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The effect of Src tyrosine kinase on the sodium/calcium exchanger NCX1

by

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ABSTRACT

Both Na^+/Ca^{2+} exchanger NCX1 and non-receptor tyrosine kinase Src are widely expressed in many tissues. A recent paper suggested that tyrosine kinase inhibitors activated membrane current generated by NCX in cardiac myocytes. Therefore, we chose to examine the potential influence of Src on NCX1 directly. When co-transfected together into HEK293 cells, Src caused an increase in the mobility of NCX1 when analyzed by SDS gel electrophoresis. The size change was caused by alteration of the structure of the N-linked glycan attached to NCX1. Surface biotinylation experiments suggested that less NCX1 was delivered to the cell surface when co-expressed with Src. Further experiments demonstrated the modification happened during biosynthesis. In summary, we have demonstrated that the co-expression of Src induced a dramatic change in glycosylation and trafficking of the NCX1 protein. These findings may provide a new and intriguing regulation pathway for NCX1.

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Dedicated to my parents

TABLE OF CONTENTS

APPROVAL PAGE ii
ABSTRACT iii
ACKNOWLEDGEMENTS iv
DEDICATION v
TABLE OF CONTENTS vi
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF ABBREVIATIONS xi

CHAPTER ONE: INTRODUCTION 1
I. The Essential Role of Ca^{2+} and Its Regulation 1
II. Mammalian Na^{+}/Ca^{2+} Exchanger 4
A. Na^{+}/Ca^{2+} exchanger family and isoforms 5
B. NCX1: a molecular perspective 7
Properties of Na^{+}/Ca^{2+} exchanger 8
Topology 8
Alternative splicing 10
Regulation of NCX1 12
1. Exchanger inhibitory peptide (XIP) 12
2. Intracellular Ca^{2+} 12
3. Intracellular Na^{+} 14
4. Intracellular H^{+} 15
5. Phosphorylation 15
6. ATP 16
7. NCX interacting proteins 17
C. Physiological role of NCX1 19
Heart muscle 19
Brain 21
Kidney 22

III. The Function and Physiological Role of Tyrosine Kinase Src 23
A. The structure of Src 23
B. Activation mechanisms 24
C. Src and cell signaling 26
IV. Protein Glycosylation 26
The process and regulation of N-linked glycosylation 27
In the endoplasmic reticulum (ER) 28
In the Golgi apparatus 29
V. Reasoning and Research Goals 31

CHAPTER TWO: EXPERIMENTAL PROCEDURES 33
I. Oligonucleotide-directed mutagenesis 33
II. Cell culture and transfection 36
III. Post-nuclear protein preparation 38
IV. Immunoprecipitation 39
V. Immunoblot 40
VI. Generation of stable inducible HEK293 cell line 41
VII. Enzymatic deglycosylation analysis 42
VIII. Biotinylation analysis of surface expression 43
IX. Immunofluorescence 44
X. Protein staining and identification 45

CHAPTER THREE: RESULTS 46
I. c-Src activity increased the mobility of NCX1 46
II. Src does not directly phosphorylate NCX1 48
III. The change in gel mobility of NCX1 was caused by alteration of the structure of the N-linked glycan 50
IV. The Src-altered NCX1 glycan was Endo H insensitive 53
V. The Src-induced alteration of NCX1-glycan happened during biosynthesis 55
VI. Trafficking of NCX1 was changed by Src activity 61
VII. Src also affected trafficking of the glycosylation defective NCX1

mutant N9Q 63
VIII. The Src-induced alteration in glycan structure showed some selectivity to the host protein 65
IX. Analysis of NCX1 and NCKX2 mutants 70
X. NCX1-associated proteins 74

CHAPTER: DISCUSSION 78
I. Overview 78
II. Src in the regulation of glycosylation 79
III. The reason for reduced glycosylation 80
IV. The reason of reduced surface expression of NCX1 81
V. Which happened first: glycosylation change or trafficking change? 82
VI. Where did the interruption in trafficking happen? 82
VII. Model of trafficking change induced by Src kinase 83
VIII. The possible mechanism - Golgi regulation 85
Golgi and trafficking 85
Quality control in the Golgi complex 86
Golgi and Src 86
IX. Specificity 87
X. Physiological implications 89
Ischemic preconditioning 89
Hypertrophy 90
Long-term potentiation (LTP) 91
XI. Future directions 92
XII. Concluding remarks 93

