A model for domain-specific regulation of src kinase by alpha-1 subunit of Na/K-ATPase

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A Dissertation

titled

A Model for Domain-Specific Regulation of Src kinase by alpha-1 subunit of Na/K-ATPase

by

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

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An Abstract of

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Our previous results indicate that alpha-1 (α1) subunit of Na/K-ATPase interacts with Src kinase via two separate domains. While the nucleotide binding domain of α1 interacts with the Src kinase domain, the second cytosolic domain (CD2) binds the Src SH2 domain. The following studies were conducted to assess the functionality of this putative CD2/SH2 interaction in cell culture. LLC-PK1 cells were transfected with either YFP or YFP-CD2. We found that expressed CD2 can bind to Src kinase in cells and prevent Src from being targeted to different signaling complexes. CD2 was found to act as a Src SH2 domain ligand in cells, increasing its basal activity but impairing its signaling function. As a consequence, Src mediated signaling pathways like those activated by ouabain and fibronectin, were inhibited. Furthermore, CD2 expression resulted in inhibition of cell spreading and proliferation, both of which require the involvement of Src.

Next, we found that CD2 is phosphorylated at tyrosine (Y260) located in its C terminal region. Since Src SH2 domain has a preference for binding to phosphorylated tyrosines, we hypothesized that this phosphorylation may facilitate its binding to Src.
Indeed, mutation of this Y to alanine (A) reduced the growth inhibitory properties of CD2. To further characterize the importance of this Y, we generated Y260A mutant rat α1 Na/K-ATPase and expressed the mutant in a α1 knockdown cell line. This mutation abolished the ouabain mediated Src-ERK signaling function of Na/K-ATPase without hindering its ion transporting function. Co-immunoprecipitation studies revealed that the Y260A mutant is compromised in its ability to bind Src kinase as compared with its normal counterpart.

Finally we have developed two Src inhibitory peptides from CD2, spanning the Y260 region- one of which is phosphorylated at Y260 (pNaSH2) and the other is unphosphorylated (NaSH2). *In vitro* assays indicated that both the peptides were successful in preventing CD2-Src interaction, although pNaSH2 was more effective. However cell studies clearly suggested that pNaSH2 was more potent as it inhibited ouabain mediated signal transduction as well as cell spreading, far better than NaSH2.

Taken together these studies indicate a very important role for CD2 in Src kinase interaction with the Na/K-ATPase for the formation of the receptor complex. Also there are indications that the Y260 and its phosphorylation might influence the dynamics of Src regulation in cells.
I am dedicating this dissertation to my family, for their love and unconditional support throughout my career and life.
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List of Abbreviations

A.................Alanine
CD2.........................Second Cytoplasmic Domain
c-Src.........................cellular Src
C terminus...........Carboxy Terminus (of protein)
DMEM....................Dulbecco’s Minimal Eagle Medium
EGFR.......................Epidermal Growth Factor Receptor
FAK.........................Focal Adhesion Kinase
MAPK.....................Mitogen Activated Protein Kinases
pY.........................Phosphorylated Tyrosine
Na/K-ATPase..........Sodium and Potassium ion transporting ATPase
N domain..............Nucleotide Binding domain
N terminus.........Amino Terminus (of protein)
PBS.......................Phosphate Buffered Saline
ROS.......................Reactive Oxygen Species
SFK.......................Src Family Kinases
SH2........................Src Homology domain 2
SH3........................Src Homology domain 3
v-Src.......................viral Src
Y.............................Tyrosine
List of Symbols

\[ \alpha \] ................................Alpha
\[ \beta \] ...............................Beta
\[ \gamma \] .............................Gamma

\[ ^\circ C \] ..............................Degrees Celsius
Chapter 1

Introduction

Na/K-ATPase or the sodium pump is an ion transporting integral membrane protein that pumps two K\(^+\) ions in and three Na\(^+\) ions out of the cell, at the expense of one molecule of ATP (Kaplan 2002). Besides the traditional role for the pump in maintaining cellular homeostasis through regulation of ion transport, it was also discovered to have signal transducing properties (Li and Xie 2009). Many independent researchers in the last decade have shown that Na/K-ATPase, in response to binding of its ligand ouabain, can transmit signal to the nucleus through phosphorylation of various protein kinases and thereby regulate cellular properties like cell growth, proliferation, attachment and survival. One of the earliest messengers, that is activated in this pathway is Src kinase, a protein which is known to function in several growth factor receptor signaling pathways. In this lab, we identified that Src kinase can directly interact with \(\alpha_1\) subunit of Na/K-ATPase and form a receptor complex quite similar to G protein coupled receptors. Upon ouabain binding to the extracellular surface of Na/K-ATPase, the associated inactive Src kinase becomes active (by autophosphorylation). The active Src can then transactivate
EGFR, activate its effector proteins including Ras/Raf/MAPKs, stimulate production of ROS through mitochondria as well as activate several transcription factors (Xie and Askari 2002; Aizman and Aperia 2003).

We became interested in revealing the mechanism by which Na/K-ATPase interacts with and thereby regulates Src kinase. Consequently, it was found through GST pull down assays that Na/K-ATPase interacts with Src via two domain-domain interactions- the second cytoplasmic domain (CD2) of the pump binds with Src SH2 domain whereas the N domain (which is a part of the third cytoplasmic domain or CD3) interacts with Src kinase domain (Tian, Li et al. 2009). Following this discovery we were able to identify a 20 amino acid sequence from the N domain that binds and inhibits Src. This peptide was then tagged with HIV-TAT sequence, to make it cell permeable and the resulting peptide called pNaKtide became an excellent Src kinase inhibitory peptide (Li, Cai et al. 2009). Studies from this lab showed that pNaKtide is able to inhibit Src kinase activity both in vivo and in vitro and can be used to inhibit the growth of cancer cells as well as xenografted tumors (Li, Zhang et al. 2011).

Following these results, we were interested in finding the exact nature of the CD2-SH2 interaction and whether it was possible to develop a novel peptide from the CD2, which inhibits Src differentially by targeting the CD2/SH2 interaction. Our previous studies indicated that the CD2-SH2 domain interaction might be even stronger than N domain-kinase domain interaction. Furthermore, the N domain/kinase domain interaction is regulated by ouabain as well as conformational states of the Na/K-ATPase but CD2/SH2 interaction is constitutive (Ye, Li et al. 2011; Ye, Lai et al. 2013). Based on
these findings, we hypothesized that CD2 regulates the formation of Na/K-ATPase/Src receptor complex in cells.

To test this hypothesis, we expressed YFP-tagged CD2 in LLC PK1 cells and tested whether expression of CD2 inhibits ouabain-mediated signal transduction. We reasoned that if CD2 is so important in formation of this receptor complex, exogenously expressed CD2 will act as a dominant negative mutant and prevent the receptor complex formation. We found that CD2 expression reduced the formation of Na/K-ATPase-Src receptor complex, resulting in the inhibition of ouabain-induced signal transduction in LLC PK1 cells. Simultaneously, CD2 expression slowed down cell proliferation, cell spreading and also affected integrin-mediated FAK-Src signaling.

Src kinase protein being the product of the first identified proto-oncogene (Martin 2004) is quite well studied and it is widely known that its SH2 domain helps Src to target and bind to specific receptor complexes (Moran, Koch et al. 1990). Therefore it is possible that besides affecting the ouabain signaling, the CD2 may also act as a SH2 domain ligand, preventing Src to be recruited to specific signaling pathways. Therefore we tested the effectiveness of CD2 as a Src SH2 domain ligand. Moreover if we could identify the specific Src SH2 binding region in CD2, we might have another peptide inhibitor of Src that functionally inhibits the SH2 domain instead of the kinase domain. Several laboratories have shown that Src SH2 domain preferentially binds to phosphorylated tyrosine residues (Cantley, Auger et al. 1991; Koch, Anderson et al. 1991). By closely examining the CD2 sequence, we found out that it contains a single tyrosine residue at position 260. Using a Na/K-ATPase phospho-Tyrosine 260 specific antibody, we detected that CD2 was tyrosine phosphorylated in LLC PK1 cells and that
mutation of this amino acid to alanine, reduced the cell growth inhibitory properties of CD2. We therefore decided to mutate this residue to alanine in rat α1 Na/K-ATPase (the whole protein) and rescue the α1 knockdown PY17 cells with this mutant. The mutant cells were unable to activate Src/MAPK in response to ouabain stimulation. Our co-immunoprecipitation studies showed that mutant α1 Na/K-ATPase binds significantly less amount of Src than its normal counterpart. Thus the tyrosine 260 seems to be extremely important for both Src binding as well as ouabain signaling.

With this knowledge, we then sought to generate a peptide inhibitor of Src SH2 domain. Songyang (Songyang, Shoelson et al. 1993) showed that there is a consensus sequence that is most optimum forSrc SH2 domain binding- pYEEI (a phosphotyrosine followed by two aspartates and an isoleucine). The tyrosine 260 in CD2 is part of a very similar sequence which is IVVY (an isoleucine followed by two valines and then a tyrosine) although in opposite orientation. We therefore synthesized an 18 amino acid sequence peptide surrounding this region in CD2 which is phosphorylated at the tyrosine and named it pNaSH2. In order to find out the significance of the phosphoryl group on tyrosine, we also generated another peptide which is unphosphorylated and named it NaSH2. Both of these peptides contain the HIV-TAT tag, which makes them cell permeable. Our preliminary studies with these two peptides reveal that the pNaSH2 (phosphorylated) is more effective than NaSH2 in terms of inhibition of cell signaling and cell spreading. We used an in vitro peptide competition assay to determine the effectiveness of the peptides in inhibiting CD2-Src interaction. Both the peptides had inhibitory effect but pNaSH2 was significantly more potent than NaSH2.
Chapter 2

Background

Na/K-ATPase

Na/K-ATPase or Na pump is a membrane protein that functions in transporting three Na\(^+\) ions out and two K\(^+\) ions into living cells at the expense of hydrolysis of one molecule of ATP and thereby maintains cellular electrochemical gradient. Jens C. Skou first identified an ATPase enzyme from crab sciatic nerve and showed that the enzyme was activated in the presence of both Na\(^+\) and K\(^+\) ions and was inhibited by the cardiac glycoside, ouabain. This enzyme was later named as Na/K-ATPase and Skou received Nobel Prize in chemistry for its discovery (Skou 1989). Na/K-ATPase belongs to a class of ATPases called P-type ATPases. This family is named P-type ATPases because of the fact that members of this family form a phosphoryl intermediate step by transferring the terminal phosphate of ATP to a specific aspartate residue in the protein, during ATP-mediated catalysis. This aspartate residue is part of a conserved sequence of amino acids namely D-K-T-G-T-[LIVM]-[TIS], which is present among all the family members (Palmgren and Nissen 2011). The most famous among these family members are
probably Na/K-ATPase, SERCA (Sarcoplasmic Reticulum Calcium pump) and gastric H/K-ATPase. It has been shown that despite having very low sequence similarity, all of these ion pumps share a high degree of three dimensional structural similarity (Bublitz, Poulsen et al. 2010).

The Na/K-ATPase is a heterodimeric integral membrane protein that consists mainly of two subunits- an alpha (α) subunit of approximately 100kDa molecular mass and a beta (β) subunit of about 50kDa. In certain cell types a third subunit called gamma (γ) subunit has also been identified which belongs to the FXYD class of proteins. The α subunit is the major functional and catalytic subunit, and contains the binding site for the cations, ATP as well as ouabain (a cardiotonic steroid that binds specifically to Na/K-ATPase). Structurally, α subunit comprises of ten transmembrane segments whose both ends reside inside the cytoplasm. The β subunit, which is posttranslationally modified to contain a number of glycosylated residues, is a single spanning membrane protein and mainly functions in packaging and directing the α subunit to the cell membrane (Geering 2001). Both α and the β subunit comprise of multiple isoforms- the α subunit is currently known to have at least 4 different isoforms namely α1, α2, α3 and α4. The β subunit on the other hand consists of three different isoforms- β1, β2 and β3 (Blanco and Mercer 1998). All of these isoforms have a tissue specific as well as developmental pattern of expression with α1β1 being the most predominant combination that is expressed almost everywhere including kidney. It has been shown that α2 and α3 isoforms constitute only 0.1% of total α isoform expression in kidney (Lucking, Nielsen et al. 1996). The α2
isoform is more prevalent in heart, muscle, adipocytes and brain (Lytton, Lin et al. 1985; Zahler, Brines et al. 1992; Lavoie, Levenson et al. 1997) whereas the α3 isoform is more predominant in the nervous system (Hieber, Siegel et al. 1991). The α4 isoform is only found in testis (Shamraj and Lingrel 1994). Among the β subunits, β1 was found to be most abundant in kidney whereas β2 was most abundant in brain (Martin-Vasallo, Dackowski et al. 1989). However, usually the different isoform expressions overlap and all different kinds of α and β isoform combinations can probably exist (Blanco, Koster et al. 1995; Blanco, Sanchez et al. 1995).

As mentioned before, the α subunit was found to contain ten transmembrane segments with mainly three large cytoplasmic regions- a N terminus tail of about 90 amino acids, an intracellular loop of 136 amino acids that connect transmembrane helices M2 and M3 (we call it the second cytoplasmic domain or CD2) and a rather large loop of about 434 amino acids also known as third cytoplasmic domain or CD3. An alternative system of naming these domains was created by C. Toyoshima, who first successfully described the 3D structure of SERCA using X-ray crystallography (Toyoshima, Nakasako et al. 2000). Since SERCA structure is quite similar to Na/K-ATPase similar nomenclature has also been followed by scientists in Na/K-ATPase field. According to Toyoshima, the NH2 terminal tail and second cytoplasmic domain form a domain called actuator domain or A domain. The CD3 domain on the other hand forms two domains- the P or phosphorylation domain contains the aspartate (D371) residue, which accepts the phosphate group from ATP catalysis, and N or nucleotide binding domain which binds the ATP (Toyoshima, Nakasako et al. 2000). During the ion pumping cycle the Na/K-
ATPase undergoes conformational transition between two unphosphorylated states namely E1, E2 and two phosphorylated states E1P and E2P (Siegel and Albers 1967; Post, Kume et al. 1969). Transition between these different conformational states is accompanied by large scale domain rearrangements. It has been suggested that during E2 stage the A, N and P domains together form a closely compacted cytoplasmic headpiece whereas on transition to E1 stage the A domain rotates about 100° to open up this compact structure (Toyoshima and Inesi 2004).

