shown in Fig. 21D, basal Src activity in F286A-11 cells was similar to that in PY-17 cells but much higher than that in AAC-19 cells.

Taken together, the above data indicate that I279A α1, an E1-like mutant, produce more inhibition of cellular Src activity than wild-type α1. On the other hand, the F286A α1, an E2-like mutant, appears to be less effective in inhibiting cellular Src. Because I279A-3 and F286A-19 express similar levels of rat α1, have the same pumping capacity, but different regulation of cellular Src activity, the following studies were conducted mainly in these cell lines to assess how expression of mutant α1 Na/K-ATPase defective in conformational transition affect Src-mediated signal transduction.

Expression of I279A and F286A Mutants Alters Ouabain-Induced Signal Transduction

Ouabain is a specific agonist of the α1 Na/K-ATPase/Src complex (Tian et al. 2006). It is also an important constituent of the α1 Na/K-ATPase mediated signaling pathway. Binding of ouabain to α1 Na/K-ATPase stimulates Src within min and increases the expression of α1 Na/K-ATPase in hours in LLC-PK1 and rescued AAC-19 cells (Liang et al. 2006; Li et al. 2009). To assess whether inhibition of E1 to E2 transition is sufficient to alter ouabain-induced signal transduction, same experiment was repeated in I279A-3 cells. As depicted in Fig. 22A, in contrast to what we observed in AAC-19 cells (Liang et al. 2006), up to 100 μM ouabain failed to stimulate Src in I279A-3 cells. Similarly, the same concentration of ouabain showed no effect on ERK and α1 Na/K-ATPase amount in I279A-3 cells (Fig. 22A and 22B). Because ouabain binding affinity was reduced in
I279A mutant (Toustrup-Jensen and Vilsen 2003a), the above defect in ouabain-induced signal transduction could be overcome by adding more ouabain if I279A mutant was capable of binding and forming a functional receptor with Src. To test this possibility, I279A-3 cells were exposed to 1 mM ouabain for 10 min and Src activity was measured. Based on the published data (Toustrup-Jensen and Vilsen 2003a), 1 mM ouabain should produce more than 50% inhibition of I279A mutant, comparable to the dose of 100 μM ouabain in AAC-19 cells. As depicted in Fig. 22C, 1 mM ouabain produced significant stimulation of Src in I279A-3 cells. Taken together, I279A mutant appears to be able to process ouabain-induced signal transduction in renal epithelia cells.

To compare and contrast, ouabain stimulation experiment was also repeated in F286A-19 cells. Unlike I279A-3 cells, no stimulation of Src by ouabain was observed even up to 1 mM in F286A-19 cells (Fig. 23A and 23C). Although it is unlikely, to be sure that the signal transduction in general was not compromised in F286A-19 cells, EGF (epidermal growth factor) signaling pathway was assessed in the cells. As shown in Fig. 22D, EGF was able to stimulate ERK in both PY-17 and F286A-19 cell lines. These findings suggest that F286A mutant is less able to assemble with Src into a functional receptor. This is consistent with the fact that F286A mutant was less effective in inhibiting the basal Src than I279A mutant (Fig. 21A and 21B).
Figure 22 Effects of Ouabain and EGF on Mutant Cells

Fig.22 A, cell were treated with indicated concentration of ouabain for 10 minutes. Cell lysates were collected and subjected to Western blot analysis with antibody against Src pY418 (Invitrogen, Carlsbad, CA) and c-Src (Santa Cruz, CA), as well as pERK (Santa Cruz Biotechnology, Santa Cruz, CA) / total ERK (Santa Cruz, CA). B, cells were treated with indicated concentration of ouabain for 24 hours, collected and probed for total α1 expression by Western blot with antibody against total α1 (α6F) (the University of Iowa, IA). C, cells were treated with 1mM ouabain for 10 minutes, harvested and probed for Src pY418 (Invitrogen, Carlsbad, CA) / total Src (Santa Cruz, CA) by Western blot. D, confluent cells were serum starved for 12 hours and treated with EGF (10ng/ml) for 2 minutes. Total cell lysates were separated by SDS-PAGE and analyzed by Western blot for pERK (Santa Cruz Biotechnology, Santa Cruz, CA) and total ERK (Santa Cruz Biotechnology, Santa Cruz, CA). A representative Western blot is shown, and the quantitative data are mean ± s.e.m. from at least three independent experiments. *, p<0.05; **, p<0.01.

Expression of Mutant α1 Produces a Partial Restoration of Caveolin-1 Expression and Allows Ouabain to Activate Akt
It has been shown that knockdown of α1 Na/K-ATPase increases endocytosis and degradation of caveolin-1 (Cai et al. 2008b). Because caveolae plays an important role in ouabain-induced signal transduction, the amount of caveolin-1 was measured in different cell lines. As depicted in Fig. 23A, while wild-type α1 could fully rescue the expression of caveolin-1 in AAC-19 cells, both I279A and F286A mutants only produced a partial restoration of caveolin-1 expression in I279A-3 and F286A-19 cells. This result suggests that changes in caveolin-1 expression could not account for the difference between I279A-3 and F286A-19 cells in response to ouabain.

To further compare and contrast I279A-3 and F286A-19 cells, the effect of 1 mM ouabain on Akt stimulation was assessed in these cell lines. As depicted in Fig. 23B, ouabain was able to activate Akt in both cell lines. However, the effect of ouabain on Akt stimulation was more pronounced in I279A-3 than that in F286A-19 cells.

**Figure 23 Effects of Mutant α1 on Caveolin-1 Expression and PI3K/pAkt Involved Signaling Pathway**

Fig. 23A, confluent cells were serum-starved and harvested. Total cell lysates collected from different cell lines were separated by SDS-PAGE and analyzed by Western blot
with anti-caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-α-tubulin (Sigma-Aldrich) (loading control). B, I279A-3 and F286A-19 cells were treated with 1 mM ouabain for 10 minutes, harvested and analyzed by Western blot with antibody against pAkt (Cell Signaling, Danvers, MA) and total Akt (Cell Signaling, Danvers, MA). A representative Western blot is shown, and the quantitative data are mean ± s.e.m from at least three independent experiments. **, p<0.01.