REFERENCE LIST 95

LIST OF TABLES

Table 1: Summary of oligonucleotide primers used 35
Table 2: List of constructs used 37
Table 3: Summary of the significant hits ($p<0.05$) from samples excised from the Coomassie-stained gels 76

LIST OF FIGURES

Figure 1: An outline of calcium pathways in and out of cells 3
Figure 2: Proposed membrane topology of NCXs 9
Figure 3: Structure and activation of Src proteins 25
Figure 4: Processing of N-linked glycan 30
Figure 5: c-Src activity increased the mobility of NCX1 47
Figure 6: NCX1 was not directly phosphorylated 49
Figure 7: Src-induced change in gel mobility of NCX1 was caused by alteration of the N-linked glycan structure 51
Figure 8: The Src-altered NCX1 changed glycan was endo H insensitive 54
Figure 9: Characterization of the tetracycline inducible cells stably expressing NCX1 56
Figure 10: The Src-induced alteration in NCX1-glycan happened during biosynthesis 59
Figure 11: Trafficking of NCX1 was changed by Src activity 62
Figure 12: Src also affected trafficking of the glycosylation defective NCX1 mutant N9Q 64
Figure 13: The Src-induced alteration of glycan chain showed some selectivity to the host protein 66
Figure 14: Neither glycan nor trafficking of mGluR1b was changed by Src activity 68
Figure 15: Alignment of rat NCX1 and mouse NCKX2 protein sequences 71
Figure 16: Analysis of mutants 72
Figure 17: Silver staining of immunoprecipitated NCX1 associated proteins 75
Figure 18: Proposed model 84

LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolone-propionate
Asn	asparagine
Asp	aspartic acid
AE	chloride/bicarbonate exchanger
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pair
BSA	bovine serum albumin
CBD	Ca^{2+} binding domain
CFTR	cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovary
CICR	calcium-induced calcium release
Cys	cysteine
DAG	diacylglycerol
DIC	differential interference contrast
DME	Dulbecco's modified Eagle's Medium
DNA	deoxyribonucleic acid
Dol	dolichol
E-C	excitation-contraction
EDTA	ethylene diamine tetra-acetic acid
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein isothiocynate
GlcNAc	N-acetylglucosamine
GlcNAc-T	N-acetylglucosaminyltransferase
GPI	glycophosphatidlyinositol
HA	hemagglutinin
HCA	homocysteic acid

HEK293	human embryonic kidney cell line 293
HRP	horseradish peroxidase
IgG	immunoglobulin G
IP	immunoprecipitation
IP_3	inositol 1,4,5-trisphosphate
kDa	kilodalton
K_d	dissociation constant
K_m	Michaelis-Menten constant
Kv	voltage-gated potassium channel
Man	mannose
mGluR	metabotropic glutamate receptor
NCX	sodium/calcium exchanger
NCKX	potassium-dependent sodium/calcium exchanger
NHE	sodium/hydrogen exchanger
NMDA	N-methyl-D-aspartate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PLM	phospholemman
PMCA	plasma membrane Ca^{2+}-ATPase
PMSF	phenylmethylsulfonyl fluoride
PnPP	p-nitrophenyphosphate
PTH	parathyroid hormone
RyR	ryanodine receptor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERCA	sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SH	Src-homology
SR	sarcoplasmic reticulum

TGN	trans-Golgi network
Thr	threonine
TM	transmembrane segment
TRP	transient receptor potential
Tyr	tyrosine
XIP	exchanger inhibitory peptide
VGCC	voltage-gated Ca^{2+} channel
VSV-G	vesicular stomatitis virus membrane glycoprotein

Plasma membrane sodium/calcium exchanger (NCX) is a key component in Ca^{2+} handling in several tissues. Non-receptor tyrosine kinase Src is ubiquitously expressed and participates in the regulation of many calcium signaling pathways. Evidence suggested tyrosine kinase inhibitors activated membrane current generated by NCX in cardiac myocytes. The research presented in this thesis is an exploration of the potential regulatory effect of Src kinase on the type 1 NCX (NCX1.1) which is expressed in the heart.