**Cardiotonic steroids**

It was known for centuries that certain digitalis compounds (first isolated from extract of the plant Foxglove) could improve heart tone and therefore was in use for treating cardiac dysfunction by clinicians. Digoxin and its derivatives (such as ouabain) chemically belong to a class of compounds called cardenolides. The other class of compounds, called bufadienolides are derived from toad skin like bufalin and marinobufagenin which were originally discovered from traditional Chinese medicine (Bagrov, Shapiro et al. 2009). The term “cardiotonic steroids” encompass both classes of these compounds. These cardiotonic steroids were found to bind specifically to the Na/K-ATPase and inhibit its function (Skou 1989). Structurally cardiac glycosides consist of a sugar (glycoside) and an aglycone-steroid ring. The position 17 on the steroid ring can either be occupied by an unsaturated butyrolactone (cardenolide group) or a pyrone (bufadienolide) thereby determining which group the glycoside falls into. Besides plant
and animal origin, very low levels of circulating cardiotonic steroids have also been
discovered in the mammalian body (brain, adrenal glands, cerebrospinal fluid, heart etc.)
(Tymiak, Norman et al. 1993). Hamlyn first identified the presence of a cardiotonic
steroid in human plasma which was structurally similar to ouabain (Hamlyn, Blaustein et
al. 1991). Subsequently, several independent studies reported presence of cardenolide or
bufadienolide compounds in human body or body fluids (Doris, Hayward-Lester et al.
1996; Komiyama, Nishimura et al. 1998).

**Cardiotonic steroid induced signaling by Na/K-ATPase**

In the classic mode of Na/K-ATPase function, ouabain binds specifically to Na/K-
ATPase and inhibits its ion pumping activity. This results in increased Na$^+$ retention in
the cells and can reverse the working mode of Na/Ca-exchanger, resulting in higher Ca$^{++}$
ion concentration inside the cells. This increased cation concentration can then stimulate
various Ca$^{++}$ signaling pathways and thereby play a role in regulation of cellular
physiology. However, recent studies indicated that very low concentrations of ouabain
that essentially do not inhibit the ion transporting function of a living cell, can also elicit
signal transduction pathways without changing cellular Na$^+$ or K$^+$ ion concentration (Liu,
Tian et al. 2000; Aizman, Uhlen et al. 2001). Two of these signaling pathways and their
role in cellular physiology are quite well established.

Aperia et al. found out that, low concentrations of ouabain that only partially
inhibits Na/K-ATPase can cause low level of Ca$^+$ oscillations in rat primary proximal
tubular cells (Aizman, Uhlen et al. 2001). These Ca\(^+\) oscillations were found to be due to IP3R, a calcium channel, using IP3R inhibitors. Consequently the same group established that Na/K-ATPase resides in close proximity with IP3R in a signaling microdomain and with the help of ouabain can regulate cellular Ca\(^+\) oscillations which can then activate the transcription factor, NF-κB (Miyakawa-Naito, Uhlen et al. 2003). Consequently it was found that nanomolar concentrations of ouabain can protect cells from apoptosis as well as enhance cell growth through NF-κB activation (Li, Zelenin et al. 2006). Similarly Saunders reported that nanomolar concentrations of ouabain induces low Ca\(^+\) oscillations, activate MAPK cascades and induce endothelial cells to produce endothelin-1 and ultimately affect cell proliferation without any inhibition of K\(^+\) ion uptake by the cells (Saunders and Scheiner-Bobis 2004). Work from this lab also supports a role for Na/K-ATPase in tethering IP3R and PLC\(\gamma\)1 in a calcium regulatory complex via direct interaction at the NH2 terminus and CD3 and is dependent on ouabain (Yuan, Cai et al. 2005).

Na/K-ATPase has also been found to interact with Src kinase to form a receptor complex for ouabain. Earlier findings suggested that ouabain treatment of various cells results in increased protein tyrosine phosphorylation (Kometiani, Li et al. 1998; Haas, Askari et al. 2000; Aydemir-Koksoy, Abramowitz et al. 2001). These multiple phosphorylation events were found to be dependent on activation of Src kinase, a tyrosine kinase that plays important role in several growth factor mediated signaling pathways. Src stimulates and subsequently activates various effector proteins like Ras/Raf/MAPKs, induces the transactivation of EGFR, and also drives PI3K/Akt signaling pathway in
response to ouabain (Liu, Zhao et al. 2007). A Src kinase inhibitor like PP2
downregulated these signaling pathways and ouabain failed to upregulate tyrosine
phosphorylation of proteins in a Src family knockout cell line (SYF) but showed good
response in the same cell line rescued with Src (SYF+Src) (Haas, Askari et al. 2000;
Haas, Wang et al. 2002; Tian, Cai et al. 2006). Consequently, it was found that Src kinase
directly interacts with α1 subunit of Na/K-ATPase to form a functional receptor complex
whose activation and inactivation depend on the binding of the ligand ouabain. This
interaction involved two domain- domain contacts. Specifically, the CD2 of Na/K-
ATPase associates with Src SH2 domain whereas the N domain binds kinase domain.
Upon ouabain binding, the N domain- kinase domain interaction gets disrupted, resulting
in the kinase domain of Src being released and activated by autophosphorylation at Tyr
418. The CD2-SH2 domain interaction remains intact and the phosphorylated kinase
domain can then phosphorylate effector proteins (Tian, Cai et al. 2006). It appears that
conformational transition of the Na/K-ATPase mediates this activation and inactivation
of Src, with E1 like stage of the pump being the inactive state of the receptor complex
and E2 like stage being the active state. Ouabain can fix the pump in E2-like stage and
hence mediate the signaling (Ye, Li et al. 2011; Ye, Lai et al. 2013). In fact, Xie and
colleagues first proposed that there are two pools of Na/K-ATPase on cell surface. One of
them being the classical ion pumping pool, whereas the other being the signaling Na/K-
ATPase, that forms a receptor complex with Src and resides in lipid rafts called caveolae
(Liang, Tian et al. 2007).
Besides mediating effect on cell phenotype via Ca/IP3R/Na/K-ATPase and Na/K-ATPase/ Src receptor complex, a series of studies showed that ouabain can also influence cell-cell adhesion. Cerejido et al. first elaborated this effect by administering MDCK cells with high doses of ouabain and showed that it disrupts tight junction resulting in a dramatic decrease in Trans Epithelial Resistance (TER) between cells. Whereas, low doses of ouabain increased the tightening of tight junctions and was dependent on Src/ERK signaling (Larre, Lazaro et al. 2010). Therefore ouabain was proposed to enhance cell-cell adhesion process by exerting its effect on Na/K-ATPase which itself acts as a cell adhesion molecule (Cereijido, Contreras et al. 2012). On the other hand, Na/K-ATPase β subunit was shown to play an important role in E-cadherin-mediated cell-cell attachment (Rajasekaran, Palmer et al. 2001).

Role of Na/K-ATPase signaling in different diseases

The role of dietary salt intake and development of essential hypertension is quite well documented (De Wardener, Mills et al. 1961; Johnson, Feig et al. 2008). Besides the regular role for renin-angiotensin-aldosterone and vasopressin in hypertension, a soluble factor in body fluid of patients was proposed to aide in the development of hypertension (Meneton, Jeunemaitre et al. 2005). This soluble factor was thought to be an inhibitor of Na/K-ATPase(Kaplan, Bourgoignie et al. 1974; Bricker, Schmidt et al. 1975). The discovery of cardiotonic steroids in human plasma and urine has lent support to these ideas. Currently there is large amount of research going on to correlate the presence of
endogenous cardiotonic steroids to the development of hypertension. Signaling function of Na/K-ATPase is thought to actively contribute to these scenario because the amount of circulating CTS in human plasma is in nanomolar or subnanomolar range, which is highly unlikely to inhibit the classical function of Na/K-ATPase (Bagrov, Shapiro et al. 2009). Besides, ouabain was reported to regulate Na/K-ATPase as well as Na\(^+\)/H\(^+\)-exchanger level in proximal tubular cells in a time and dose dependent manner (Liu and Xie 2010). Na/K-ATPase and Na\(^+\)/H\(^+\)-exchanger are known to coordinately regulate Na\(^+\) reabsorption in kidney. Ouabain was shown to stimulate endocytosis of \(\alpha_1\) subunit of Na/K-ATPase and Na\(^+\)/H\(^+\)-exchanger in LLC PK1 cells, a pig kidney proximal tubule cell line. This was dependent on the signaling function of Na/K-ATPase and required activation of Src and PI3K (Oweis, Wu et al. 2006; Cai, Wu et al. 2008). Thus it is possible that ouabain could regulate renal salt handling by regulating the amount of Na/K-ATPase and Na\(^+\)/H\(^+\)-exchanger on cell surface by controlling the signaling function.

There is a large body of evidence showing the alteration in level of either the Na/K-ATPase subunits or the whole sodium pump in different types of cancer (Akopyanz, Broude et al. 1991; Sakai, Suzuki et al. 2004; Mijatovic, Roland et al. 2007). One of the hallmarks of a cell becoming metastatic is, escape from its normal tissue microenvironment. A metastatic cell usually acquires its metastatic ability by loosening up its attachment with the surrounding cells. Several studies indicate the importance of Na/K-ATPase in regulating cell-cell adhesions (Contreras, Shoshani et al. 1999; Rajasekaran, Palmer et al. 2001; Rajasekaran, Hu et al. 2003). Thus it is possible that
change in Na/K-ATPase expression might enable cancer cells to acquire a more aggressive phenotype. Thus currently there is an increased interest in utilizing cardiotonic steroids for treatment of particular types of cancer including breast cancer (Stenkvist, Bengtsson et al. 1980; Stenkvist, Bengtsson et al. 1982). Clinical studies have demonstrated better survival rate among breast cancer patients treated with digitalis (Stenkvist, Bengtsson et al. 1982). Cellular studies also demonstrated that cardenolide analogs promote apoptosis of prostate cancer cells, but not normal cells and is independent of change in cellular Ca\(^+\) level (Mijatovic, De Neve et al. 2008). However one of the concerns for developing cardiac glycosides as anti-cancer therapeutics is their potential cardiac toxicity. Thus efforts are under way to identify novel Na/K-ATPase ligands for treatment of cancer. In this aspect it is important to mention the development of a peptide inhibitor of Src kinase (a protein tyrosine kinase whose activity is elevated in many forms of cancer) from Na/K-ATPase in this lab, which showed antiproliferative effect against prostate cancer cell lines and also inhibited xenografted tumor formation in mice (Li, Zhang et al. 2011).

**Src kinases – historical perspective (the discovery)**

Src family kinases (SFK) are nonreceptor tyrosine kinases that are found in all eukaryotic life forms. SFKs mediate highly specialized signaling functions and have widespread biological effects. Perhaps the most important of this 9 member family is Src kinase which is ubiquitously expressed. Peyton Rous in 1909 first discovered that cancer
may be spread by a filterable transmissible agent (virus). Although initially this idea was met with enough skepticism, it was found that the viral oncogenic properties can in fact be attributed to specific viral counterpart of normal cellular genes. This was followed by discoveries from numerous other scientists who showed that oncogenic transformation may indeed be caused by specific gene malfunctions. V-Src or the viral counterpart of Src was discovered by Brugge and Erikson in 1977, who and others also confirmed it to be a protein kinase. Based on the discovery that the gene was not significant for viral lifecycle, Varmus and Bishop proposed and consequently identified c-Src or cellular Src to be the cellular counterpart of v-Src in normal cells. Thus came the idea of proto-oncogenes, that is, cellular genes which when deregulated, can give rise to cancer (Martin 2004).

**Src kinase family-structure and function**

As mentioned before, SFKs comprise of a nine member family whose pattern of expression and function are summarized briefly in table 1. (Parsons and Parsons 2004).

Src family kinases have been implicated to regulate many fundamental cellular functions like cell division, signaling, apoptosis, attachment, migration and survival. As shown in the table above, most Src family members are expressed in cells of hematopoietic lineage. However, Src, Fyn and Yes are expressed in almost all types of cells although the level of expression may vary from cell to cell. For example, Src is expressed in very high level in
Table 1. Tissue specific distribution pattern of Src family kinases.

<table>
<thead>
<tr>
<th>Src family member</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Src</td>
<td>Ubiquitous, highest in brain, platelets and osteoclasts.</td>
</tr>
<tr>
<td>Fyn</td>
<td>Ubiquitous : brain isoform in brain, fibroblasts, endothelial cells and keratinocytes; thymocyte isoform in T and B cells.</td>
</tr>
<tr>
<td>Lyn</td>
<td>Myeloid cells, brain, B-cells</td>
</tr>
<tr>
<td>Yes</td>
<td>Ubiquitous: highest in brain, fibroblasts and endothelial cells. Also found in platelets and T cells</td>
</tr>
<tr>
<td>Yrk</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>Hck</td>
<td>Myeloid cells</td>
</tr>
<tr>
<td>Fgr</td>
<td>Myeloid cells, B cells</td>
</tr>
</tbody>
</table>
Lck | T cells, NK cells, brain
---|---
Blk | B cells

platelets and osteoclasts (Brown and Cooper 1996). This is manifested by the finding that, Src-/− knockout mice manifest only one disease phenotype which is defectiveness in bone resorption, resulting in osteoporosis. It is suggested that multiple Src kinases may be responsible for carrying out signaling function in particular pathways, and knockout of single gene such as Src is therefore usually tolerated. Multiple Src family member knockouts usually exhibit a more severe phenotype. However, lyn or lck single knockouts has been shown to severely affect immune system and brain function. For example, loss of Fyn was shown to cause neural defects, whereas Lyn and Lck was suggested to play a role in thymocyte maturation and development (Thomas and Brugge 1997).

Src family members usually exhibit highly conserved domain organization having six well defined functional domains: a short amino terminal membrane anchoring **SH4** domain, a **unique** region, a **SH3** domain which binds polyproline motifs, followed by a **SH2** domain which binds tyrosine phosphorylated residues, a **SH1/kinase** domain which catalyzes tyrosine phosphorylation and a **carboxy terminal tail** which contains a
regulatory phosphotyrosine (Cooper, Gould et al. 1986). The domain structure and function of these various domains are discussed individually below-

**SH4 domain**- The SH4 (SH stands for Src homology) domain comprises of N-terminal 15 amino acids and contains membrane localization signal. Usually there are two lipid modification sites in this region which serves to target Src kinases for membrane association. A glycine at second position usually serves as myristoylation site and in some SFKs, a palmitoylated cystiene residue also serve to tether the protein to cellular membrane. Palmitoylation signal is absent in Src and Blk. SFKs not only associate with plasma membrane but are also found in endosomal region, RER (Rough Endoplasmic Reticulum), secretory vesicles and caveloae. There have also been reports of Src and Fgr localization in nuclear region. Upon membrane localization, Src kinases usually associate with cytoplasmic tail of various growth factor receptors and proteins in signaling modules to carry out robust signaling events (Resh 1993).