Expression of I279A and F286A Mutants Attenuates the Ability of Cells to Regulate Cellular Functions Using Src-Mediated Signal Transduction

Src is known to play an important role in cell attachment and spreading (Sabe et al. 1994; Schaller et al. 1994; Webb et al. 2004). Because the expression of I279A α1 Na/K-ATPase inhibits cellular Src activity and attenuates the ouabain-mediated signaling pathway, it is tempting to test whether I279A affects signal transduction pathway activated by cell attachment. Plating of AAC-19 cells caused a significant activation of Src and FAK in 60 min. However, when I279A-3 cells were plated, no activation was observed (Fig. 24A). With the help of my co-worker Lai, the time-dependent cell spreading assay was conducted, and consistently, it was found that expression of I279A greatly slowed down cell spreading (Fig. 25). For example, 45% inhibition in spreading was observed at 4 hour time point.

Interestingly, when the same experiments were done in F286A-19 cells where basal Src was higher than that in I279A-3 cells, cell spreading was also inhibited, but to a less degree (Fig. 25). For example, only 30% inhibition was recorded in F286A-19 after 4 hour of plating. Moreover, cell attachment-induced activation of Src and FAK was also inhibited in F286A-19 cells as did in I279A-3 cells (Fig. 24B). It is important to note that not every kinase pathway associated with cell attachment was inhibited in mutant-
expressing cell lines. When ERK was probed, the activation of ERK was preserved in all three cell lines (Fig. 24C).

**Figure 24 Effects of Mutant α1 on Integrin-Induced Signaling Pathway**

Fig. 24 A, B and C, cells were plated and collected as described under “Material and Methods”. Total cell lysates were separated by SDS-PAGE and analyzed by Western blot with antibody against Src pY418 (Invitrogen, Carlsbad, CA)/total Src (Santa Cruz Biotechnology, Santa Cruz, CA), pY576/577 FAK (Cell Signaling, Danvers, MA)/total FAK (Santa Cruz Biotechnology, Santa Cruz, CA), and pERK (Santa Cruz Biotechnology, Santa Cruz, CA)/total ERK (Santa Cruz Biotechnology, Santa Cruz, CA). A representative Western blot is shown, and the quantitative data are mean ± s.e.m. from at least three independent experiments. *, p<0.05; **, p<0.01.
**Figure 25 Inhibition of Cell Spreading by the Expression of Mutant α1**

Fig. 25 Cells were plated on 60 mm dishes for indicated time and the phase-contrast images were photographed as described under “Material and Methods”. The number of spread cells was counted, and the values are mean ± s.e.m. from at least three independent experiments. *, p<0.05 vs AAC-19; #, p<0.05 vs I279A-3.

**Expression of I279A and F286A Mutants Inhibits Cell Growth**

Since Src-mediated pathways are known to play an important role in cell proliferation (Thomas and Brugge 1997), cells were cultured in full medium and the number of cells was counted at different time points. As shown in **Fig. 26A**, expression of either F286A or I279A caused a significant inhibition of cell growth. Moreover, when compared between the two mutant-expressing cell lines, the growth of I279A-3 cells was much slower than that of F286A-19 cells. To exclude the possibility that the inhibition is due to the defect in pumping activity, cell growth of PY-17 cells was also measured. As depicted in **Fig. 26A**, even F286A-19 cells grew slower than PY-17 cells. Moreover, the proliferation rate of I279A-2 cells, which expressed less I279A, was higher than that of I279A-3 (**Fig. 26B**).
Figure 26 Effects of Mutant α1 Expression on Cell Growth

![Graph A](image1)

Fig. 26 A, growth curves of different cell lines. AAC-19, PY-17, I279A-3 and F286A-19 cells were plated in 12-well plates (20,000 cells/well), cultured for different time periods as indicated, then collected and counted as described under “Material and Methods”. The values are mean ± s.e.m. from four independent experiments. *, p<0.05 vs F286A-19 cells. B, comparison of I29A-2 cell growth with that of I279A-3 cells. The values are mean ± s.e.m. from at least three independent experiments. *, p<0.05; **, p<0.01.

Part II Exploration of the Ligands of Na/K-ATPase/Src Receptor Complex

Identification of K⁺ as a Ligand for Na/K-ATPase/Src Complex

The substrates of the Na/K-ATPase, such as Na⁺, K⁺ and ATP, are known to alter the balance of E1/E2 Na/K-ATPase. To verify the above findings, as well as to explore the physiological relevance of this conformation-dependent regulation of α1 Na/K-ATPase-associated Src, purified Na/K-ATPase/Src complex was incubated in the presence of different ions to induce the accumulation of certain conformation, and ionic effect on Src activity was assessed. For example, physiologically, the cellular Na/K-ATPase is exposed to around 15 mM intracellular Na⁺ and 5 mM extracellular K⁺. Under these ionic conditions, most Src was inactive. Lowering K⁺ from 5 mM to 1 mM produced a robust stimulation of Src (Fig. 27A) (Ye et al. 2011). It is important to mention that although the
concentration of Na\(^+\) and K\(^+\) is identical on both extracellular and intracellular sides in the purified enzyme, the internal and external ionic effects can be distinguished based on the different binding preferences of Na\(^+\) and K\(^+\). Cytoplasmic site displays a much higher affinity for Na\(^+\) over K\(^+\), and extracellular site vice versa (Robinson and Flashner 1979).