CHAPTER ONE

INTRODUCTION

I. The Essential Role of Ca^{2+} and Its Regulation

Calcium is essential to normal cell life. It functions as a versatile intracellular signaling element that regulates a host of diverse physiological processes. To achieve this versatility, the concentration of ionized calcium, $[Ca^{2+}]$, is fine-tuned with very different spatial and temporal dynamics. Fast events like exocytosis and contraction are regulated by rapid highly localized Ca^{2+} spikes, whereas slower responses like gene transcription and proliferation are controlled by repetitive global Ca^{2+} transients or intracellular Ca^{2+} waves (Berridge et al., 2000; Bootman et al., 2001; Berridge et al., 2003).

Unlike other second messengers, Ca^{2+} is not metabolized, thus intracellular [Ca^{2+}] is wholly determined by a balance between introducing Ca^{2+} into and removing Ca^{2+} from the cytoplasm (Figure 1). To generate a Ca^{2+} signal, cytoplasm Ca^{2+} increases through either the entry of external Ca^{2+} or the release of Ca^{2+} stored within the endoplasmic/sarcoplasmic reticulum (ER/SR). External Ca^{2+} enters the cell through various channels located in the plasma membrane, such as voltage-gated channels, receptor-operated channels, store-operated channels, transient receptor protein ion-channels, etc. Internal Ca^{2+} is released through inositol-1, 4, 5-trisphophate (IP_3) receptors and ryanodine receptors. Once inside the cell, Ca^{2+} is sensed by various targets, which interpret this signal into appropriate cellular responses. When the signaling function has been fulfilled, the Ca^{2+} must be removed. Two systems contribute to the removal of Ca^{2+}: Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchanger. The plasma membrane Ca^{2+}-ATPase (PMCA) and Na^{+}/Ca^{2+} exchange extrude Ca^{2+} out of the cell, whereas the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps Ca^{2+} back into sarco(endo)plasmic reticulum. The Ca^{2+}-ATPases have a relatively high affinity for Ca^{2+}, but a low transport capacity, and are thus generally thought to fine-tune cytoplasmic Ca^{2+} during the resting state. In contrast, Na^{+}/Ca^{2+} exchangers have a lower affinity, but a much larger capacity, and are thus believed to play a major role during times when Ca^{2+} concentration is elevated (Guerini et al., 2005).

Src tyrosine kinase has a significant effect on Ca^{2+} signaling. It can modulate the activity of other Ca^{2+}-handling proteins like voltage-dependent L-type Ca^{2+} channels (Wijetunge et al., 2000), store-operated Ca^{2+} channels (Babnigg et al., 1997), etc. In addition, Src