**Unique region**- The unique region is named because its sequence is distinct among Src family members and is thought to confer specificity to particular Src kinases in mediating interaction with proteins unique to its signaling function. A few serine and threonine phosphorylation sites have also been identified in Src kinase, whose precise function has not been elucidated yet but may confer its specificity.

**SH3 domain**- SH3 domains are 50 amino acid long intra- and intermolecular interaction domains that are important for Src regulation as well as functioning in signaling pathways. Structurally, SH3 domains adopt a barrel shaped structure
comprising of five antiparallel $\beta$-strands and two loops namely RT and n-Src loop. The P-X-X-P (polyproline) motif recognition region is composed of several aromatic and hydrophobic residues spanned on either side by the two loops. SH3 domains in general exhibit diversity in terms of both sequence as well as binding modes, which indicates that this might be one of the ways to confer substrate binding specificity. They mediate protein–protein interactions in signaling cascades. For example, it allows the interaction of Src with its downstream effector protein Paxillin. SH3 domain also serves to maintain Src in its inactive form by interacting with a polyproline motif in the SH2-kinase linker region (Thomas and Brugge 1997).

**SH2 domain**- SH2 domains are 100 amino acid long modular protein–protein interaction domains that specifically recognize phosphotyrosine containing protein sequences (Pawson 1995; Pawson and Nash 2003). Like SH3 domains, SH2 domains also function in recognizing and helping in recruitment of Src to different signaling complexes like PDGFR and EGFR signaling complexes (Erpel and Courtneidge 1995; Thomas, Ellis et al. 1998). It also plays a dominant role in Src regulation by interacting with the C terminal tail phosphotyrosine and keeping Src in a closely compact and inactive form. Structurally SH2 domains are quite conserved with most adopting a common fold consisting of a central $\beta$-sheet region flanked by two $\alpha$-helices. Although SH2 domains exhibit a general affinity for phosphotyrosine containing sequences, higher order of specificity is achieved by the amino acid residues surrounding the phosphotyrosine residue (Waksman, Kominos et al. 1992). For example Src SH2 domain was shown to bind preferentially to the pYEEI optimal peptide sequence (Johnson, Perich et al. 1997).
The pYEEI was revealed to bind to the crystallized Src SH2 domain in two pronged socket kind of mechanism, with the phosphoTyr (pY) forming one set of interaction with arginine residues in the SH2 domain whereas the Ile (I) fits snugly into another pocket formed by hydrophobic residues. Until now, three SH2 domain mutations have been directly implicated to cause human diseases. A SH2 domain mutation in SAP (SLAM associated protein) has been shown to cause X-linked lymphoproliferative disease characterized by heightened sensitivity to Epstein-Barr virus. Also a point mutation in the Btk SH2 domain has been shown to result in X-linked agammaglobulinaemia. Thirdly, a point mutation in SH2 domain of Shp-2 has been linked with Noonan’s syndrome (Thomas and Brugge 1997).

**Kinase domain**- The catalytic/kinase domain mediates phosphate transfer reactions and thereby is responsible for its tyrosine kinase activity. The kinase domain is comprised of two lobes-a) N lobe containing five β-strands and a C helix and b) C lobe containing primarily α-helices including the activation loop (Boggon and Eck 2004). ATP is bound into a cleft between these two lobes. This bi-lobed structure of the catalytic domain is evolutionary conserved among protein tyrosine kinases. Several studies have shown that the tyrosine kinase activity is mediated by an autophosphorylation site in the kinase domain, specifically a tyrosine at position 418, also known as Tyr418/Y418. Phosphorylation of this Y418 is indicative of an active form of Src, and inactive wild type Src is dephosphorylated at this site (Parsons and Weber 1989). For example, mutational studies indicate that the autophosphorylation at Y418 is necessary for full activation of Src (Kmiecik and Shalloway 1987). The Y418 is located on a C lobe loop
called activation loop which when phosphorylated, is stabilized in an open and extended conformation. This activation loop undergoes major structural changes when the kinase is switching from an inactive to active state and vice versa. Helix $\alpha$C located in the C lobe also functions in repositioning the activation loop by making direct contact with its N terminal region. A conserved Glu 310 residue on $\alpha$C helix forms a salt bridge with Lys 295 which in turn is responsible for chelating the $\alpha$- and $\beta$- phosphates of ATP. These structural conformational changes during the active state, stabilizes the kinase domain structure and also mediates nucleotide binding. Any variation from this structurally conserved catalytic conformation results in structural collapse leading to Src inactivation (Xu, Harrison et al. 1997; Huse and Kuriyan 2002).

Most protein kinases (including serine/threonine and tyrosine kinases) typically adopt the same “catalytic scaffold” during their activation. However the mechanisms for regulating these biologically important kinases are diverse and unique to each family (Huse and Kuriyan 2002).

**Carboxy terminal tail**- The C terminus short tail contains another regulatory Tyr (Tyr 529) phosphorylation of Tyr529 keeps the Src in an inactive conformation by interacting with the SH2 domain. The SH2/phospho Tyr 529 interaction is rather weak and can be easily displaced by Protein tyrosine phosphatases like SHP-1 and PTP- $\alpha$ which dephosphorylate Y529 and stimulate Src (Xu, Doshi et al. 1999; Zheng, Resnick et al. 2000) or by competition with a SH2/SH3 domain ligand (Liu, Brodeur et al. 1993). Mutagenesis studies have shown that this low affinity interaction is determined by the
residues surrounding the phosphotyrosine. For example if the Tyr 529 and its surrounding sequence is replaced with a high affinity sequence like pYEEI, then the mutant protein can no longer be activated by exogenous SH2 ligands (Porter, Schindler et al. 2000).

Thus, domain organization of Src family kinases is primarily important in determining its activation status.

![Figure 1. Schematic diagram of domain organization and key amino acid residues of Src kinase.](image)

**Insights into activation and inactivation of Src kinases**

As mentioned before, the SH2 and SH3 domains play a major role in determining Src targeting to protein complexes as well as function in regulating Src activity. Besides that, Src also contain two regulatory tyrosine residues whose phosphorylation statuses are important in determining its activity. In the inactive form, the C terminal tail tyrosine (Tyr 529) is phosphorylated by other protein kinases, Csk (Okada 2012) and CHK. The SH2 domain interacts with this phosphorylated Tyr529 to keep the Src in a closely
compacted inactive conformation. Further level of regulation is achieved by the SH3 domain binding tightly with a short poly-proline sequence located on the SH2-kinase linker region. This interaction is rather important because interaction of SH3-polyproline linker hinders the formation of the Glu310-Lys 295 salt bridge that is absolutely necessary for the active state. It has been proposed that the SH2-pTyr 529 interaction facilitates the SH3-polyproline binding which on the other hand prevents the kinase region from becoming active by inhibiting formation of some crucial structures necessary for kinase activity (Hubbard 1999). Most of the cellular Src is in inactive form under basal conditions. Stimulation with growth factors or other stimuli like stress can recruit Src to specific signaling complexes and activate it. Src then phosphorylates protein substrates specific to that signaling complex resulting in a downstream signaling process that ultimately is manifested as phenotypic or morphological changes. Protein phosphatases like PTP-α can dephosphorylate the pTyr529 and thereby displace its interaction with the SH2 domain (Zheng, Resnick et al. 2000). As a result, the SH3-polyproline linker region constraint is removed and the αC helix can reposition itself to aid the formation of the salt bridge, help in ATP binding and the activation loop becomes primed for acceptance of phosphate group from bound ATP on the positive regulatory tyrosine (Tyr 418). Hubbard et al. 1998 proposed a trans-phosphorylation mechanism for auto-activation of tyrosine kinases (Hubbard, Mohammadi et al. 1998). In short, binding of PDGF to its receptor, results in PDGF receptor dimerization and autophosphorylation on a number of cytoplasmic tyrosine residues. Some of these phosphorylated tyrosine residues (for example pTyr 579) can serve as a recruitment site for SH2 domain of Src kinases. The stronger pTyr579-SH2 domain interaction can displace the weaker C
terminal tail pTyr-SH2 binding and remove the regulatory constraint imposed upon kinase domain. Therefore Src can then be autophosphorylated on its activation loop tyrosine by a trans-phosphorylation and become fully active (Yamaguchi and Hendrickson 1996). Thus SH2 and SH3 domain ligands can also activate Src by competing for SH2/SH3 domain binding.

**Src signaling**

**Role for Src in receptor tyrosine kinase signaling**- The first evidence that SFKs are involved in signal transduction came from the observation that Src was activated in response to PDGF stimulation of quiescent fibroblasts (Ralston and Bishop 1985). Consequently numerous studies have shown that Src kinases play an important role in transducing signal from receptor tyrosine kinases in response to growth factor stimulation including platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR) insulin like growth factor receptor (IGF-1R), colony stimulating factor (CSF), nerve growth factor receptor (NGFR) etc(Abram and Courtneidge 2000; Bromann, Korkaya et al. 2004).
Figure 2. Schematic diagram of Src kinase activation and inactivation by various mechanisms.

One of the most well characterized receptor tyrosine kinase signaling involving Src is probably the PDGF receptor. Src kinase binds to phosphorylated Tyr 579 and 861 in the justamembrane region of PDGFR in response to PDGF stimulation. However, full activation may require participation of the SH3 domain as well. Src on the other hand phosphorylates PDGFR at the C terminal part of its kinase domain (Tyr 934). Cell lines expressing a mutant form of Tyr934 (in PDGFR) was shown to exhibit reduced cell division but increased cell motility (Hansen, Johnell et al. 1996). Thus, Src appears to be important in driving PDGF induced mitogenesis and chemotaxis in cells. Besides, Src has also been proposed to function in PDGF receptor endocytosis and degradation through its
substrate Cbl (Deckert, Elly et al. 1998; Miyake, Lupher et al. 1998). In comparison, the role of Src in EGFR signaling seems to be rather ambiguous. Increased Src expression has been reported to be tightly coupled to increased EGFR expression in certain human cancers (Abram and Courtneidge 2000). Also coexpression of Src and EGFR leads to increased tumorigenesis in nude mice (Maa, Leu et al. 1995). Dominant negative mutants of Src when expressed in cells can inhibit EGF induced DNA synthesis (Wilson, Luttrell et al. 1989). All of these findings are supportive of a mechanism of direct cooperative interaction between Src and EGFR for efficient signaling. In fact, Biscardi et al. showed that c-Src can form a “heteroduplex complex” with EGFR as well as phosphorylate it at two new tyrosine residues (Biscardi, Maa et al. 1999). Similarly, there have been reports of SFK activation and ligand dependent association with FGFR (Courtneidge, Dhand et al. 1993), CSF-1R (Courtneidge, Dhand et al. 1993). Besides playing a role in ligand-induced receptor tyrosine kinase signaling, Src kinases have also been implicated in regulation of adhesion signaling like cell-cell and cell-matrix adhesion. Some of these important signaling pathways are integrin-mediated cell-matrix adhesion and cadherin-mediated cell-cell attachment, deregulations of both of which are thought to be important in tumor progression and metastasis. The integrin-mediated Src signaling would be discussed in further details in the next section.

**Role of Src in integrin and cadherin mediated cell adhesion signaling**

Attachment to the matrix and also between cells is one of the hallmarks of normal cells residing in a tissue microenvironment. Integrin, cadherin, selectin and CAMs are classes of membrane receptor molecules that mediate these kind of interactions (Thomas and
Brugge 1997). To date, Src kinase has been known to be a key mediator in some of these signaling pathways.

Integrins are a class of heterodimeric membrane receptors that help cells attach to extracellular matrices with the help of specific contact points called focal adhesions. These focal adhesions connect the cell actin cytoskeleton to the substratum and their assembly/disassembly is required for cells to undergo adhesion/proliferation/migration. Cell migration on a specific substratum is dependent on efficient focal adhesion turnover involving formation of new contacts on the projecting end and dissolution of previously existing contacts at the retracting end. Src plays a role in both the processes by helping in formation of focal adhesion through linking focal adhesion kinase (FAK) protein to various substrate proteins as well as in activating calpain, that cleaves focal adhesion associated proteins. Deregulation of FAK/Src complex has been linked to a number of metastatic cancer phenotype (Playford and Schaller 2004; Mitra and Schlaepfer 2006).

Cells in tissues form contacts with each other with the help of a number of different junctions, one of which is the cadherin-based adherens junction. Cadherin-based junctions are typically calcium dependent homophilic cell-cell adhesion joints that are also of particular importance in understanding how metastatic cells escape their micro-environment. Studies have shown that v-Src expression in cells can reduce cadherin-based adhesive forces by tyrosine phosphorylation of junctional complex proteins (Yap, Brieher et al. 1997). Src has also been implicated in phosphorylation of β-catenin, a key component of cadherin based junctions, and thereby disrupt cell-cell adhesion (Matsuyoshi, Hamaguchi et al. 1992; Hamaguchi, Matsuyoshi et al. 1993).
Since Src is known to function in both of these adhesion signaling pathways, a crosstalk between integrin and cadherin mediated signaling is also possible (Weber, Bjerke et al. 2011).