**Figure 27 Effects of K\(^+\) on Src**

A. The α1 Na/K-ATPase was incubated with purified Src in the presence of different ligands for 30 minutes, followed by addition of 2 mM Mg\(^{2+}\)/ATP. The reaction continued for 10 minutes, and the preparation was subjected to Western blot analysis and assayed for Src Tyr 418 phosphorylation with anti-pTyr 418 (Invitrogen, Carlsbad, CA). Representative Western blots are shown and quantitative data are presented as mean ± s.e.m. of at least three independent experiments. *, p<0.05. B, LLC-PK1 cells were exposed to the normal (K\(^+\) 5 mM) or low K\(^+\) (K\(^+\) 1 mM) medium for 12 hours, and stained by anti-pTyr 418 antibody (Invitrogen, Carlsbad, CA). The scale bar represents 20 μm. C, LLC-PK1 cells were exposed to medium containing 5 mM or 1 mM K\(^+\) for 12 hours, and cell lysates were subjected to Western blot analysis with antibody against Src pY418 (Invitrogen, Carlsbad, CA)/total Src (Santa Cruz Biotechnology, Santa Cruz, CA), and pERK (Santa Cruz Biotechnology, Santa Cruz, CA)/total ERK (Santa Cruz Biotechnology, Santa Cruz, CA). Kinase activity was presented as a ratio of phosphorylated protein over total protein, and quantitative data are presented as mean ± s.e.m. of at least three independent experiments. *, p<0.05; **, p<0.01.
Because changes in Na\(^+\)/K\(^+\) concentration could also alter the Na/K-ATPase activity and ATP/ADP concentration in the test tube, the extracellular K\(^+\) effect was verified in live cells. As depicted in Fig. 27B, confocal imaging analysis indicated that lowering K\(^+\) from 5 mM to 1 mM significantly increased active Src in both peripheral and intracellular compartments in LLC-PK1 cells (Ye et al. 2011). This low K\(^+\) induced Src activation was confirmed by my co-worker Dr. Li with immunoblot showing a 70% increase in Src activity (Fig. 28C) (Ye et al. 2011). Moreover, ERK, a Src downstream effector, was also found to be activated to the similar level (Fig. 27C) (Ye et al. 2011). Like ouabain, this up-regulation of ERK by low K\(^+\) was derived from Na/K-ATPase/Src complex-mediated EGFR signaling pathway, as suggested by the abolishment of induction by pNaKtide, a specific Na/K-ATPase/Src inhibitor discovered in this lab, as well as by the inhibitor of EGFR (AG1478) (Fig. 28A) (Ye et al. 2011).

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**Figure 28 Regulation of Src and ERK by low K\(^+\)**

Fig. 28 A, LLC-PK1 cells were exposed to medium containing 5 mM or 1 mM K\(^+\) for 12 hours, and in the presence of 2 μM pNaKtide or 1μM AG1478 as indicated. Cell lysates were subjected to Western blot analysis with antibody against pERK (Santa Cruz Biotechnology, Santa Cruz, CA)/total ERK (Santa Cruz Biotechnology, Santa Cruz, CA). B, control cells (P-11), Na/K-ATPase α1 knock-down cells (PY-17), and Na/K-ATPase α1-rescued cells (AAC-19) were exposed to medium containing 5 mM or 1 mM K\(^+\) for
12 hours, and cell lysates were subjected to Western blot analysis with antibody against Src pY418 (Invitrogen, Carlsbad, CA)/total Src (Santa Cruz Biotechnology, Santa Cruz, CA). Kinase activity was presented as a ratio of phosphorylated protein over total protein, and quantitative data are presented as mean ± s.e.m. of at least three independent experiments. *, p<0.05; **, p<0.01.

To further test this low K\(^+\)-induced activation, the above experiment was also performed in LLC-PK1 derived cell lines expressing different amount of α1 Na/K-ATPase. As expected, in PY-17 cells which contain little signaling Na/K-ATPase, low K\(^+\) response was abolished (Fig. 28B) (Ye et al. 2011). However, Src induction by low K\(^+\) was restored in AAC-19 cells as shown in Fig. 28B (Ye et al. 2011). The above experiments suggest that low K\(^+\) could regulate Na/K-ATPase/Src complex.

**Identification of Na\(^+\) as a Ligand for Na/K-ATPase/Src complex**

Because Na\(^+\) is another substrate of Na/K-ATPase, its effect on Src activity is also of interest to test. Similarly, purified Na/K-ATPase/Src complex was incubated in the presence of various Na\(^+\) concentrations (Fig. 29A and B). In comparison to choline (the organic ion employed to maintain ionic strength), Na\(^+\) was more potent in converting E2 to E1 in the presence of ATP (Skou and Esmann 1983). Accordingly, addition of 150 mM Na\(^+\) produced a significant inhibition of Src in the presence of either 0 mM or 5 mM K\(^+\) (Fig. 29A and B).
Figure 29 Regulation of \(\alpha_1\) Na/K-ATPase-Associated Src by Na\(^+\) in the Purified Preparations

Fig. 29 A and B, \(\alpha_1\) Na/K-ATPase was incubated with purified Src in the presence of different ligands for 30 minutes, followed by addition of 2 mM Mg\(^{2+}\)/ATP. The reaction continued for 10 minutes, and the preparation was subjected to Western blot analysis and assayed for Src Tyr 418 phosphorylation with anti-pTyr 418 (Invitrogen, Carlsbad, CA). Representative Western blots are shown and quantitative data are presented as mean ± s.e.m. of at least three independent experiments. **, p<0.01.

The Na\(^+\) effect was further studied in cell culture. LLC-PK1 cells were incubated in medium containing different Na\(^+\) concentration in the presence of monensin. Monensin is a Na\(^+\) ionophore derived from streptomyces cinnamonensis, and can clamp the intracellular Na\(^+\) concentration to the same level as the extracellular concentration (Harootunian et al. 1989). As depicted in Fig. 30A, when intracellular Na\(^+\) was increased by addition of monensin to the culture medium, a stimulation of Src was detected. This was in accordance with the accumulation of E2-Na/K-ATPase under this experimental condition. Another way to increase intracellular Na\(^+\) concentration is to stimulate Na\(^+\) influx by activating the Na\(^+\)-dependent glucose transporter (Schultz and Zalusky 1964). As depicted in Fig. 30B, LLC-PK1 cells were cultured in Transwell plates until they formed a monolayer. Upon addition of 10 mM D-glucose from the apical side, Src activity was up-regulated. However, increasing intracellular Na\(^+\) failed to stimulate
cellular Src in Na/K-ATPase knock-down PY-17 cells, suggesting that the effects were α1 Na/K-ATPase specific.

The extracellular Na⁺ effect was also examined in LLC-PK1 cells. It is known that reducing extracellular Na⁺ would favor the formation of E1 Na/K-ATPase due to its competition with K⁺ at the extracellular binding site (Beauge and Adragna 1971). Indeed, lowering extracellular Na⁺ from 150 mM to 15 mM caused a reduction in cellular Src activity (Fig. 30C) (Ye et al. 2011).