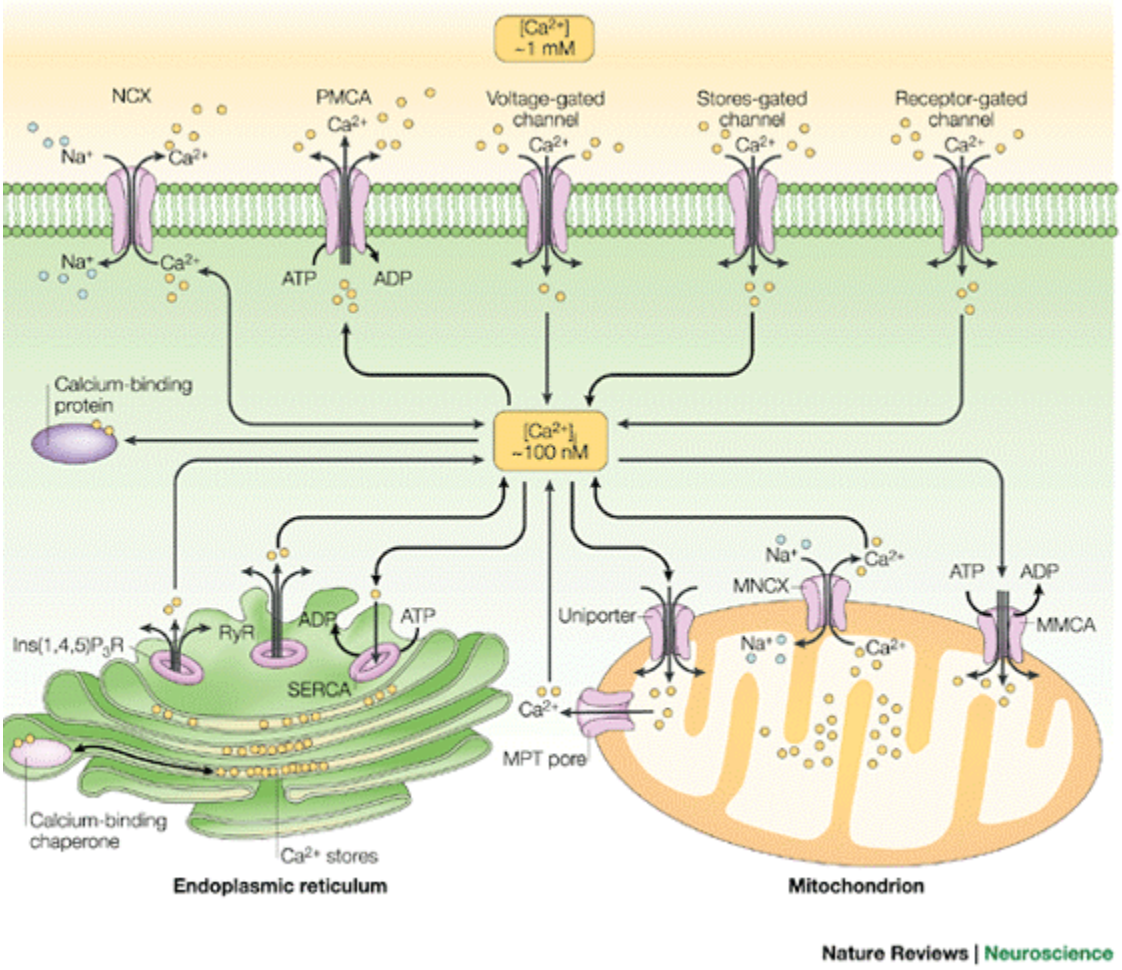


Figure 1: An outline of calcium pathways in and out of cells. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience (Syntichaki and Tavernarakis, 2003)

indirectly affects IP_3-induced Ca^{2+} release from the ER in smooth muscle cells by regulating the level of phosphatidylinositol 4,5-bisphosphate (PIP_2) (Tolloczko et al., 2002). PIP_2 is the substrate of phospholipase C (PLC), whose activation leads to the production of second messengers IP_3 and diacylglycerol (DAG).

II. Mammalian Na^+/Ca^{2+} Exchanger

The $Na^{+/}Ca^{2+}$ exchanger was first described in cardiac muscle and squid axon in the late 1960s (Reuter and Seitz, 1968; Baker et al., 1969). Since then it has been identified in many other tissues (smooth muscle, skeletal muscle, nervous system, blood cells, kidney, eye, secretory cell, etc) and plays an important physiological role (Blaustein and Lederer, 1999). The function of NCX1 is best defined in the heart, where it contributes to Ca^{2+} efflux during diastole (Philipson and Nicoll, 2000). The Na^+/Ca^{2+} exchanger is also related to several pathophysiological conditions. For example, salt-sensitive hypertension is triggered by Ca^{2+} entry via Na^+/Ca^{2+} exchanger in vascular smooth muscle (Iwamoto et al., 2004b). Alzheimer's disease brain tissues show increased Na^+/Ca^{2+} exchanger activity (Colvin et al., 1994; Colvin et al., 1991). The exchanger is tightly regulated in a spatial-temporal manner to fulfill its diverse physiological roles. Exploring the regulation mechanism of the exchanger would contribute to better understanding the important physiological role of the exchanger.