**Role of Src in cell cycle regulation** - As described earlier, Src kinase is required for growth factor-induced mitogenesis. Injection of a Src specific antibody or kinase inactive form of Src has been shown to prevent CSF-1 or EGF stimulated cell growth (Weber, Bjerke et al. 2011). The oncogenic form of c-Src, v-Src can induce cyclins D1, E and A expression via a PI3K dependent mechanism thereby enabling serum starved cells to enter S phase of cell cycle (Riley, Carragher et al. 2001). In 1993, Twamley-Stein et al. demonstrated that Src family kinases mediate PDGF-stimulated entry of NIH3T3 cells into S phase (Twamley-Stein, Pepperkok et al. 1993). In 1995, Roche et al. from the same group, demonstrated that Src family kinases, namely Src, Fyn and Lyn became 2-3 times more active during mitotic phase and were necessary for the cells to divide. Furthermore, SH2 domain of Fyn was shown to be inhibitory to cell division suggesting that SH2 domain might be important in recruitment of Src to appropriate signaling complexes (Roche, Fumagalli et al. 1995). Taken together these data suggest that Src plays an important role in mitotic progression of normal cells. However some of the key features of this Src-mediated cell cycle regulation are still missing.
**Src kinase in various diseases**

Src family kinases have been implicated to function in various signaling pathways, leading to the regulation of numerous cellular processes like survival, apoptosis, cell division, migration and attachment. Consequently it is obvious that deregulation of such an important kinase would result in various disease phenotypes. Indeed it was found that that viral form of Src or v-Src can induce malignant transformation of various cell types. Src family kinases have been frequently found to be over expressed or deregulated in a number of different types of cancer (Jacobs and Rubsamne 1983; Rosen, Bolen et al. 1986; Loganzo, Dosik et al. 1993). Most evident among these, is the colorectal carcinoma where Src kinase activity was found to be very high, and its activity correlated with metastatic progression (Bolen, Veillette et al. 1987). Irby et al. (1999) reported that a mutation in the C terminal tail of Src resulting in loss of negative regulatory tyrosine (Y529), was found in approximately 12% of colon cancers (Irby, Mao et al. 1999), thus providing direct evidence. However this mutation might not only be the contributing factor, but lots of other events like upregulation of growth factor receptors, downregulation of Src regulatory proteins like Csk, changes in important signaling pathways may cooperate with increased Src kinase activity to produce a cancerous and metastatic phenotype. Breast cancer is another form of cancer where considerable amount of work has been done supporting the role of Src in disease progression (Ottenhoff-Kalff, Rijksen et al. 1992). In comparison to breast and colon cancer, relatively less is known about the role of Src in other forms of cancer. There is some literature suggesting Src activity might be important in melanoma, pancreatic

Being a proto-oncogene, the role of Src in cancer was the major area of study by many scientists in this field. Surprisingly in 1991, Soriano et al. published a paper in Cell showing that mice harboring a null mutation in Src gene, shows a prominent defect in function of bone resorbing cells called osteoclasts, thereby leading to “osteopetrosis” (Soriano, Montgomery et al. 1991). Further studies showed that Src kinase activity was also important for the function of osteoblasts, bone cells that mediate osteogenesis (Marzia, Sims et al. 2000). Currently there are many laboratories focused on developing Src specific inhibitors for treatment of osteoporosis.

**Interest in developing Src kinase inhibitors**

In context to the importance of Src kinase in development of so many types of cancer, there is an active ongoing interest in developing Src inhibitors for single or combinatorial cancer therapeutics. There are mainly three mechanisms of action by which these inhibitors could potentially inhibit Src function- 1) by inhibiting ATP binding to the ATP binding pocket of Src 2) by inhibiting protein substrate binding and 3) by inhibiting Src binding/targeting to specific receptor complexes by SH2 or SH3 ligands (Susva, Missbach et al. 2000). Dasatinib, a drug that has been approved by the FDA for treatment of Chronic Myelogenous Leukemia (CML) is an ATP competitive inhibitor and potently inhibits Src family kinases (Konig, Copland et al. 2008) but has the disadvantage of
having multiple targets. pNaKtide, which is a Na/K-ATPase derived Src inhibitory peptide developed in this lab probably belongs to the second class of inhibitors as mentioned above. pNaKtide prevents ouabain-mediated Src kinase activation and has been shown to be independent of ATP. A general criterion for development and selection of these kind of compounds are –that they are either peptides or peptide-mimetics, capable of inhibiting one or few Src family kinase activity selectively and have very low IC$_{50}$ value (Alfaro-Lopez, Yuan et al. 1998). Finally the third class of Src inhibitors are mainly Src SH2/SH3 domain ligands that theoretically do not inhibit Src kinase activity but hinder Src function by not allowing it to bind to target signaling complexes. There has been a high interest in this field because these inhibitors could confer much higher order of specificity than generic Src inhibitors by virtue of the precise nature of sequence preferred by SH2 domain of different Src family kinases (Liu, Jablonowski et al. 2010). The high affinity Src SH2 ligand peptide pYEEI has an IC$_{50}$ value of about 6.5 µM in vitro (Songyang, Shoelson et al. 1993). However, development of peptide inhibitors of SH2 domain has proven to be challenging due to their susceptibility to cellular proteases and very low cell permeability. Therefore to improve their pharmacokinetic properties, several laboratories have focused on developing peptidemimetic compounds as SH2 domain inhibitors (Nam, Ye et al. 2004; Kraskouskaya, Duodu et al. 2013). One such peptidomimetic compound AP22408 showed promising result in an in vivo model of osteoclast mediated bone resorption (IC$_{50} =$ 0.30 µM in vitro and 57µM in a cell based assay), although there is not sufficient in vivo data to determine its efficacy as an inhibitor (Shakespeare, Yang et al. 2000). Development of SH3 domain inhibitors have been more challenging due to structural constraints.
Chapter 3

Materials and Methods

Materials

The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA)- monoclonal anti-Src antibody (B12), polyclonal anti-ERK1/2 antibody, monoclonal anti-phosphoERK1/2 antibody, monoclonal anti-FAK antibody, goat anti-mouse IgG HRP and goat anti-rabbit IgG HRP secondary antibodies. Anti-Src (pY418) polyclonal antibody and anti-Src (pY529) polyclonal antibody were from Invitrogen (Carlsbad, CA) and anti-phospho FAK (576/577) rabbit polyclonal antibody was from Cell Signaling Technologies (Danvers, MA). The monoclonal anti-α1 Na/K-ATPase subunit antibody (α6f) was obtained from Developmental Studies Hybridoma Bank at The University of Iowa (Iowa City, IA). Monoclonal anti-Src GD11 antibody, anti-phosphotyrosine 4G10 antibody, purified recombinant human Src and polyclonal anti-Na/K-ATPase α1 antibody were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal phosphotyrosine 260 α1 Na/K-ATPase antibody and anti-GFP
rabbit polyclonal antibody were from Abcam (Cambridge, MA). $^{86}\text{Rb}^+$ was obtained from PerkinElmer Life Science Products (Boston, MA). The CMV promoter driven pEYFP-C1 vector was purchased from Clontech (Palo Alto, CA) and pGEX-4T-1 was from GE Healthcare (Buckinghamshire, England). Glutathione beads were purchased from Amersham Biosciences (Uppsala, Sweden). *Escherichia coli* BL21 and DH5α were purchased from Invitrogen. Transfection kits used were Lipofectamine 2000 and Lipofectamine PLUS LTX from Invitrogen. Retroviral vectors (pQCXIP and pVSVG) as well as packaging cell line GP2-293 were purchased from Clontech (Mountain View, CA). QuickChange mutagenesis kit was purchased from Stratagene (La Jolla, CA). Polyclonal rat α1 specific antibody (anti-NASE) was kindly provided by Dr. Thomas Pressley (Texas Tech University, Lubbock, TX). All peptides (purity~95%) were synthesized by HD Biosciences (Shanghai, China) and their purity was confirmed by HPLC.

All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Plasmid Constructs**

Plasmid construct bearing GST fusion protein CD2 (amino acid residues 152-288) was prepared as previously described (Tian, Li et al. 2009). YFP tagged CD2 construct was generated by PCR amplifying CD2 segment and inserting it at the C terminus of YFP in pEYFP-C1 vector. Similarly, CD2N (amino acid residues 152-230) and CD2C (amino acid residues 231-288) were amplified and inserted into the C terminus of YFP sequence.
in pEYFP-C1 vector. The α2 CD2 construct was synthesized in a similar manner as the α1 CD2. Point mutation of tyrosine was performed using Quickchange mutagenesis kit using either pEYFP-C1- YFP-CD2 construct or pRC/CMV -α1AAC m1 vector as template. For retroviral constructs, YFP-CD2 was PCR amplified from pEYFP-C1- YFP-CD2 and inserted into multiple cloning site of pQCXIP vector.

All constructs were verified by DNA sequencing.

Cell Culture

Pig kidney epithelial LLC-PK1 cells and mouse fibroblast SYF and SYF+Src cells were purchased from ATCC (Mannassa, VA) and cultured in DMEM containing 10% (v/v) FBS with 100U/ml penicillin and 100µg/ml streptomycin in a 5% CO₂-humidified chamber. LLC-PK1 cells were allowed to reach 95-100% confluence and then serum starved overnight for experiments. SYF and SYF +Src cells were serum starved overnight in 0.5% FBS containing medium before experiments. GP2-293 cells were cultured in the same manner as LLC PK1 cells. AAC19, PY17, A4-11 and mutant Y260A clonal cell lines were all cultured in DMEM medium containing 10% FBS and penicillin (100U/ml)/streptomycin (100µg/ml). However since the mutant cell lines were highly sensitive to complete serum starvation, all of these cell lines were serum starved in 0.5% FBS containing medium for 24 hrs before any experiment.
**Transfections and Generation of Cell Lines**

LLC-PK1 cells were transfected (Lipofectamine 2000) with pEYFP-C1 vector containing α1 CD2 construct or pEYFP-C1 empty vector. After verifying YFP expression visually, the cells were selected with 1mg/ml G418 for one week. G418 resistant colonies were selected and expanded. Cells were then cultured without G418 for at least three generations before being used for experiments. CD2N and CD2C cells were generated in the same way. Alpha2 CD2 cells were also generated in similar manner. SYF and SYF+Src cells were transiently transfected with pEYFP-C1 or pEYFP-C1-α1CD2 construct using Lipofectamine PLUS LTX reagent. Transfection efficiency was more than 50% both SYF and SYF +Src cells.

Retroviral transfections were achieved by cotransfecting packaging cell line GP2-293 with pQCXIP–YFP-α1 CD2 or pQCXIP- YFP-Y260A mutant α1 CD2, along with the packaging plasmid pVSVG, using Lipofectamine PLUS LTX reagent. After 48 hrs, supernatant were collected and used to infect LLC PK1 cells. Cells were visually inspected for fluorescence and then selected for 2 days with Puromycin (1ug/ml) before doing any experiments.

Y260A mutant rat α1 Na/K-ATPase was generated by transfecting PY17 cells with pRC/CMV –α1 AACm1 vector harboring mutation at Y260 and selected with ouabain (3µM) for 2 days. Ouabain resistant colonies were isolated and expanded in to stable cell lines. The cells were cultured for at least 3 generations without ouabain before being used for any experiments.
Immunoprecipitation and Immunoblot Analysis

Cells were washed with ice cold PBS, and then solubilized in modified radio immunoprecipitation assay buffer containing 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.4). Cell lysates were centrifuged at 14000rpm for 15 minutes; supernatants were collected and subjected to immunoprecipitation or Western blot analysis as described before (Tian, Cai et al. 2006).

\[ ^3\text{H}-\text{Ouabain Binding Assay and Ouabain Sensitive } ^{86}\text{Rb}^+ \text{ Uptake} \]

\(^3\text{H}-\text{Ouabain binding assay was performed as described previously (Tian, Li et al. 2009). The transport function of Na/K-ATPase was assessed by measuring the ouabain-sensitive uptake of the K}^+ \text{ congener, } ^{86}\text{Rb}^+, \text{ as described (Tian, Li et al. 2009) with minor modifications. 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 ouabain (1mM) over 10 minutes at 37 °C. } ^{86}\text{Rb}^+ \text{ (1µCi/well) was then added for 10 minutes at 37 °C and the reaction was stopped by washing three times with ice-cold 0.1M MgCl}_2. \text{ Then cells were incubated in 10\% trichloroacetic acid (TCA) and TCA soluble } ^{86}\text{Rb}^+ \text{ was counted in a Beckman scintillation counter. TCA-precipitated proteins were dissolved in 0.1N NaOH and 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 Growth Assay**

Cell growth assay was performed as described before (Liang, Tian et al. 2007). Briefly, 20,000 cells/well were seeded in triplicates in 12 well plates in DMEM containing 10% FBS. At indicated time points, cells were trypsinized and counted.

**Cell Spreading Assay**

Cell spreading assay was performed as described by Richardson et al (Richardson, Malik et al. 1997). Cells were harvested by trypsinization and 200,000 cells were plated in 60 mm dish containing 4 ml of DMEM with 10% FBS. Cells were then allowed to spread at 37°C for the indicated time points. Images of cell spreading were recorded by phase contrast microscope and for each experiment 5 random fields were photographed. At least 300 cells per experimental condition were counted. Spread cells are defined as those which had extended processes, lacked a rounded morphology and were not phase bright. In order to analyze the spreading of transfected SYF and SYF+ Src cells, 400,000 cells were seeded in 60 mm dishes and cell spreading was recorded using fluorescence microscope at indicated time points. Spreading of untransfected SYF and SYF+Src cells was measured in the same way and the activity was used for comparison.
Cell Spreading Associated Kinase Activity Assay

Cells were grown up to 80-90% confluence and serum starved with DMEM +0.5% FBS for 24 hours. Dishes (100 mm) were coated overnight with 10ug/ml fibronectin (in PBS) at 4°C on a shaker. On the day of the experiment, dishes were first washed with PBS solution and incubated with serum free media at 37°C incubator for 1 hour. Cells were harvested using 0.05% trypsin +0.53mM EDTA and then the 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 in 15 ml Falcon tubes for 1 hour at 37°C. An aliquot of cells (6,000,000) were plated in the fibronectin-coated dishes and allowed to attach/spread for 0, 30 or 60 minutes. At the indicated time points, dishes were removed from incubator, washed once with ice-cold PBS, and lysed in ice-cold lysis buffer as previously described (Richardson, Malik et al. 1997). Cell lysates were collected and subjected to Western blot analyses.

GST Pulldown Assay

GST fused proteins (10µg) conjugated on Glutathione beads were incubated with recombinant human Src in 500µl of PBS (Phosphate Buffered Saline) for 30 minutes at room temperature in the presence of 0.5% Triton X-100. The beads were then washed with PBS with the same buffer for 3 times. The bound Src was then resolved using 10% SDS-PAGE and detected by Western blot using a Src specific antibody.
Peptide Competition Assay

One unit of recombinant human Src was incubated with 0, 0.5, 1 and 5 µM of either NaSH2 or pNaSH2 at in 500 µl PBS (0.5% Triton X-100) for one hour at 4°C. Glutathione bead-bound GST or GST CD2 were then added and incubated at 4°C for another hour. The beads were then washed three times with PBS containing 0.5% Triton X-100. Bound Src was then detected in the same way as described above.