**Figure 30 Regulation of α1 Na/K-ATPase-Associated Src by Na⁺ in Cells**

Fig. 30 A, confluent LLC-PK1 cells were incubated in medium containing indicated Na⁺ concentration in the presence of 10 μM monensin for 10 minutes. B, cells were cultured in Transwell plates for 5-6 days until they formed a monolayer. The cells were then incubated in glucose-free medium for 10 minutes, and 10 mM glucose was added back to the cells from the apical side. Cell lysates were collected 5 minutes after the addition of glucose. C, LLC-PK1 cells were incubated in medium containing either 150 mM Na⁺ or 15 mM Na⁺ plus 135mM choline chloride. After indicated treatment, cell lysates were subjected to Western blot analysis of Src Tyr418 phosphorylation with antibody against...
Identification of Cholesterol as a Ligand for Na/K-ATPase/Src Complex

The above data suggest that α1 Na/K-ATPase-associated Src could be regulated by substrate-induced conformational changes. To further test this hypothesis and explore more potential ligands, other factors which are known to modulate the balance of E1/E2 were tested. It has been shown that removal of cholesterol promotes the E2-Na/K-ATPase (Cornelius 2008). To test whether the cholesterol effect on Na/K-ATPase conformation balance will affect Src activity, LLC-PK1 cells was treated with Methyl-β-cyclodextrin (Mβ-CD), a direct cholesterol extractor, in order to decrease the cholesterol content (Fig. 31A) (Chen et al. 2011). Concomitantly, Src was stimulated as a function of time (Fig. 32B) (Chen et al. 2011). These effects were fast with significant changes being detected within 5 minutes. Repletion of cholesterol prevented Src activation, suggesting that the effect of Mβ-CD on Src was mediated by the reduction in cholesterol (Fig. 31D) (Chen et al. 2011). Consistently, exposure of LLC-PK1 cells to U18666A, an intracellular cholesterol transport inhibitor which could also reduce the plasma membrane cholesterol content but with a slower kinetics (Lange et al. 1998; Lange et al. 2002), induced Src activation after 30 minutes (Fig. 31C) (Chen et al. 2011). To be sure that the Src stimulation was through α1 Na/K-ATPase, the same experiment was repeated in PY-17 cells. As shown in Fig. 31E, after Mβ-CD treatment, Src induction was not detected in PY-17 cells which contains little signaling Na/K-ATPase. However, in rescued AAC-19 cells, this stimulation was restored (Fig. 31E). It is important to mention that the effect of
Na⁺, K⁺ and cholesterol could be a result of altered ouabain binding by these factors, not necessarily the E1/E2 states at least in cultured cells in view of the recent study by Lichtstein’s lab (Dvela et al. 2012).

Figure 31 Regulation of α1 Na/K-ATPase-Associated Src by Cholesterol in Cells
Fig. 31 A and B, LLC-PK1 cells were treated with 10mM Mβ-CD for different times. Cholesterol content in the cell lysates from different time points were measured, adjusted to protein level, and compared (A). Representative Western blots are shown on the levels of pY418 Src (Invitrogen, Carlsbad, CA) and c-Src (Santa Cruz Biotechnology, Santa Cruz, CA) (B). C, LLC-PK1 cells were treated with 10g/ml U18666A for different times. Representative Western blots are shown on the levels of pY418 Src (Invitrogen, Carlsbad, CA) and c-Src (Santa Cruz Biotechnology, Santa Cruz, CA). D, LLC-PK1 cells were treated with 10mM Mβ-CD or 10mM Mβ-CD/0.1mM cholesterol for 10 min. Representative Western blots are shown on the levels of pY418 Src (Invitrogen, Carlsbad, CA) and c-Src (Santa Cruz Biotechnology, Santa Cruz, CA). E, AAC-19 and PY-17 cells were treated with 10mM Mβ-CD for 5 minutes. Quantitative data are presented as mean ± s.e.m. of at least three independent experiments. *, p<0.05; **, p<0.01.
Discussion

The recent studies on the interaction between α1 Na/K-ATPase and Src have greatly expanded the understanding of the non-pumping function of Na/K-ATPase. Since the discovery of low concentration of ouabain induced hypertrophy in myocytes (Peng et al. 1996), α1 Na/K-ATPase has been shown to integrate multiple proteins, i.e., IP3R, PLC-γ, PI3K, caveolin-1, etc., and form a signalosome which is involved in regulation of intracellular Ca^{2+}, trafficking of caveolae and cholesterol, as well as cell proliferation (Xie et al. 1999; Liu et al. 2000; Yuan et al. 2005; Liu et al. 2007b; Cai et al. 2008b; Chen et al. 2008; Chen et al. 2009). Moreover, through the association with Src in caveolae, α1 Na/K-ATPase converts the ouabain binding to activation of Src, which further amplifies the signal to multiple down-stream pathways through protein phosphorylation. Recently, it has been shown that two pairs of direct intermolecular interactions between Src and α1Na/K-ATPase contribute to the formation of ouabain-regulatory α1 Na/K-ATPase signalosome (Tian et al. 2006). Moreover, one of the binding site has been successfully mapped down to a 20 amino acid peptide (NaKtide) which is localized at the N domain of α1-Na/K-ATPase (Li et al. 2009).

Regarding to the significance of α1 Na/K-ATPase-mediated signaling, it is important to further explore the molecular mechanism of how α1 Na/K-ATPase interacts and regulates Src. So herein, utilizing cell lines expressing α1 mutant in which the α helix in NaKtide is disrupted showed that the helical structure of NaKtide was an important determinant in inhibition of Src. Moreover, results from both in vitro cell-free assays as well as cell models implied that the activity of Src was regulated by its coupled α1 Na/K-ATPase in a conformation-dependent manner. Specifically, E1-Na/K-ATPase inhibited
Src while E2-Na/K-ATPase might be less effective. These findings made it possible to further assess the importance of Src-regulatory function of α1 Na/K-ATPase. Finally, the recognition of the α1 Na/K-ATPase conformation-dependent regulation of Src led to the exploration of multiple potential ligands of this receptor complex.

**Na/K-ATPase Acts as a Src Inhibitor**

Low concentration of ouabain has been found to induce hypertrophic growth in rat neonatal myocytes by stimulating multiple growth-related signaling pathways including Ras/Raf/MEK/MAPK cascade (Kometiani et al. 1998; Xie et al. 1999). Further analysis implies that Src may be the most proximal kinase in this ouabain-induced Ras/MAPK pathway. For example, Src is found to be coimmunoprecipitated with α1 Na-K/ATPase in a ouabain-regulatory manner (Haas et al. 2002). In LLC-PK1 cells, confocal imaging shows that α1 Na/K-ATPase co-localizes with Src in the plasma membrane (Li et al. 2009). This close proximity between α1 Na/K-ATPase and Src is further confirmed by FRET analysis (Tian et al. 2006). Moreover, GST pull-down assay indicates that the association between α1 Na/K-ATPase and Src is specific through two intermolecular interacting pairs: one is between Src SH2 domain and Na/K-ATPase CD2 domain, and the other is between Src kinase domain and Na/K-ATPase N domain (Li et al. 2009). GST pull-down assay further showed that the CD2/SH2 interaction had an even higher affinity than that of the N domain/kinase domain interaction (Fig. 13A). The specific region involved in this intermolecular interaction is still under investigation.