A. Na^+/Ca^{2+} exchanger family and isoforms

Na^+/Ca^{2+} exchanger protein belongs to cation/Ca^{2+} exchanger superfamily, whose members are found in organisms ranging from bacteria to man (Philipson and Nicoll, 2000). In 1997, Schwarz and Benzer noted two internally similar regions from a *Drosophila* homolog of NCX1. The two regions, now known as α-1 and α-2 repeats, are highly conserved among known cation/Ca^{2+} exchanger proteins (Schwarz and Benzer, 1997). In higher eukaryotes, the Na^+/Ca^{2+} exchanger is encoded by two multigene families: the K^+-independent *SLC8* Na^+/Ca^{2+} exchanger (NCX) and the K^+-dependent *SLC24* $Na^+/Ca^{2+}+K^+$ exchanger (NCKX) (Cai and Lytton, 2004; Quednau et al., 2004; Schnetkamp, 2004).

The mammalian NCX exchanger gene family encompasses three proteins, NCX1, NCX2, and NCX3, which are thought to exchange either 3 or 4 extracellular Na^+ ions for 1 intracellular Ca^{2+} ion (Blaustein and Lederer, 1999; Fujioka et al., 2000). NCX1 is expressed in many tissues, with high levels in cardiac muscle, brain and kidney (Lee et al., 1994; Quednau et al., 1997). NCX2 and NCX3, in contrast, are predominantly expressed in the brain and skeletal muscle (Li et al., 1994; Nicoll et al., 1996b). In rat brain, NCX1, NCX2 and NCX3 are differentially expressed in a cell - specific manner (Thurneysen et al., 2002; Papa et al., 2003), suggesting the different subtypes may play specific functional roles. Since the different isoforms show very similar functional properties when expressed in recombinant cell lines, distinct *in vivo* functional roles are likely to be clue to differences in regulation of expression, function or subcellular

location. The three NCX isoforms share about 70% overall amino acid sequence identity and more than 80% identity within the predicted transmembrane segments (Nicoll et al., 1996b). The large intracellular loop of NCX1 and NCX3, but not NCX2, undergoes extensive alternative splicing in a tissue-specific manner (Quednau et al., 1997).

For the NCKX gene family, five members, NCKX1 through NCKX5, have been identified so far. The NCKX proteins are distinguished from the NCX proteins by their absolute requirement for K^+ for their function. NCKX proteins transport 4 Na^+ in exchange for one Ca^{2+} and one K^+ (Dong et al., 2001). A key acidic residue, aspartic acid, corresponding to amino acid 575 of human NCKX2, appears to be responsible for the K^+-dependence. Asp^{575} is conserved in the NCKX1–5 proteins whereas an asparagine is found at this position in the NCX1-3 proteins. Substitution of Asp^{575} abolishes the functional requirement for potassium of NCKX2 (Kang et al., 2005). NCKX1 is only found in retina and platelets and is the major Ca^{2+} extrusion means in photoreceptors. It plays a crucial role in visual transduction (Kimura et al., 1999; Fain et al., 2001). NCKX2 is mainly expressed in brain and shows an important role in motor learning and memory (Li et al., 2006; Tsoi et al., 1998). NCKX3 and NCKX4 are more broadly expressed but their physiological roles have not been well-established (Kraev et al., 2001; Li et al., 2002; Tsoi et al., 1998). NCKX5 is primarily expressed in eye and skin and is genetically linked to skin pigmentation (Lamason et al., 2005). Interestingly, NCKX5 seems to be located on intracellular membranes whereas other NCKX proteins are expressed on the cell surface. The function of NCKX5 has not been determined yet. Alternative splicing variants are found in NCKX1, NCKX2, NCKX3 and NCKX4. Most alternative splicing

regions are within the large cytosolic loop that separates two sets of transmembrane spanning segments. However, the functional impact of these isoforms is largely unknown.

Although little sequence identity exists between the NCX and NCKX families, there is high sequence identity within the α-repeat regions, implying that they may have diverged from a common ancestor (Cai and Lytton, 2004).