Data Analysis

Data are given as mean± SEM. Statistical analysis were performed using Students T test and significance was accepted at p<0.05.
Chapter 4

Results (Part 1)

As mentioned before, our previous findings indicated that the $\alpha_1$ subunit of Na/K-ATPase interacts with Src kinase via two domain-domain interactions- the CD2 interacts with SH2 domain of Src kinase and the N domain interacts with kinase domain of Src. Furthermore, the CD2-SH2 interaction seems to be much stronger than that of the N-kinase domain interaction (Fig. 3- data taken from (Ye, Li et al. 2011)). However all of these findings were based on in vitro GST pull down assays. Thus we wanted to validate that CD2-SH2 interaction in live cell model and tested whether CD2 expression can inhibit Na/K-ATPase/Src receptor complex formation.

Characterization of CD2 expressing cells

LLC PK1 (pig kidney epithelial) cells were transfected with either pEYFP-C1 or pEYFP-C1-pig $\alpha_1$ CD2 vector, and selected by G418. Numerous G418 resistant clones were generated. After both Western blot analyses and fluorescence microscopic imaging,
we picked and expanded four stable cell lines (CD2-1, CD2-2, CD2-4 and CD2-5) that expressed different amount of YFP-CD2 (Fig. 4A). We also generated two stable empty vector-transfected cell lines (YFP-1 and YFP-2) for use as controls. As depicted in Fig. 4A, clone CD2-2 expressed the highest amount of YFP-CD2, followed by clones CD2-1, CD2-5 and CD2-4. The same experiments showed that YFP expression was higher in YFP-1 cells than that in YFP-2 cells. Thus, YFP-1 and CD2-2 cell lines were used for most of the following experiments, and other cell lines were used to verify the main findings or as a control.
To assess the cellular distribution of the expressed YFP-CD2, fluorescence microscopic images were taken, showing that YFP-CD2 and YFP had similar pattern of cellular distribution as soluble proteins (Fig. 4B).

![Figure 4. Generation of CD2 expressing cells](image)

A) Stable cell lines expressing either YFP (YFP-1 and YFP-2) or YFP tagged CD2 (CD2-1, CD2-2, CD2-4 and CD2-5) were created from pig kidney epithelial cell line LLC-PK1. Equal amount of cell lysates from all the seven cell lines were analyzed by Western blot for YFP expression. B) Fluorescent images of two representative cell lines shown (images are at same scale).
To test whether the expression of YFP-CD2 altered the pumping function of Na/K-ATPase, we first determined the expression of Na/K-ATPase α1 subunit. As shown in Fig. 5A, there was no detectable difference between the control and CD2 expressing cells. We also assessed the expression pattern of Na/K-ATPase β1 subunit and found no difference among the cells. However, about 15% increase in the surface expression of α1 Na/K-ATPase as measured by $^3$H-Ouabain binding assay was noted in both CD2-1 and CD2-2 cells as compared with that control LLC PK1 cells (Fig. 5B). There was no difference in surface expression of Na/K-ATPase in YFP cells. To verify that this increase in surface expression due to CD2 was real, we tested the pumping activity of two of these cell lines. Rubidium is a congener for K$^+$ ions and ouabain-sensitive Rb$^+$ uptake is a standard assay for measuring Na/K-ATPase ion transporting activity. Consistent with the previous data, we found a 17% increase in Ouabain-sensitive $^{86}$Rb$^+$ uptake in the CD2 cells (Fig. 5C).

**Effect of CD2 on cellular Src activity**

To test whether CD2 interacts with Src, we first conducted a co-immunoprecipitation analysis. Cell lysates from both control YFP and CD2-2 cells were immunoprecipitated by a monoclonal anti-Src antibody. Immunoprecipitates were then analyzed by Western blot using anti-Src and anti-YFP antibodies. As shown in Fig. 6A, anti-Src antibody co-precipitated YFP-CD2, but not YFP, in the cell lysates, supporting the notion that CD2 is capable of interacting with Src.
Figure 5. Characterization of CD2 expressing cells

A) Na/K-ATPase α1 and β1 subunit expression. Total cell lysates from LLC PK1, YFP, CD2-1 and CD2-2 cell lines were analyzed for expression of α1 and β1 subunit of Na/K-ATPase. A representative western blot is shown and quantitative data was analyzed from at least three independent experiments.

B) Cells were grown up to 100% confluence and ouabain binding site was measured according to protocol described in “Materials and Methods” section. The values are mean± SEM from at least three independent experiments.* p<0.05 compared with LLC PK1, **p<0.01 compared with LLC PK1.

C) Ouabain dependent ⁸⁶-Rubidium uptake assay was done to compare the ion transport activity of Na/K-ATPase present on cell membrane. The values are mean± SEM from at least three independent experiments.* p<0.05 compared with YFP.
To further test whether CD2-Src interaction regulates Src activity, we measured basal Src activity in CD2-2 and the control YFP cell lines. Should CD2 interact with the Src SH2, we would expect an increase in basal Src activity because such an interaction could displace the intra-molecular SH2/pY529 interaction, resulting in Src activation as done by many other SH2 ligands (Mandine, Jean-Baptiste et al. 2002; Yadav and Miller 2007). Indeed, as depicted in Fig. 6B, the expression of YFP-CD2 in CD2-2 increased basal Src activity as measured by Western blot analyses of Src tyrosine phosphorylation at Y418 (pY418). To verify that expression of CD2 increases basal Src activity, we also measured Src activity in CD2-1 cell lysates. The data confirmed an increase in Src activity in CD2-expressing cells (Fig. 6B). When the phosphorylation of Y529 was measured, there was no detectable difference in pY529 between the control and CD2-2 cells (Fig. 6C), indicating that the increase in Src activity by the expression of YFP-CD2 is not because of a decrease in Y529 phosphorylation.

To test whether the activation of Src would lead to an increase in protein tyrosine phosphorylation, we analyzed total cell lysates by Western blot using an anti-phospho-tyrosine antibody. As depicted in Fig. 7A, tyrosine phosphorylations of several proteins were significantly increased in CD2-2 cells. Because ERKs are known downstream effectors of Na/K-ATPase/Src signaling, we also measured ERK activity in the cell lysates. A significant increase in ERK activity was noted (Fig. 7B). Again, in order to verify the changes, we performed the same assays using cell lysates from CD2-1 cells.
Figure 6. **CD2 binds and activates Src kinase**  
A) Five hundred µg of total cell lysate was immunoprecipitated with 10µg anti-Src antibody and immunoprecipitates were subjected to Western blot analysis of YFP and Src. A representative blot of at least three independent experiments is shown. 
B) YFP and CD2-2 cells were grown up to 90% confluence, serum-starved overnight and total cell lysates were analyzed for pY418 Src and total Src. A representative Western blot is shown and the data are mean± SEM of at least three independent experiments. *, p<0.05. 
C) Total cell lysate was analyzed for pY529 Src and total Src in the same way as described in B. Quantitative analysis of at least three independent experiments is shown.
Figure 7. Effect of CD2 expression on protein phosphorylation A) Total cell lysate from different cell lines were collected, separated by SDS-PAGE and analyzed for total protein phosphorylation. The same membrane was stripped and reprobed for α-tubulin. A representative Western blot of at least three independent experiments is shown. To show the increase in phosphorylation in both higher and lower molecular weight proteins, higher and lower exposure images of same blot shown. B) The expression of phospho-ERK1/2 and total ERK1/2 was detected by Western blot. A representative Western blot is shown. The values are mean± SEM from at least three independent experiments. * p<0.05 compared with YFP, ** p<0.01 compared with YFP.
Effects of CD2 expression on ouabain-induced signal transduction

We have proposed that the interaction between the $\alpha_1$ CD2 and Src SH2 is important for the formation of a functional Na/K-ATPase/Src receptor complex (Tian, Cai et al. 2006; Ye, Li et al. 2011). It is known that ouabain activates Src and ERK in LLC-PK1 cells through the functional Na/K-ATPase/Src receptor complex (Li and Xie 2009). Thus, to further address the functionality of YFP-CD2, we exposed the control YFP and CD2-2 cells to different concentrations of ouabain and then measured the activity of cellular Src and ERK. As depicted in Fig. 8A and B, expression of CD2 significantly attenuated ouabain-induced Src and ERK activation. To probe whether this inhibition is because of reduced formation of Na/K-ATPase/Src complex by YFP-CD2-mediated competition of Src interaction, we immunoprecipitated Src using a monoclonal anti-Src antibody from CD2-2 cell lysates and measured co-precipitated $\alpha_1$ subunit of Na/K-ATPase. Cell lysates from control YFP-1 cells were subjected to the same measurements and used as a control. As depicted in Fig. 8D, expression of YFP-CD2 significantly reduced the co-precipitated $\alpha_1$ subunit of Na/K-ATPase from CD2-2 cell lysates in comparison to that in YFP-1 cell lysates, which is in accordance with the finding that YFP-CD2, not YFP, co-precipitated with Src as depicted in Fig. 6A.

It is known that ouabain also stimulates PI3K/Akt pathways in LLC-PK1 and other cells, and that the activation of Src is important for the full activation of Akt (Liu, Zhao et al. 2007; Tian, Li et al. 2009). To further verify the inhibitory effects of YFP-CD2 on ouabain-induced signal transduction, we exposed both control and CD2-2 cells to different concentrations of ouabain and measured Akt activation by Western blot analysis.
of cell lysates. As depicted in Fig. 8C, ouabain-induced Akt activation was significantly attenuated by the expression of YFP-CD2. Thus, YFP-CD2 appears to work as a dominant negative mutant, capable of blocking ouabain-induced signal transduction including the activation of Src, ERKs and Akt. These findings also support the postulation that the CD2/SH2 interaction is essential for the formation of a functional Na/K-ATPase/Src receptor complex.

To determine that the inhibition of ouabain signaling as observed in CD2-2 was not due to clonal effect, we also exposed the CD2-1 cells to ouabain and measured ERK activation. CD2-1 cells also failed to show ouabain-mediated ERK phosphorylation as shown in Fig. 8D.

Expression of YFP-CD2 attenuates cellular activities involving the activation of Src-mediated signal transduction pathways

It is known that the cell attachment and spreading requires controlled activation and in-activation of Src kinase (Calalb, Polte et al. 1995; Mitra and Schlaepfer 2006; Huveneers and Danen 2009; Sen and Johnson 2011). We have suggested that the Na/K-ATPase/Src interaction plays an important role in this dynamic process of Src regulation (Ye, Lai et al. 2013). Thus, we anticipate that the expression of YFP-CD2 will also affect cell attachment and spreading if YFP-CD2 truly disrupts the interaction between the Na/K-ATPase and Src as implied by the findings shown in Figs. 6 and 8. Indeed, as shown in Fig. 9A, cell spreading was significantly reduced in CD2-2 cells. Moreover,
Figure 8. CD2 inhibits ouabain mediated signal transduction. Cell lines were treated with indicated concentration of ouabain for 10 minutes. Cell lysates were collected and
analyzed for A) pY418 Src and total Src, B) phospho-ERK1/2 and total ERK1/2 C) phospho- Akt and total Akt. Representative Western blots are shown and quantitative data are mean± SEM of atleast three independent experiments. * p<0.05 compared with 0nM control, #, p<0.05 compared with different cell line. D) Five hundred µg of total cell lysate was immunoprecipitated with 10µg anti-Src antibody and analyzed for Na/K ATPase α1 co-immunoprecipitation. A representative Western blot is shown. Quantitative data is shown below. The values are mean ± SE from at least three independent experiments. **, p<0.01. E) YFP and CD2-1 cells were treated with ouabain in a similar manner as described above. Cell lysates were analyzed for phospho-ERK1/2 and ERK1/2. Representative Western blot of at least three independent experiments is shown. * p<0.05 compared with 0nM control, # p<0.05 compared with different cell line.

this defect was also observed in other YFP-CD2 expressing cells and the inhibitory effects appeared to be dependent on the amount of expressed YFP-CD2 (Figs. 9A and B).

In order to verify that this defect was Src-specific, we measured cell spreading activity of SYF and SYF+Src cell lines that were transiently transfected with either YFP or YFP-CD2. SYF cells are mouse fibroblasts isolated from Src, Yes, and Fyn knock-out mouse embryo and SYF+Src are Src-rescued SYF cells. As depicted in Fig. 9D, transient transfection of YFP-CD2 inhibited cell spreading in SYF+Src, but not SYF, cells. We also compared the cell spreading of SYF and SYF+Src cells and found the former to be significantly deficient in cell spreading (Fig. 9C).
Figure 9. CD2 inhibits cellular properties by inhibiting Src kinase

A) Top panel shows images of cell spreading after plating them on dishes for 120 minutes at 37°C.

B) % of cells spread over time (in minutes) for different cell lines: LLC-PK1, YFP-1, YFP-2, CD2-1, CD2-2, CD2-3.

C) Graph showing % of cells spread over time (in minutes) for SYF and SYF + Src.

D) Graph showing fold change in cell proliferation with 10% FBS for different cell lines: LLC-PK1, YFP, CD2-1, CD2-2, CD2-4, CD2-5.
Inset panel show larger image of cell spreading of each cell line. Quantitative data of percentage of cell spread at indicated time points against total number of cells in each field is shown below. The experiment was done as described in “Materials and Methods” section. Values are mean± SEM and of three independent experiments. ** p<0.01 vs. YFP-1. C) Cell spreading in SYF and SYF+Src cells. **p<0.01 vs. SYF at same timepoint. D) SYF and SYF+Src cell lines were transiently transfected with YFP (mock transfected) or YFP-CD2. After visually confirming YFP expression, cells were harvested for cell spreading assay. Values are mean± SEM of atleast three independent experiments. ** p<0.01 and *p<0.05. E) Cell growth curve. Different cell lines were seeded at a density of 20,000 cells/well in 12 well plates, collected at indicated timepoints and counted as described under “Materials and Methods” section. The values are mean± SEM of at least three independent experiments. **p<0.01 vs YFP.

To further demonstrate the importance of CD2/Src SH2 interaction in the regulation of cellular activity, we next examined whether CD2 expression inhibited cell proliferation (Pugacheva, Roegiers et al. 2006). As depicted in Fig. 9E, the expression of YFP-CD2 significantly reduced cell proliferation and this effect seemed to be correlated with the amount of YFP-CD2 expressed in different cell lines. For example, CD2-2 cell line having the highest expression of YFP-CD2 exhibited the slowest growth.