Src activity is positively correlated with Tyr 418 phosphorylation, whereas it is inhibited by Tyr 529 phosphorylation (Williams et al. 1997; Xu et al. 1999). Interestingly, ouabain increases Tyr 418 phosphorylation without affecting the phosphorylation of Tyr
529 in cell culture (Haas et al. 2002). This mode of regulation is verified in cell-free system (Tian et al. 2006). Recently, Li et. al reported that NaKtide derived from the Na/K-ATPase N domain inhibited Src Tyr 418 phosphorylation without affecting Tyr 529 phosphorylation (Li et al. 2009). Therefore, ouabain and Na/K-ATPase represent another important regulation mechanism of Src instead of Tyr 529 phosphorylation.

Functionally, α1 Na/K-ATPase appears to be an important inhibitor of Src in live cells. This is supported by several lines of evidence. First of all, when α1 Na/K-ATPase is specifically knocked down by Si-RNA, a stimulation of basal Src activity is observed (Liang et al. 2006). Rescuing of PY-17 cells by wild-type rat α1 or pump-null mutant rat α1 restores basal Src activity (Liang et al. 2006). In vitro cell-free assay with purified α1 Na/K-ATPase and Src also confirms this inhibitory effect (Tian et al. 2006). Further analysis suggests that this inhibition is mainly due to α1 Na/K-ATPase N domain / Src kinase domain association because GST-ND construct is as effective as intact full length α1 in inhibiting Src (Tian et al. 2006). Moreover, addition of Src kinase domain to compete for binding is sufficient to activate the pump-associated Src (Tian et al. 2006). This inhibitory binding site has been pinpointed to a 20 amino acid peptide (NaKtide) located at the N terminus of the N domain (Li et al. 2009). pNaKtide, a cell permeable NaKtide, inhibits Src in α1 Na/K-ATPase knockdown PY-17 cells, but not parental LLC-PK1 cells (Li et al. 2009).

To further understand the importance of α1 Na/K-ATPase in Src regulation, it is necessary to study the molecular mechanism of how NaKtide interacts with Src kinase domain. In vitro mutagenesis studies from my co-workers indicate that disruption of the helical structure by A to P mutation reduced the inhibitory effect of NaKtide on Src.
Therefore, several stable cell lines expressing A420P and A425P mutant α1 were generated accordingly. As shown in Fig. 6 and 7, expression of A420P and A425P restored the membrane α1 amount to the level comparable to that in AAC-19 cells. Moreover, they could assemble with β1 subunit (Fig. 7C) to form a functional Na/K-ATPase which retained the similar pumping activity as wild-type α1 as demonstrated by $^{86}$Rb$^+$ uptake assay (Fig. 8A) and vanadate-dependent Na/K-ATPase activity assay (Fig. 8B). However, these mutants were unable to effectively inhibit Src as intact α1 (Fig. 9A and B). These data suggest that the helix in NaKtide is an important structural determinant in associating with Src and inhibiting its activity. Moreover, these mutant-expressed cell lines provide an important tool to study the signaling function of α1 Na/K-ATPase without interference with the pumping function.

**Na/K-ATPase regulates Src in a conformation-dependent manner**

The idea of α1 Na/K-ATPase conformation-dependent regulation of Src originates from early studies which suggest that ouabain could regulate the two interacting pairs differently: ouabain binding releases the Src kinase domain from α1 Na/K-ATPase N domain in both cell-free system and live cells, whereas the association between SH2 domain and CD2 domain remains intact (Tian et al. 2006). The dissociation of kinase domain from Na/K-ATPase seems to be essential for Src activation (Tian et al. 2006; Li et al. 2009). However, the overall binding affinity between Src and α1 Na/K-ATPase remains unchanged (Tian et al. 2006), indicating that the CD2/SH2 association may be constant during the whole process, and may facilitate the formation of Na/K-ATPase/Src complex. This is consistent with the GST pull-down assay showing that the CD2/SH2 interaction had a higher affinity than that of the N domain/kinase domain interaction (Fig.
Combing the crystal structures of Src, Na/K-ATPase, and SERCA (Xu et al. 1999; Toyoshima et al. 2004; Cowan-Jacob et al. 2005; Morth et al. 2007), it is therefore speculated that Src associates with E1-like Na/K-ATPase through two interacting pairs; while Src kinase domain is released from the Na/K-ATPase in an E2-like conformation due to the rotation of the A domain and the inward movement of the N domain. This speculation is also consistent with the finding that no inhibitory effect of Src on Na/K-ATPase activity was detected even at a 1:1 molecular ratio (Fig. 13B). Therefore, we hypothesized that Src might be regulated by the conformational transition of α1 Na/K-ATPase (Fig. 14).

To experimentally test this hypothesis, the Na/K-ATPase was treated with E1 stabilizer (NEM/oligomycin) and E2 stabilizer (BeF$_3$ and AlF$_4$). Although all of these compounds inhibit Na/K-ATPase activity, they produced an opposite effect on Src activity (Fig. 15 and 16). In line with the hypothesis, E1-Na/K-ATPase inhibited Src while E2-Na/K-ATPase stimulated it. Moreover, two mutant α1 cell lines whose Na/K-ATPase is defective in conformation transition were generated (Fig. 18) (Toustrup-Jensen and Vilsen 2003a). Both I279A and F286A mutants could rescue the membrane expression of α1 Na/K-ATPase (Fig. 19A and B). They could also assemble with β1 subunit to form a functional pump, although the overall Na/K-ATPase activity was reduced based on the Na/K-ATPase activity assay and $^{86}$Rb$^+$ uptake analysis (Table 1). The reduction is expected because both mutants are defective in conformational transition as determined by the vanadate - sensitive Na/K-ATPase activity assay (Fig. 20). Similar to the in vitro findings, I279A-rescued cells displayed a much lower Src activity than F286A cells (Fig. 21A and B).
Moreover, Src stimulation by either ouabain or Na$^+$ was abolished in chymotrypsinized or trypsinized Na/K-ATPase (Fig. 17). Therefore, coordinated domain movements during E1 to E2 transition may be essential in activating Src. This notion is further supported by the findings presented in Fig. 22A and B showing that ouabain-induced Src activation was altered in mutant-rescued cells.

It is not so sure how to model this α1 Na/K-ATPase conformation-dependent regulation of Src based on the new findings. However, this E1/E2 mode of regulation could provide cells a dynamic switch of Src activity, which is important for certain cellular processes. For example, during adhesion process, Src is stimulated and then repressed. Constant activation of Src (i.e., v-Src) is associated with cell detachment (Sabe et al. 1994; Fincham et al. 1995). Therefore, defects in conformational transition are likely to cause disordered dynamic Src regulation, which could lead to changes in Src/FAK signaling (Fig. 26), and consequently cell attachment/spreading (Fig. 27), as well as proliferation (Fig. 28).

It is important to mention that there is about four to five fold more α1 Na/K-ATPase than that of Src in renal epithelial cells (Fig. 4). Most of α1 Na/K-ATPase resides in the plasma membrane interacting with Src. Therefore, as discussed above, α1 Na/K-ATPase could represent another major mechanism of Src regulation in addition to control of Tyr 529 phosphorylation, especially in the compartment of plasma membrane. This is consistent with the previous publications (Liang et al. 2006; Tian et al. 2006; Li and Xie 2009) and with the new findings presented in Fig. 9, 10, 21 and 22.

Uncertainties
A recent publication showed that regulation of Src by purified α1 Na/K-ATPase in the cell-free system was related to changes in ATP/ADP ratio instead of a direct α1 Na/K-ATPase/Src interaction (Weigand et al. 2012). It is known that Src autophosphorylation (both Tyr 418 and Tyr 529) is regulated by ATP concentration (Osusky et al. 1995). While low μmol of ATP is required for Tyr 418 phosphorylation, high μmol ATP promotes more phosphorylation of Tyr 529. The possibility of ATP exhaustion exists in the cell-free experiments (Figs. 15, 17C, 27A and 29) as suggested by Weigand et. al (Weigand et al. 2012) could explain the effect of ouabain or Na/K-ATPase on Src activity instead of the E1/E2 model proposed here. Therefore it would be important to determine the ATP amount under these experimental conditions.

However, the uncertainties in the cell-free experiments mentioned above could not rule out the direct interaction between α1 Na/K-ATPase and Src based on the studies from fragments (GST-ND1 and NaKtide) (Tian et al. 2006; Li et al. 2009) and cells (Liang et al. 2006). Moreover, literature shows that the majority of cellular ATP is consumed by Na/K-ATPase in kidney epithelial cells, and ATP content is correlated to the pumping activity of Na/K-ATPase (i.e., inhibiting Na/K-ATPase will reduce ATP level) (Whittam and Willis 1963; Balaban and Mandel 1980; Mandel and Balaban 1981; Soltoff and Mandel 1984). Therefore, ATP concentration might not be a critical factor in regulation of Src by α1 Na/K-ATPase in cells, which is consistent with the fact that Src is activated by ouabain in cultured cells (Liang et al. 2006).

The same publication (Weigand et al. 2012) also showed that inhibition of Na/K-ATPase by vanadate was sufficient to stimulate Src, which was in contrast to what was
reported by Tian (Tian et al. 2006). The reason for this discrepancy is not known. If α1 Na/K-ATPase associated Src was regulated by the conformational transition of Na/K-ATPase as proposed in Fig. 14, vanadate should stimulate Src as Weigand reported (Weigand et al. 2012). If vanadate fails to stimulate Src as reported by Tian (Tian et al. 2006), it would indicate that the vanadate-bound E2 form differs from ouabain-bound E2 form and is capable of binding and inhibiting Src kinase should the Na/K-ATPase and Src interact to form a receptor complex as proposed in Fig. 14. Therefore, it is important to clarify the effects of vanadate on Src in the near future.

Although the evidence, as discussed in the above paragraphs, on a direct Na/K-ATPase/Src interaction is strong, indirect interaction is still a possibility because of the following uncertainties. First, the so-called purified Na/K-ATPase is not pure and it contains lipids and many proteins including Src. Second, most of the binding assays performed in this lab are conducted with expressed protein fragments such as His-kinase domain and His-SH2/SH3 domain. Moreover, some of these experiments are done in the presence of detergent which may promote the interaction (Fig. 13). Third, FRET analysis and immunoprecipitation have limitations in detecting direct potential protein interaction.

Moreover, experimental data are insufficient to describe the kinetic properties of this E1/E2 mode of regulation. The apparent turnover rate of Src is ranged from 26 to 744 per min (Boerner et al. 1996), much slower than that of α1 Na/K-ATPase, which is 8,000-10,000 cycles per minute for purified enzyme and 1,500-5,000 cycles per minute in cultured cells or in isolated renal tubules (Lamb and McCall 1971; Pollack et al. 1981; El Mernissi and Doucet 1984). Although it is difficult to experimentally demonstrate due to
the limitation of techniques, it is inferred that the on/off of Na/K-ATPase-associated Src is determined by the time when Na/K-ATPase is in E2-like conformation during each pumping cycle. Therefore, the overall signal strength will be determined by the number of Na/K-ATPase/Src units in E2-like conformation at a given time point, which could be influenced by the expression levels of Na/K-ATPase and Src as well as the cellular distribution of these proteins.