**Expression of YFP-CD2 inhibits Src-mediated integrin/FAK signaling**
Src is an important player in the integrin/FAK signaling (Fig 10A), that is required for cell attachment and spreading (Schaller, Hildebrand et al. 1994; Fincham and Frame 1998; Thomas, Ellis et al. 1998). To understand the molecular mechanism of YFP-CD2-induced inhibition of cell spreading, we plated control YFP-1 and CD2-2 cells in fibronectin-coated dishes and measured the time-dependent changes in cellular Src and FAK activity. As shown in Fig.10B, a time-dependent activation of Src was observed in control cells after plating. This activation was significantly reduced in CD2-2 cells. Moreover, when FAK, a known Src effector in integrin signaling (Schlaepfer, Hauck et al. 1999), was probed for the tyrosine phosphorylation, an increase in Y576/577 phosphorylation (Fig. 10C) was observed in control cells. Again, this increase was attenuated in CD2-2 cells. Interestingly, when ERK activation was assessed, we found that cell attachment-induced ERK activation was not reduced by the expression of YFP-CD2 (Fig. 10D).

Taken together the above findings indicated that 1) CD2 can interact with Src when expressed in cells 2) CD2 acts as a dominant negative mutant of Na/K-ATPase/Src receptor complex and reduces the formation of this complex on cell surface 3) CD2 acts as a Src SH2 domain ligand in cells and thereby can inhibit cellular properties like proliferation and spreading and 4) CD2 expression may also affect other Src-mediated signaling pathways by preventing the targeting of Src via SH2 domain mediated interaction.
Figure 10. CD2 inhibits fibronectin mediated integrin signaling

A) Schematic diagram showing Src mediated Integrin-FAK signaling pathway that regulates cell spreading. Figure was adapted from (Westhoff, Serrels et al. 2004).

B) C) and D) Serum-
starved YFP and CD2-2 cells were harvested and plated for indicated time points as described under “Materials and Methods” section. Total cell lysates were analyzed for pY418 Src/ total Src, pFAK 576/577/FAK and pERK/ERK. Representative Western blots are shown and quantitative data are mean± SEM of at least three independent experiments. * p<0.05 vs. 0 time point, # p<0.04 compared with different cell line.
Chapter 5

Results (Part 2)

In the previous section, we have shown that CD2 interacts with Src and can act as a Src regulator when expressed in cells. We then became interested in locating the exact Src binding region on CD2. Historically, Src SH2 domain is known to sequentially prefer specific phosphotyrosine containing motifs for binding (Liu, Engelmann et al. 2012). These motifs usually consist of a sequence like pY-X-X-I (a phosphotyrosine followed by two amino acids and then an Isoleucine)(Songyang, Shoelson et al. 1993). We therefore wanted to determine whether the CD2-SH2 interaction was also phosphotyrosine-dependent.

C terminus of CD2 contains the Src binding region

The CD2 of α1 Na/K-ATPase contains a single Tyr residue at position 260 (Y260) as shown in Figure 11A. We hypothesized that this tyrosine might be important in
Src SH2 domain binding and its phosphorylation might regulate the kinetics of Src binding in cells. However our previous GST pulldown assays (Ye, Li et al. 2011) suggested that unphosphorylated CD2 is able to bind to Src kinase. Since the purified CD2 used for the experiments was expressed in a bacterial system, in view of the dogma that bacteria do not have a post translational modification system. However, it is important to note that this may not be true (Ge and Shan 2011).

We therefore decided to divide the CD2 region in two parts and determine whether the N terminal (CD2N) or C terminal part (CD2C) is important for Src regulation. In the first set of control experiments, we made GST-fused CD2N containing N-terminal 80 amino acids of CD2 (from amino acid residue of 150 to 230), and GST-fused CD2C containing C-terminal 59 amino acids of CD2 (from amino acid residue of 231 to 280). Using *in vitro* GST pull-down analysis, we found that the GST-fused CD2 and CD2C, but not CD2N, were able to pull down Src (data not shown).

Based on the above *in vitro* pull-down assay, we transfected LLC-PK1 cells with either YFP-CD2N or CD2C, and generated several stable cell lines. As shown in Fig. 11B, two clones of each expressed comparable amount of YFP-fusion proteins. When these cells were analyzed for cell growth, we found that the expression of YFP-CD2C slowed cell growth but not YFP-CD2N (Fig. 11C). Moreover, ouabain-induced ERK activation was attenuated in CD2C cells (Fig. 11D). Finally, expression of CD2C was able to reduce cell attachment-induced activation of Src (data not shown) and FAK (Fig. 11E) as did YFP-CD2.
Figure 11. C terminus but not N terminus contains the Src binding sequence A)

Schematic Diagram showing the whole sequence of CD2 and the sequence design used in constructing CD2N (blue) and CD2C (red) parts. Y260 showed in bold in CD2C region (Note that the sequence shown above is pig Na/K-ATPase α1 sequence, since the original CD2 construct used in designing all of the cell lines was derived from pig). B) LLC-PK1 cells were transfected with either YFP-CD2N (N6 or N1) or YFP-CD2C (C5 or C9) and stable cell lines were generated as described in “Materials and Methods” section. A
representative blot showing YFP expression in different cell lines is shown. C) Cell growth curve of CD2N or CD2C cell lines were done as described in “Materials and Methods” section. D) N1 and C9 cells were grown up to 90% confluence, serum-starved and treated with indicated concentrations of ouabain for 10 minutes. Cell lysates were analyzed by Western blot for pERK/ERK. Quantitative data from three independent experiments are presented as mean± SEM. * p<0.05 vs. 0 nM same cell line. # p<0.05 vs. different cell line. E) N1 and C9 cell lysates were analyzed for attachment-induced phosphorylation of FAK (pFAK 576/7/FAK). Values are mean± SEM from three independent experiments. * p<0.05 vs 0 time point control. # p<0.05 vs different cell line.

**CD2 is phosphorylated at Tyr 260 when expressed in cells**

The previous data showed that the C terminal fragment of CD2, which contains Y260 can regulate Src. We therefore wanted to determine whether this tyrosine residue is phosphorylated. Cell lysates from both YFP and CD2 expressing cells were immunoprecipitated with a YFP specific antibody. Immunoprecipitates were then probed with the anti-phosphotyrosine antibody 4G10 which detects phosphorylated tyrosine residues. As shown in figure 12B, we detected a faint phosphotyrosine band in CD2 immunoprecipitate. We then used a phosphotyrosine antibody that specifically detects Y260 phosphorylation of α1 Na/K-ATPase, to verify the finding. Sequence comparison indicates that Y260 is conserved in mammalian α1 subunit of Na/K-ATPase, but not
present in the other isoforms (Fig. 12A). Thus, we generated a α2 CD2 expressing cell line and used it as a control. As shown in figure 12C, the phosphotyrosine 260 specific antibody detected a phosphotyrosine band from cell lysate of α1 CD2, but not in α2 CD2 expressing cells.

**Figure 12. Y260 in C terminus is phosphorylated** A) Amino acid sequence comparison between α1, α2, α3 and α4 human Na/K-ATPase as well as α1 subunit from different species- mammalian (Pig, Rat), bird (Chicken), amphibian (Frog), Fish (White Sucker Fish) and insect (Drosophila). Software used - CLUSTAL W2 software from EMBL-
EBI. B) 500 μg of cell lysate were immunoprecipitated with a YFP specific antibody as described in “Materials and Methods” section. The immunoprecipitates were then probed for phosphotyrosine using the antiphosphotyrosine antibody 4G10. The same membrane was stripped and probed for YFP. Representative Western blot shown. B) The same amount of cell lysate from YFP, CD2-2 and α2 CD2 cells were probed with a phosphotyrosine antibody specific for phosphorylated Y260 of α1 subunit of Na/K-ATPase. The same membrane was stripped and probed with YFP antibody. Representative Western blot is shown.

α2 CD2 cannot regulate Src like α1 CD2

As mentioned before, the α2 subunit of Na/K-ATPase does not contain the tyrosine which seems to be so important for Src binding. A comparison between pig α1 and α2 CD2 sequence (using the CLUSTAL W2 software from EMBL-EBI) shows the sequence variation between C terminal region of α1 and α2 CD2(Fig. 13A). Most of the variation is at the Y 260 and its surrounding sequence. Therefore to investigate the potential role of the Y260 on Src regulation, we transfected LLC PK1 cells with rat α2 CD2 and generated a stable cell line which expresses comparable amount of α2 CD2 as CD2-2 cells (Fig 12C). Cell growth studies (Fig 13B) indicated that, although α2 CD2 expression slowed down cell growth, it was significantly less effective than that of α1 CD2. Similarly, α2 CD2 failed to inhibit ouabain stimulated ERK phosphorylation (Fig
13C) as compared to α1 CD2 (Fig 8). Taken together these findings indicated that the absence of a single tyrosine residue in α2 CD2 significantly compromised its ability to regulate Src and Src-mediated signal transduction.

**Figure 13. α2 CD2 cannot regulate Src kinase like α1 CD2**

A) Schematic diagram showing C terminal region of α1 and α2 CD2 aligned against each other. The sequence diversity thought to influence difference in Src regulation by the different isoforms is marked with a red box (software used for alignment CLUSTALW2 from EMBL EBI). B) Cell growth curve of YFP, CD2-2 (α1 CD2) and α2 CD2 were performed as described in “Materials and Methods” section. C) YFP and a2 CD2 cells were stimulated with indicated concentrations of ouabain for 10 minutes. Cell lysates were analyzed for

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pERK/ERK by Western blot. Values are mean± SEM from three independent experiments. * p<0.05 vs 0nM.

Generation of Y260 mutant CD2 and its characterization using cell growth studies

To assess the importance of Tyr 260 in Src regulation, we mutated the Y260 in α1 CD2 to Ala and generated a Y260A mutant α1 CD2 expressing cell line, using retroviral transfection system (Fig. 14A). As a control, we also generated normal α1 CD2 and α2 CD2 expressing cell line and compared their cell growth inhibitory properties. As shown in Fig 14B, α1 CD2 cells exhibited the highest growth inhibition whereas α2 CD2 was less effective, which confirmed the data in Fig. 13B. Moreover the Y260A mutation reduced the growth inhibitory capability of α1 CD2. This further affirmed an important role for Y260 in Src regulation.

Generation and characterization of Y260A mutant rat α1 Na/K-ATPase rescued PY17 cells

To further verify the role of Y260 in regulation of Src kinase, we generated a mutant rat Na/K-ATPase α1 subunit construct in which the Y260 was mutated to A. To reduce interference from endogenous Na/K ATPase in cells, the mutant was transfected into a Na/K-ATPase knockdown PY17 cells, generated in this lab (Liang, Cai et al. 2006). PY17 is a LLC PK1 derived cell line in which the expression of Na/K-ATPase α1 subunit has been knocked down more than 90% by using siRNA technique.
Figure 14. Y260A mutation reduces growth inhibitory effect of α1 CD2  A) YFP
tagged α1CD2, Y260A mutant α1 CD2 and α2 CD2 were expressed in LLC PK1 cells
using a retroviral system as described in “Materials and Methods” section. Equal amount
cell lysate were analyzed for YFP expression. Sam membrane was stripped and probed
for α-tubulin. B) Cell growth curve. Different cell lines were seeded at a density of
20,000 cells/well in 12 well plates, collected at indicated timepoints and counted as
described under “Materials and Methods” section. The values are mean± SEM of at least three independent experiments.

Transfection of Y260A rat α1 into PY17 cells resulted in several clones (Y260A clone 1, 21, 24, 13) by ouabain selection. Wild type rat α1 Na/K-ATPase-rescued PY17 cell line, also called AAC19, was used as a positive control.

A rat α1 specific antibody was used to characterize these clones against various PY17 derived cell lines- AAC19 and A4-11 (Liang, Cai et al. 2006). We also used the parental cell line LLC PK1 (pig kidney cells) as negative control since it does not contain any rat α1 Na/K-ATPase. As shown in Fig. 15A, clone 21 seemed to express comparable amount of rat α1 as AAC19 and was used mainly for the following experiments. The clone 1 which expressed ~75% of rat Na/K ATPase was used to verify some of the findings.

To verify total α1 expression, Y260A clone 21 was compared with AAC19, using a Na/K-ATPase α1 antibody which can recognize both pig and rat α1. Total α1 expression was similar in both cell lines. To verify the functionality of the mutant Na/K-ATPase, we also checked the expression of β1 subunit. As shown in Fig. 15B. Y260A mutant was able to rescue the expression and glycosylation of β1 to a comparable level with wild type α1.
Because ouabain dissociates from rat α1 very rapidly as compared with pig α1, we used a $^3$H-ouabain binding assay to determine the level of endogenous Na/K-ATPase in the mutant cells. As depicted in Fig. 15C, endogenous pig α1 expression was further reduced in the mutant cells to about 10% and 50% of PY17 cells for clone 21 and 1 respectively. Since PY17 cells express only 10% of total Na/K-ATPase as compared with control AAC19 cells, it is fair to conclude that about 99% of α1 expressed in Y260A clone 21 was from mutant rat α1 cDNA. This finding indicates that interference from endogenous Na/K-ATPase is insignificant in the following experiments.

**Regulation of protein phosphorylation by Y260A mutants**

As depicted in Fig. 4B, CD2 expression increased basal Src activity by acting as a Src SH2 ligand in cells. However this seems to be quite unlikely in case of the Y260A mutant Na/K-ATPase because we would expect Src kinase not to bind to the mutant Na/K-ATPase and remain in the cell in an inactive form. Indeed we failed to detect any upregulation in Src activity as basal Src activity in the mutant cells was found to be similar to that in AAC19 cells (Fig. 16A) by western blot.

We however noticed a significant increase in ERK (Fig. 14B) as well as total protein phosphorylation level in the mutant cells (Fig 16C).
Figure 15. Generation of Y260A mutant rat α1 cells  A) Y260A mutant cells were generated by transfecting rat α1 Na/K-ATPase that has been mutated at Y260 to A into PY17 cells. Total cell lysates from different cells were collected and analyzed for Na/K-ATPase α1 subunit expression using a rat α1 specific antibody (anti-NASE). Representative Western blot shown. B) AAC19, PY17 and Y260A clone 21 were analyzed for total α1 and β1 expression using α6f and β1 antibodies respectively. Representative Western blot shown of three independent experiments shown. C) Cells were grown up to 100% confluence and ouabain binding site was measured according to protocol described in “Materials and Methods” section. The values are mean± SEM from at least three independent experiments.** p<0.01 compared with PY17.
Figure 16. Regulation of protein phosphorylation by Y260A Cells were serum starved with 0.5% FBS for 24 hours and equal amount of cell lysates were analyzed for A) pY418Src/c-Src, B) pERK/ERK and C) total phosphotyrosine/ α-tubulin. Representative Western blots are shown. Quantitative data are mean± SEM of at least three independent experiments. * p<0.05.