Furthermore, F286A mutant cells produced a phenotype somehow different from what we expected according to the working model. For example, it did not significantly elevate the cellular Src activity compared to the wild-type α1 (Fig. 21D). Moreover, it did not respond to ouabain stimulation as I279A mutant did (Fig. 22C). The exact mechanism causing these defects is not clear. It is reported that F286A mutant has an increased conversion rate from E2P to E2(K₂) (Toustrup-Jensen and Vilsen 2003a), which implies that F286A may stay longer in E2(K₂). Therefore the specific kinetic step where F286A mutant is altered is different from the E2-ouabain form which may give the highest stimulation of Src. Actually, in the E2P:ouabain crystal structure, the A domain rotates about 10° to the N/P domain compared to the (K₂)E2P:ouabain structure (Ogawa et al. 2009; Yatime et al. 2011), which suggests that in the E2P:ouabain state the three cytoplasmic domains form the most compact configuration. Therefore, the E2P:ouabain conformation has the least possibility to accommodate the two intermolecular interactions simultaneously, and may be the optimal conformation in terms of Src activation. This possibility could also explain the stimulation of Src by ouabain after Na/K-ATPase was pretreated with vanadate (Tian et al. 2006). As discussed above, more enzymatic studies on vanadate are needed in order to resolve these important issues.
According to the literature, a number of mutations in α1 subunit affecting E1/E2 equilibrium have been identified (Toustrup-Jensen and Vilsen 2003a; Toustrup-Jensen and Vilsen 2003c; Toustrup-Jensen and Vilsen 2003b; Vilsen and Toustrup-Jensen 2003; Einholm et al. 2005; Toustrup-Jensen and Vilsen 2005). Due to the complex kinetic properties of α1 Na/K-ATPase mutants we used, it is important to test other mutants to verify the above findings and to identify mutant-specific defects in signal transduction. It has been suggested that mutations in α2 and α3 Na/K-ATPase may contribute to neuronal diseases such as FHM2 (familial hemiplegic migraine type 2), sporadic hemiplegic migraine, rapid-onset dystonia parkinsonism and bipolar disorder (Segall et al. 2005; Rodacker et al. 2006; Clapcote et al. 2009; Morth et al. 2009; Einholm et al. 2010; Kirshenbaum et al. 2011a; Kirshenbaum et al. 2011b; Schack et al. 2012). Interestingly, several mutants identified in FHM2 patients such as R202Q and T263M shift E1/E2 transition in favor of E1 as illustrated by the lower E1P→E2P conversion rate and the accumulation of more E1 over E2 (Schack et al. 2012). Moreover, R689Q and M731T have also been found to favor E1 form as evidenced by their insensitivity to vanadate (Segall et al. 2005). Although the fundamental pathological mechanism for FHM2 has not been clarified, the E1/E2 mode of regulation implies that changes in E1/E2 equilibrium by these mutations not only affect the pumping but also the signaling function of Na/K-ATPase. Needless to say, whether α2 Na/K-ATPase in the brain shares signaling function as α1 Na/K-ATPase in the kidney needs to be experimentally tested.

Na/K-ATPase has been suggested to exist as a dimer, tetramer or even higher oligomer forms, and the formation of these oligomers also affect the E1/E2 conformational transition. For example, some reports suggest that higher oligomer form
(dimer or tetramer) prefers E2 conformation (Hayashi et al. 1997; Kobayashi et al. 2007). We have not looked into the effect of oligomer formation on Src activity yet. But it is of interest to study whether any physiological or pathophysiological conditions are related to the formation of higher oligomer, and how Src will be affected under these conditions. This will add another layer of complexity to α1 Na/K-ATPase/Src signaling. Moreover, Src activity is regulated by cellular phosphatases. The effect of Na/K-ATPase on tyrosine phosphatases needs further investigation.

**Does the E1/E2 Transition Affect the Signaling Function of α1 Na/K-ATPase Other than Src?**

As mentioned before, Na/K-ATPase functions as a signal integrator by recruiting Src as well as other protein partners, including IP3R, PI3K, caveolin-1, and ankyrin (Jordan et al. 1995; Yuan et al. 2005; Zhang et al. 2006; Cai et al. 2008b). It is important to address the issue whether the interaction between Na/K-ATPase and Src affects and regulates the formation and function of the Na/K-ATPase signalosome. Moreover, does this E1/E2-dependent mode of regulation also apply to other signaling partners? As shown in **Fig. 23A**, both I279A-3 and F286A-19 could not fully restored caveolin-1 expression, which suggests that caveolin-1 activity is not directly dependent on the conformation of α1 Na/K-ATPase. However, the failure to restore caveolin-1 indicates that the disregulation of Src by mutant α1 may affect the phosphorylation and trafficking of caveolin-1, as well as the formation of caveolae. On the other hand, as shown in **Fig. 12** and **23B**, ouabain could induce Akt activation in A416P/A420P and I279A-3/F286A-19 cells as it did in other epithelial cells or myocytes (Aizman et al. 2001; Liu et al. 2007b). However, the extent of stimulation appeared lower in A420P/F286A-19 cells
than that in A416P/I279A-3 cells. This partial activation in A420P/F286A-19 cells may be due to a cross-talk between PI3K/Akt pathway and Src/Ras/ERK pathway as suggested by Liu (Liu et al. 2007b). More experiments are needed to address these issues. Regarding to the cellular signaling pathways other than α1 Na/K-ATPase, no effects were observed when mutant α1 was transfected, as evidenced by the unaltered EGF signaling cascade shown in Fig. 22D. This indicates a specific role of α1 Na/K-ATPase in the overall cellular signaling picture.

**Ligands of the Na/K-ATPase/Src Receptor Complex: Pathophysiological Implication**

As discussed above, there are many uncertainties regarding to the model of α1 Na/K-ATPase-mediated regulation of Src. However, it is important to propose this E1/E2-dependent Src regulation model for directing further study. Moreover, the equilibrium between E1/E2 states in live cells is affected by many cellular factors in addition to ouabain. For example, it is known that increase in intracellular Na\(^+\) or decrease in extracellular K\(^+\) promotes more E2-like state as evidenced by the increased apparent ouabain binding affinity (Akera and Ng 1991). Therefore it is of interest to test the effect of ions on Src regulation. As shown in Fig. 27, consistent with the hypothesis, reducing extracellular K\(^+\) produced more E2-Na/K-ATPase, and stimulated Src and its downstream effectors. This ionic regulation seems to be specific to α1 Na/K-ATPase/Src complex, because it was blocked by pNaKtide (Fig. 28A) or abrogated in PY-17 cell (Fig. 28B). Similarly, changes in intracellular or extracellular Na\(^+\) were found to modulate Src activity (Fig. 29 and 30). For example, increasing intracellular Na\(^+\) through addition of monensin or glucose elevated Src activity (Fig. 30A and B). Besides the substrates of
Na/K-ATPase, cholesterol also affects the conformational conversion of Na pump (Cornelius 2008). Accordingly, it exerted similar effect on Na/K-ATPase-associated Src. Removal of cholesterol accumulated more E2-Na/K-ATPase and stimulated Src activity in a time-dependent manner (Fig. 31B and C). This effect was counteracted by restoration of cholesterol amount or abrogated by reduction of the signaling α1 Na/K-ATPase (Fig. 31D and E).

Therefore, α1 Na/K-ATPase/Src complex may be capable of sensing ouabain, intracellular (e.g., Na\(^+\) or cholesterol) and extracellular (e.g., K\(^+\)) cues, and converting the conformational transition of α1 Na/K-ATPase to the regulation of its associated Src. This mode of regulation could have important physiological and pathophysiological relevance as discussed below if it turned out to be true.

First of all, because the activation/inhibition of protein kinases is essential for regulating activity and trafficking of many membrane transporters (Schultz 1981), the Src-coupled α1 Na/K-ATPase receptor could be responsible for the coordination of transporter activities (pump/leak coupling) across the cell membrane. The pump/leak coupling is an intrinsic property of cells to maintain cellular ionic strength, as well as to prevent the over burst/shrinkage of the cell. The importance of pump/leak coupling was recognized even before the identification of Na/K-ATPase (Krogh 1946). Hoffman and Tosteson explained this concept using the red blood cell as a model (Hoffman and Tosteson 1971). This pump/leak coupling is now well documented in epithelial cells as well as in other types of cells (Schultz 1981). For example, expression of basolateral α1 Na/K-ATPase and apical NHE3 is coordinated in renal epithelial cells through a Src-dependent pathway (Cai et al. 2008a). Changes in the expression or activity of Na/K-
ATPase are also correlated with the expression and activity of apical and basolateral K\(^+\) channels (Dawson and Richards 1990; Wang 2004). Moreover, K\(^+\) deficiency stimulates renal Src activity \textit{in vivo}, resulting in a decrease in surface expression of ROMK (renal outer medullary potassium channel) (Lin et al. 2004; Wang 2004).

Second, new steady states of E1/E2 balance would contribute to increased/decreased Src activity under certain pathological conditions such as tissue ischemia, hypo/hyperkalemia and hypo/hypernatremia, as well as neurodegenerative disease like Niemann-Pick disease type C1 (NPC1). Indeed, Src is found to be activated in ischemia, hypokalemia, high salt intake and NPC1 (Akera and Ng 1991; Garver et al. 1999; Sinha et al. 2003; Wang 2004; Liu et al. 2011; Blaustein et al. 2012).

Third, the Src-coupled α1 Na/K-ATPase receptor could explain the complexity and the physiological interplay among endogenous CTS, extracellular K\(^+\) and intracellular Na\(^+\) and cholesterol. Specifically, any deviation from the steady state of E1/E2 balance could affect the receptor function, which can be triggered by changes in any of these ligands and enhanced or antagonized by the other ligands. For example, low extracellular K\(^+\) would work in concert with the increase in intracellular Na\(^+\), favoring the formation of E2 Na/K-ATPase, which in turn could enhance ouabain binding, further stabilizing the E2 conformation and consequently maximizing the activation of Src. Similarly, this type of feed-forward regulation could be initiated by the binding of CTS to Src-coupled α1 Na/K-ATPase. Needless to say, whether this newly described signaling mechanism occurs under pathological or physiological conditions \textit{in vivo} needs further investigation.
Conclusions

1. The α1 Na/K-ATPase may regulate its coupled Src activity in an E1/E2 conformation-dependent manner.

2. Factors which modulate the E1/E2 equilibrium (intracellular Na\(^{+}\), cholesterol, extracellular K\(^{+}\)) can serve as ligands for α1 Na/K-ATPase/Src complex.
Summary

It has been suggested that α1 Na/K-ATPase possesses both pumping and signaling functions. Here, a unique interaction between α1 Na/K-ATPase and Src is presented. In the cell-free experiments it is found that α1 Na/K-ATPase may interact with and regulate Src in a conformation-dependent manner: specifically, E1-Na/K-ATPase (stabilized with NEM or oligomycin) inhibits Src while E2-Na/K-ATPase (stabilized with fluoride compounds or ouabain) activates it. To further test the α1 Na/K-ATPase conformation-dependent regulation of Src and Src-related signal transduction in cell culture, several stable cell lines that express dominantly (over 90%) mutant α1 Na/K-ATPase defective in conformational transition are generated. While I279A (E1-like) and F286A (E2-like) mutants have resulted in the same degree of pump inhibition, they produce opposite effects on cellular Src activity, supporting the hypothesis that α1 Na/K-ATPase can regulate Src in a conformation-dependent manner. This kind of regulation is different from the C-tail phosphorylation regulatory mechanism of Src. Moreover, this is the first demonstration that E1/E2 transition defective mutants can affect both pumping and signaling functions of Na/K-ATPase. In accordance, ouabain-induced Src activation is altered. While mM concentration of ouabain is required to activate Src in I279A cells, F286A cells fail to respond to ouabain stimulation. The expression of these mutants also abrogates integrin-induced activation of Src and FAK, as well as inhibits cell spreading and growth, with I279A being the most effective. Furthermore, binding of Na⁺, K⁺ and cholesterol to the Na/K-ATPase is known to alter its conformation. Accordingly, Na⁺, K⁺ and cholesterol binding-induced conformational change of Na/K-ATPase affects Src and Src effectors.
Taken together, the study proposes a α1 Na/K-ATPase conformation-dependent Src regulatory mechanism, which enables α1 Na/K-ATPase to be a key player in dynamic regulation of cellular Src and Src mediated pathways. These findings are significant since they imply that the capability of normal conformational transition is essential for both pumping and signaling functions of α1 Na/K-ATPase. Moreover, α1 Na/K-ATPase could serve as a broad receptor mechanism for other ligands of Na/K-ATPase (such as salt) to signal through Src. Finally, the identified E1/E2 mutants will allow us to further assess this newly appreciated signaling mechanism in animal physiology.
References


and Na-K-ATPase: implication for regulation by FXYD proteins."

"The Npc1 mutation causes an altered expression of caveolin-1, annexin II and protein
kinases and phosphorylation of caveolin-1 and annexin II in murine livers."


"Ouabain stimulates a Na+/K+-
ATPase-mediated SFK-activated signalling pathway that regulates tight junction

13-55.

sodium flux through the sodium pump: evidence for allosteric effects of

Goldin, S. M. (1977). "Active transport of sodium and potassium ions by the sodium and
potassium ion-activated adenosine triphosphatase from renal medulla.
Reconstitution of the purified enzyme into a well defined in vitro transport

factor receptor in the signal-transducing function of Na+/K+-ATPase." J Biol

between the Na+/K+-ATPase and the epidermal growth factor receptor relays the


100