We also noticed that the mutant cells were extremely sensitive to either serum starvation or increasing passage number. Complete serum starvation was noticed to trigger a rather rapid change in cellular phenotype or degradation (Fig 17). The same phenotype changes were observed when cells grew for more than eight generations. Thus
all the studies were done in the presence of 0.5% FBS and at very early passages to eliminate the effect of cell phenotype/death on cellular signaling.

Figure 17. Phenotypic changes due to Y260A mutation Phenotypic changes manifested by mutant cells. AAC19 cells at passage 13 are shown in upper panel. The images of Y260A clone 1 and 21 in upper panels are from passage 4. Same cell lines at passage 8 are shown in lower panels. Images are at same scale. Similar changes were also observed if the cells were serum starved with 0% FBS for overnight.
**Y260A mutants do not respond to ouabain stimulation**

As shown previously ouabain stimulated Src and ERK phosphorylation in YFP expressing cells at 10 and 100 nM concentration. These effects were however downregulated in YFP-CD2 expressing cells (Fig.8). Thus, if Y260 is important for Src SH2 domain binding, we would expect the mutant rat α1 to also have defect in this signaling pathway. As shown in Fig. 18A, ouabain stimulated Src and consequently ERK phosphorylation in AAC19 cells at 10 and 100µM concentration. However no ouabain stimulation of Src and ERK was observed in the mutant cells. This suggested that the ouabain signaling pathway in the mutant Na/K-ATPase was completely abolished due to the mutation of Y260.

It is possible that the Y260A mutant hinders Src kinase binding to α1 Na/K-ATPase and prevents the formation of Na/K-ATPase/Src receptor complex. Therefore to be sure that abolition of ouabain signaling in the mutant is due to inhibition of Src binding to Na/K-ATPase, the same amount of cell lysates from both AAC19 and Y260A clone 21 were immunoprecipitated with a monoclonal Src antibody. As depicted in Fig. 18B, significantly less amount of Na/K-ATPase was co-precipitated by Src from the mutant cell lysates than AAC19.

**The Y260A mutant has no apparent defect in ion transporting function**

Thus the data presented so far show that a single point mutation in second cytoplasmic domain could completely inhibit the signaling function of Na/K-ATPase. However, it is also possible that the observed effect on signaling was due to a change in
Figure 18. Y260A mutation reduces Na/K-ATPase/Src receptor complex formation

A) AAC19 and Y260A clone 21 cells were treated with indicated concentrations of ouabain for 10 minutes. Cell lysates were analyzed for pY418Src/c-Src and pERK/ERK. Quantitative data are mean± SEM from at least three independent experiments. * p<0.05 and **p<0.01 compared with 0nM. #p<0.05 compared with different cell line. B) Cell lysates (500 µg) were immunoprecipitated with a monoclonal Src antibody as described in “Materials and Methods” section. The immunoprecipitates were then probed with a rat α1 specific antibody (anti-NASE) and a Src specific antibody. As a control, 60µg of cell
lysates were loaded in the same gel and analyzed in the same way. Representative Western blot of three independent experiments is shown.

altered ion concentration in cells, or in other words, due to impaired ion transporting by the mutant Na/K-ATPase. We therefore compared the pumping function of Y260A mutants (clone 1 and 21) with AAC19 by using ouabain sensitive $^{86}$Rb$^+$-uptake assay and found them to be similar (Fig.19). Thus it is fair to conclude that the ouabain desensitization of the mutants is independent of intracellular ion concentration and is completely dependent on the signaling function.

Figure 19. Y260A mutant α1 do not have any defect in ion transportation  Ouabain dependent $^{86}$-Rubidium uptake assay was done to compare the ion transport activity of Na/K-ATPase present on cell membrane. The values are mean± SEM from at least three independent experiments.

The above findings indicate that 1) Y260 is important for CD2-SH2 binding and 2) Y260 might be phosphorylated in cells.
Chapter 6

Results (Part 3)

Src SH2 domain inhibitors are currently the subject of investigation by several laboratories because of their potential as a novel inhibitor of Src-mediated signal transduction. In this regard, we also attempted to develop a peptide inhibitor spanning the Y260 region in CD2, after discovering its importance in Src regulation. Data presented in the previous section suggest that the Y260 might be phosphorylated but it does not clearly define the role of this phosphorylation in cells. We therefore sought to address this question by designing two peptides from the Src SH2 binding region of CD2, one of which is phosphorylated and the other is unphosphorylated.

Development of NaSH2 and pNaSH2

We synthesized two 18 amino acid peptides spanning the Y260 region of CD2-NaSH2 (unphosphorylated) and pNaSH2 (phosphorylated at Y260) and purified them
using HPLC. In order to make them cell permeable, they were tagged with HIV-TAT sequence (a sequence we have used before to make cell permeable peptides). The sequence and properties of both the peptides are listed in the table 2.

Table 2. The properties and sequence of NaSH2 and pNaSH2.

Peptide inhibitors from CD2 domain

- **NaSH2**- control peptide
  - Nonphosphorylated
  - Contains HIV-TAT sequence for cell permeability
  - Length -31 amino acids
  - Sequence- \textit{GRKKRRQRRR PPQSTNCVEG TARGIVVTGD}

- **pNaSH2**- test peptide
  - Phosphorylated at Tyrosine residue
  - Contains HIV-TAT sequence for cell permeability
  - Length -31 amino acids
  - Sequence- \textit{GRKKRRQRRR PPQSTNCVEG TARGIVVPYTYGD}

Peptide competition assay

In order to determine the effectiveness of the peptides, we developed an \textit{in vitro} peptide competition assay (Liu, Brodeur et al. 1993; Liang, Cai et al. 2006). Briefly, purified Src was incubated with either NaSH2 or pNaSH2 and then glutathione bead bound GST CD2 was used to pull down the available Src. Our \textit{in vitro} peptide
competition assay (Fig. 21) showed that, pNaSH2 was more potent and effective in blocking Src/CD2 interaction.

![Diagram](image)

**Figure 20. In vitro competition assay** One unit of purified Src was incubated with indicated concentrations of peptides for one hour and then glutathione bead bound GST-CD2 was used to pull down available Src (protocol described in “Materials and Methods” section). The bound Src was then resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with a Src specific antibody. Upper panel shows amount of Src precipitated, whereas lower panel shows amount of GST bound protein.
used for each condition (SDS-PAGE image). A quantitative data analysis showing dose dependent Src inhibition is also included. Values are mean ± SEM of four independent experiments.

**Inhibition of cell signaling by the peptides**

As mentioned before, the peptides were tagged with HIV-TAT sequence in order to increase their cell permeability. We have used this technique before to make our NaKtide cell permeable (Li, Cai et al. 2009). Furthermore, pNaKtide (after addition of TAT sequence to NaKtide it is called pNaKtide) specifically inhibited ouabain mediated signal transduction but not IGF-1 mediated ERK phosphorylation (Li, Cai et al. 2009).

Therefore to verify that the NaSH2 and pNaSH2 are cell permeable and are able to inhibit cell signaling, we preincubated LLC PK1 cells with 0.5 µM of both the peptides for 1 hour and then exposed the cells to 100 nM ouabain for 10 minutes. As positive control, LLC PK1 cells preincubated with 1 µM pNaKtide was also used for the experiment. As depicted in Fig. 22A, pNaKtide was effective in inhibiting ouabain induced ERK activation. Similarly, 0.5 µM of pNaSH2, but not NaSH2, completely abolished ouabain mediated ERK phosphorylation. Moreover pNaSH2, like pNaKtide, had no effect on EGF mediated ERK phosphorylation (Fig. 22B), indicating that like pNaKtide, pNaSH2 also may be quite specific to ouabain signaling pathway.
Figure 21. pNaSH2 inhibits ouabain signaling specifically  A) Serum starved cells were preincubated with or without indicated concentrations of different peptides and then exposed to 100nM ouabain for 10 minutes. Cell lysates were collected and analyzed for pERK/ERK. Quantitative data are mean± SEM of at least three independent experiments. Representative blots are shown. B) Similarly cells were treated with 10ng/ml of EGF and analyzed for ERK phosphorylation. Representative Western blots are shown and data are mean± SEM of at least three independent experiments.

Inhibition of cell spreading by the peptides

We have shown before that CD2 expressed in LLC PK1 cells can significantly slowed down cell spreading and eventually cell growth. We therefore tested whether the two
peptides, tested so far, can also inhibit cell spreading. We also included pNaKtide for this assay. The data indicate that pNaSH2 is the most effective among the three peptides tested. It inhibited ~40% and 30% of cell spreading at second and fourth hour after plating, respectively. In contrast, NaSH2 exhibited only 15% and 20% inhibition of cell spreading, whereas pNaKtide inhibited 30% and 20% of cell spreading at the respective time points. (Fig. 23).

**Figure 22. pNaSH2 inhibits cell spreading more potently** A) and B) LLC PK1 cells were preincubated with or without different peptides at 0.1, 0.3 and 1µM concentrations for one hour. Cells were then trypsinized and allowed to spread for two and four hours respectively. Images were taken as described in “Materials and Methods” section. Quantitative data of inhibition of cell spreading by the different peptides is shown. n=3.
Taken together, these results indicate that 1) both phosphorylated and non-phosphorylated peptide might have inhibitory effect depending upon the assay used 2) pNaSH2 is more effective than NaSH2 in cellular assays.
Chapter 7

Discussion section

Discussions

The role of Na/K-ATPase in regulating cellular physiology has become well appreciated since its discovery in the early 1950s. Since then lots of research into its structure, function, metabolism and expression has revealed valuable insights into the molecular mechanism of its regulation of cellular physiology. In the past decade, another role for the Na/K-ATPase besides its classical ion transporting function has emerged. Recent studies suggest that the Na/K-ATPase also acts as a signal transducing molecule in response to low ouabain concentrations and can regulate cell growth, adhesion, survival by relaying the signal to the nucleus (Aperia 2007; Li and Xie 2009). Work from this lab and some other laboratories have shown that the non pumping pool of Na/K-ATPase physically interacts with Src kinase in lipid rafts called caveolae and can regulate Src kinase activation/inactivation through a conformational transition by the binding of ligands such as ouabain (Jiang Tian, Ting Cai et al. 2006; Liang, Tian et al. 2007; Ye, Li et al. 2011). Besides Src kinase, Na/K-ATPase can directly interact with several other proteins like PLCγ, IP3R, ankyrin, PI3K, AP1 to form a large signaling complex
Importantly, Src kinase was found to be an essential component of this signalosome and Src kinase inhibition resulted in significant attenuation of the signaling (Xie and Cai 2003). It is therefore of prime importance to determine the molecular mechanism of formation of this Na/K–ATPase/Src mediated signaling complex, as it will allow us to understand the exact nature of signaling function played by the sodium pump.

Our previous GST-pulldown assays indicate that the second cytosolic domain (CD2) of α1 subunit of Na/K-ATPase interacts with the Src SH2 domain (Tian, Cai et al. 2006; Ye, Li et al. 2011). To further explore the functionality of this putative interaction, we have characterized Src-mediated signal transduction in stable cell lines expressing YFP-fused CD2. Our new findings suggest the following. First, CD2 appears to be a functional ligand of Src SH2. Second, the interaction between the CD2 and Src SH2 domain appears to be essential for cardiotonic steroids to activate protein kinase cascades. Finally, as a functional ligand of Src SH2, YFP-CD2 acts as a dominant negative mutant, capable of inhibiting cellular pathways and activities where Src plays an important signaling role.

Furthermore, a single tyrosine residue in CD2 of α1 Na/K-ATPase was found to be the most critical factor in Src binding. This finding is supported by the following evidence- 1) Src SH2 domain is quite well known for its preference in binding phosphotyrosine residues, and CD2 expressed in cells was phosphorylated at Y260. 2) Y260A mutation in α1 Na/K-ATPase abrogated its ability to activate Src and ERK in response to ouabain. 3) Src binding affinity of Y260A Na/K-ATPase was significantly
reduced and 4) a peptide derived from the CD2 spanning Y260 region abolishes ouabain-mediated signaling in live cells. These and other important findings are further discussed.

**CD2/SH2 interaction and its role in Src regulation:** We have proposed that the α1 Na/K-ATPase interacts with Src to form a functional receptor complex for cardiotonic steroids to regulate protein kinase cascades (Tian, Cai et al. 2006; Ye, Li et al. 2011; Ye, Lai et al. 2013). In support of our model of direct interaction between Na/K-ATPase and Src kinase, we have shown that a GST-fused CD2 interacts with Src SH2 domain whereas the N domain binds Src kinase domain (Tian, Cai et al. 2006). Based on the latter, we have successfully delineated a 20 amino acid peptide (NaKtide) region in α1 subunit of Na/K-ATPase that is able to bind and inhibit Src kinase activity both *in vitro* and *in vivo* (Li, Cai et al. 2009; Li, Zhang et al. 2011). Furthermore, when key residues in NaKtide sequence were mutated, it gave rise to α1 mutants that pump normally but are defective in Src regulation (Lai, Madan et al.). In this report we have assessed the significance of the CD2-SH2 interaction. First, we found that the ectopically expressed YFP-CD2 interacted with Src as demonstrated by co-immunoprecipitation analyses. Second, expression of YFP-CD2 reduced the number of available receptor complexes, resulting in the inhibition of ouabain-induced signal transduction in LLC-PK1 cells. Therefore, it is concluded that CD2 domain of the α1 subunit of Na/K-ATPase is likely to be involved in the direct interaction with Src kinase in live cells. We further speculate that this interaction is important for the formation of a functional Na/K-ATPase/Src receptor complex through the following two possible mechanisms: (a) the binding of Src
SH2 to the CD2 might facilitate the association of the Src kinase domain to the α1 N domain, thus helping the formation of the receptor Na/K-ATPase/Src complex; (b) because our in vitro binding assays indicate a stronger interaction between the SH2 and CD2 than that of kinase/N domain, the CD2-SH2 association might remain even after the release of Src kinase domain from the Na/K-ATPase, resulting in a localized activation of Src and subsequent recruitment and assembly of signaling cascades in the vicinity of receptor Na/K-ATPase. A schematic diagram for CD2 mediated Src regulation is included below-

Figure 23. Schematic diagram showing CD2 mediated regulation of Src in CD2 expressing cells.
Moreover, CD2 expression was found to upregulate Na/K-ATPase cell surface expression by about 15%, as suggested by both $^3$H-ouabain binding as well as $^{86}$Rb+ uptake assay. It is well known that receptor-endocytosis is one of the principal mechanisms used by cells to suppress ligand induced receptor activation. Clathrin-mediated endocytosis is perhaps the most well characterized among different endocytosis pathways (Cavalli, Corti et al. 2001). We have previously shown that long term exposure (12 hours) of LLC PK1 cells on basal surface to low doses of ouabain can induce clathrin-dependent internalization of Na/K-ATPase into endosomal vesicles. This endocytosis process required activation of PI3K as well as the participation of Src kinase. This process is reversible and removal of ouabain can restore plasma membrane Na/K-ATPase content (Liu 2005). Moreover caveolin was also suggested to play a role in receptor endocytosis, indicating a role for scaffolding and signaling function of Na/K-ATPase as well (Liu, Liang et al. 2005). Here, we have shown that CD2 can inhibit ouabain induced Src as well as PI3K activation in LLC PK1 cells, by preventing Na/K-ATPase/Src receptor complex formation. We speculate that, constitutive inhibition of the Na/K-ATPase/Src receptor complex-mediated signaling pathways by CD2, could affect clathrin and caveolin mediated endocytosis of Na/K-ATPase at very low levels. This may result in the observed ~15% increase in surface expression of $\alpha_1$ Na/K-ATPase as observed in the CD2 expressing cells. Therefore, it is tempting to propose that, CD2 could also act as a functional inhibitor of ouabain-mediated signaling inside cells and upregulate Na/K-ATPase expression on cell surface by chronically inhibiting the endocytosis of receptor complex.
**CD2 as a Src SH2 ligand:** It is known that Src-mediated signal transduction is dependent not only on its kinase activity, but also on its recruitment to specific membrane assemblies (Anderson, Koch et al. 1990; Moran, Koch et al. 1990; Schaller, Hildebrand et al. 1994; Lombardo, Consler et al. 1995). For example, mutagenesis analysis indicate that the kinase domain and the SH2 domain cooperate in the membrane targeting of Src (Shvartsman, Donaldson et al. 2007). Blocking either kinase activity or SH2-mediated targeting to the membrane assemblies could inhibit Src-mediated signal transduction, resulting in alterations in cell spreading and growth (Owen, Ruest et al. 1999; Brunton, Avizienyte et al. 2005; Yeo, Partridge et al. 2006). Based on the new findings reported here, we suggest that CD2 works as a putative Src SH2 domain ligand (Stover, Furet et al. 1996; Moarefi, LaFevre-Bernt et al. 1997). While expression of CD2 increased basal Src activity and total protein phosphorylation, it impeded Src-mediated signal transduction and consequently inhibited cell spreading and growth. It might be important to mention here that pYEEI peptide (Songyang, Shoelson et al. 1993) which has been shown to have the highest affinity for Src SH2 domain induces only ~2.5 fold basal Src family activation in terms of substrate phosphorylation (Moarefi, LaFevre-Bernt et al. 1997) in an *in vitro* system. In comparison, CD2 exhibited ~1.5 fold increase in basal Src activity (measured as Y418 phosphorylation) and ~2 fold elevation in ERK phosphorylation, which is a downstream effector for Src.

Moreover, it is important to note the following unique properties of CD2 as a Src pathway inhibitor. First, CD2 is not an ATP analog like PP2 (Al-Obeidi and Lam 2000). Second, CD2 is also different from NaKtide because it does not interact with the Src
kinase domain (Tian, Cai et al. 2006) although both are derived from the α1 subunit of Na/K-ATPase (Li, Cai et al. 2009). Finally, CD2 does not inhibit Src kinase activity. If anything, it increased Src activity, probably by disrupting the intra-molecular interaction between the pY529 and the SH2 domain, by acting as a SH2 domain ligand.

Thus from the data presented demonstrate that CD2 displays all the characteristics of a classical Src SH2 domain inhibitor.

**Importance of Y260 in Src binding:** Src kinase is negatively regulated by its C terminal phosphotyrosine (pY529). This inactive state can be relieved through competition by a ligand with higher affinity for the SH2 domain, which displaces the weak pY529 –SH2 interaction. As mentioned before, the sequence with highest affinity for Src SH2 domain was discovered to be pYEEI. Based on sequence comparison, we hypothesized that the exclusive tyrosine in CD2 (Y260) and its phosphorylation might be critical for Src binding. However, there was one concern in this regard. Our GST pulldown assays, which showed CD2-SH2 interaction, were performed with proteins expressed and purified from a bacterial system. It was well known that bacteria cannot post translationally modify proteins by tyrosine phosphorylation. Recent literature however indicates that bacteria can phosphorylate proteins at serine, threonine as well as tyrosine residues (Grangeasse, Nessler et al. 2012). Therefore we wanted to determine whether the Y260 could be phosphorylated in live cells and if so, whether pY260 is important for Na/K-ATPase to regulate Src.
We found that CD2 expressed in cells is tyrosine phosphorylated using the anti-phosphotyrosine 4G10 antibody. We further verified site of the tyrosine phosphorylation to be Y260 using a phosphotyrosine Y260-specific antibody.

To test whether the Y260 plays a critical role in Na/K-ATPase/Src receptor complex, we made Y260A mutants. Our studies using Y260A mutant CD2 revealed that this Tyr is critical for CD2-mediated Src regulatory effects in cells. We also confirmed these findings using α2 CD2 expressing cells. Interestingly while α2-CD2 cells failed to respond to ouabain stimulation, the expression of α2-CD2 appeared to be inhibitory to cell proliferation as the expression of α1-CD2 Y260A mutant. Taken together, these findings suggest that Y260 is important and its mutation to Ala reduced its ability to regulate Src. However, Y260A mutant still may possess some Src inhibitory effect although it is not as potent as the phosphorylated Y260.

To further assess the importance of Y260, we generated stable cell lines expressing Y260A mutant Na/K-ATPase. Y260A mutant α1 could assemble with β1 subunit to form a fully functional ion pump, as depicted by ouabain binding and ouabain-sensitive $^{86}$Rb$^+$ uptake assays. However ouabain induced Src/ERK phosphorylation was completely abolished in these mutant cells, as compared to normal α1 expressing cells. This was supported by the co-immunoprecipitation analysis, which revealed that the mutant α1 is impaired in their ability to form a functional signaling complex with Src kinase. Thus Y260A mutation resulted in a fully functional ion pump that is defective in
signal transduction, clearly dissociating the ion transporting from signaling function of the Na/K-ATPase.

Mutant Y260A α1 expression also increased total protein tyrosine phosphorylation as well as ERK phosphorylation in cells, in accordance with the CD2 expressing cells. As mentioned before, Na/K-ATPase acts as a scaffold on cell membrane, by binding and inhibiting significant amount of Src kinase molecules in functional signalosomes. As a result, basal Src activity is reduced in normal cells but elevated in cancer cells where Na/K-ATPase expression is downregulated. We speculate that expression of Y260A mutant should completely abolish this regulation and result in increased protein phosphorylation as well as basal ERK activation.

Surprisingly, we did not observe any increase in basal Src activation (pY418 phosphorylation) in Y260A mutant cells. Our previous data indicates a role for Na/K-ATPase to compete for Src binding by displacing the Src SH2/pY529 interaction in cells, because Y529 phosphorylation is not changed either in CD2 expressing cells or because of ouabain stimulation (Tian, Cai et al. 2006). Therefore in normal cells, most of cellular Src is inactivated either by SH2/pY529 interaction or Na/K-ATPase/Src interaction. Our co-immunoprecipitation studies indicate that Y260A mutation reduces the ability of Na/K-ATPase to recruit and bind Src kinase. The unchained Src kinase thus would be expected to adopt an inactive conformation, by autoregulation (Roskoski 2004). This may explain, why we did not observe any significant change in basal Src kinase activity in Y260A mutant cells. The expressed CD2 however, itself binds to Src kinase like a Src
SH2 domain ligand and thereby increases Src Y418 phosphorylation, by domain displacement.

However, data from both α2 CD2 cells as well as Y260A mutants suggest that some level of Src regulation may still be achieved in the absence of this tyrosine. Data presented here are not sufficient enough to come to an exact conclusion. Further studies with other isoforms of Na/K-ATPase will be necessary to address this issue, but are out of scope in this dissertation. Interestingly, a paper published in 2007 specifically shows ouabain-induced ERK activation mediated by α1, α3 and α4, but not α2, isoform of Na/K-ATPase expressed in Sf9 cells (Pierre, Sottejeau et al. 2008). Thus further clarification of the importance of this Y260 in signaling function of Na/K-ATPase, could also pinpoint the role of isoform specific differences in ouabain-mediated signaling.

To better address the issue of tyrosine phosphorylation in CD2 and the role it plays in Src regulation, we generated the peptide inhibitors- NaSH2 (unphosphorylated) and pNaSH2 (phosphorylated). From the data presented so far, it is clear that the phosphorylated peptide acts as a far better inhibitor of Src than the unphosphorylated peptide. Our preliminary data (not shown) suggests that LLC PK1 cells exhibit a time and dose dependent Y260 phosphorylation of α1 Na/K-ATPase on ouabain treatment. Taken together these findings signify an important role for Y260 phosphorylation in regulating the dynamics of Src kinase in normal cells. PhosphoSitePlus, an online repository for protein post translational modification (registered under Cell Signaling Technology), lists
this phosphorylation site as the most frequently found phosphorylation site in α1 Na/K-ATPase.

(http://www.phosphosite.org/homeAction.do;jsessionid=BEEC4F45E0AD1817EE921314298B9E9)

**Importance of developing a Src SH2 domain inhibitory peptide** - Src kinase, is a promising drug target for various cancers as well as bone diseases. In this regard, small molecule inhibitors of Src SH2 domain are of great interest as they provide the potential for blocking specific intracellular signaling pathways that are implicated in the development of certain diseases (Sundaramoorthi, Kawahata et al. 2003; Nam, Pitts et al. 2004). However, low cellular uptake, increased degradation of the phosphotyrosine moiety by cellular phosphatases and poor in vivo results have been some of the problems encountered by researchers in this field.

In this regard development of pNaSH2 as specific Src SH2 domain inhibitor is highly significant. The 18 amino acid peptide itself, is primarily hydrophilic in nature due to the presence of the Phosphotyrosine moiety and is therefore cell impermeable. Therefore, this peptide was tagged with the cell permeable peptide sequence, TAT from HIV to make it cell permeable (Li, Cai et al. 2009). Here we have shown that pNaSH2 is able to inhibit CD2-SH2 association using an *in vitro* assay. Second, the peptide is cell permeable, as it can inhibit ouabain mediated ERK phosphorylation in cells at about 0.5µM concentration. Third, it is very specific to particular signaling pathways as it did not inhibit EGF mediated ERK phosphorylation. Fourth, it also successfully inhibited cell
spreading, as was observed with CD2 expressing cells, indicating its ability to regulate cell phenotype. Further studies such as cell proliferation and survival assays are needed to establish this peptide into a functional antagonist of Src kinase. It is also important to compare the ability of the peptide to inhibit cancer and normal cell growth. Also, all the cellular studies performed here were short term studies. Long term studies are necessary to test the stability of the peptide inside cells.

As a SH2 domain ligand peptide, pNaSH2 could be used to slow down proliferation of particular cancer types and subtypes where Src activity is quite high. Furthermore it would be also interesting to test the additive effect of pNaSH2 in conjunction with pNaKtide in inhibiting cancer cells where α1 Na/K-ATPase expression is low. There is also potential for application of this peptide for treatment of osteoporosis, as done by other groups (Shakespeare, Yang et al. 2000). Finally, pNaSH2 as a specific inhibitor, could also be used to probe the physiological importance of Na/K-ATPase/Src receptor complex signaling and their role in pathogenesis of different diseases.

Uncertainties- We have inferred that the CD2 of Na/K-ATPase α1 subunit can act as a Src SH2 domain ligand and thereby change the phenotype of cells expressing this peptide. It is however difficult to determine whether any other Src family kinase or membrane/soluble protein is affected directly due to the expression of CD2 or its derived peptide. Furthermore the IVVY region has been shown to be a binding site for AP1 (Adaptor Protein-1) by Efendiev et al. (Efendiev, Budu et al. 2008). The IVVY region was shown to be a binding site for AP-1 protein in response to angiotensin-dependent trafficking of Na/K-ATPase by G-protein coupled receptors. Whereas it could be possible
that the same site serves as binding site for AP-1 and Src indicating a cell and ligand specific signaling event, it is not possible to rule out the presence and influence of endogenous Na/K-ATPase in the supporting experiments shown in the above paper. First, the OK cells used in the experiments were assumed to have no endogenous Na/K-ATPase but it was not verified experimentally. In our mutant cells expression of endogenous Na/K-ATPase was ~1% of total expression of Na/K-ATPase based on 3-H ouabain binding, indicating practically zero interference. Second, co-immunoprecipitation and FRET analysis only indicates the possibility of direct interaction between this site and AP-1. It is not possible to rule out the interference of other proteins in these assays.

We observed an increase in basal ERK and tyrosine phosphorylation in Y260A mutant cells, without any increase in basal Src activity. For CD2 expressing cells, the increase in basal ERK and protein phosphorylation was attributed to Src activation because Src activation (v-Src) has been shown to increase total protein tyrosine phosphorylation in cells (Seidel-Dugan, Meyer et al. 1992). We however do not know whether very subtle increases in Src activation could occur in the mutant cells, due to experimental limitations. We can only speculate that the increase in protein phosphorylation is due to transient increases in Src kinase activity or deregulation of Src kinase due to this mutation.

Our new findings therefore provide further support for the hypothesis that the α1 Na/K-ATPase plays a critical role in the dynamic regulation of Src signaling pathways in LLC-PK1 cells (Ye, Lai et al. 2013). We have recently reported that the α1 Na/K-
ATPase regulates Src in a conformation-dependent manner (Ye, Li et al. 2011). Mutations that accumulate the α1 Na/K-ATPase at either E1 or E2 conformation cause defect in Src regulation. Like the expression of CD2, the expression of E1 or E2 mutants is capable of inhibiting cell spreading and cell attachment-induced activation of Src/FAK pathways (Ye, Lai et al. 2013). Our new cells, as well as peptide data are in support of these findings and indicate a scaffolding role for Na/K-ATPase in regulating Src kinase dynamics on cell surface.

Conclusions

1) CD2-SH2 interaction is necessary for Na/K-ATPase/Src receptor complex formation.
2) Y260 in CD2 of α1 Na/K-ATPase is the most critical residue for Src binding.
3) pNaSH2, a peptide developed from CD2, acts like a Src SH2 domain ligand and is capable of blocking Src mediated signaling in cells.
References