B. NCX1: a molecular perspective

NCX1, which was first cloned in 1990 by screening an expression library with a polyclonal antibody (Nicoll et al., 1990), is the best studied member in the NCX protein family. It is composed of 970 amino acids including an N-terminal signal peptide of 32 amino acids, which is not functionally important (Sahin-Toth et al., 1995). So the full-length mature cardiac exchanger is 938 amino acids long, with a predicted molecular mass of 110 kDa. However, SDS-PAGE reveals two prominent protein bands at 70 and 120 kDa under reducing conditions (Nicoll et al., 1990; Philipson et al., 1988). The 120 kDa band is the mature glycosylated protein while the 70 kDa band is a proteolytic fragment which is partially functional (Gabellini et al., 1996; Li and Lytton, 1999; Saba et al., 1999; Van et al., 2001). When run on SDS gels under non-reducing conditions, the truncated protein as well as the full length NCX1 run at the size of ~140 kDa and ~220 kDa, respectively, which suggests that NCX1 may form a dimer in the membrane (Li and Lytton, 1999).

Properties of Na^+/Ca^{2+} exchanger

The exchanger activity is associated with a current since 3 Na^+ or 4 Na^+ ions are transported in exchange for 1 Ca^{2+} ion. The divalent cation Ni^{2+} blocks the current and thus it is often used to identify the Na^+/Ca^{2+} exchanger-mediated currents in electrophysiological experiments when other Ni^{2+}-sensitive ionic currents have been already inhibited by other agents (Kimura et al., 1987; Blaustein and Lederer, 1999). The Na^+-Ca^{2+} exchange process is reversible, with the direction of Ca^{2+} movement depending upon the thermodynamic driving force as determined by the membrane potential and the concentrations of Na^+ and Ca^{2+} sensed by the exchanger. Thus, Na^+/Ca^{2+} exchanger can mediate Ca^{2+} influx (reverse mode) as well as Ca^{2+} efflux (forward mode) (Blaustein and Lederer, 1999; Kang and Hilgemann, 2004). Common selective inhibitors include KB-R7943 and SEA0400, both of which preferentially inhibit the reverse mode of NCX (Iwamoto and Kita, 2004). Compared to KB-R7943, SEA0400 is more potent and specific.

Topology

The most recent topological model of the mature NCX protein predicts nine transmembrane segments (TMs) with extracellular N-terminus and intracellular C-terminus (Figure 2). The N-terminal extracellular domain contains the only N-linked glycosylation site at position 9 (Asn9) (Hryshko et al., 1993). The nine transmembrane segments (TMs) are organized into two clusters. A large intracellular loop links these two

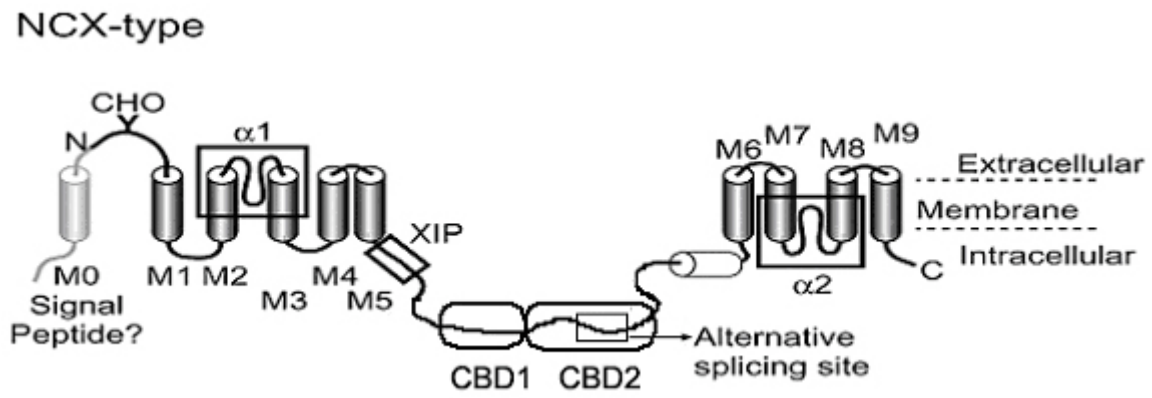


Figure 2: Proposed membrane topology of NCXs. CHO: site of glycosylation; XIP: exchanger inhibitory peptide; CBD: Ca^{2+} binding domain. Figure modified from Lytton, J. Membrane Transporters: Na^{+}/Ca^{2+} exchanger. In Lennarz, W.J. & Lane M.D., eds. *Encyclopedia of Biological Chemistry* Elsevier, Oxford, 2004; Vol. 2; pp. 631-636

clusters by connecting TM5 and TM6. Although the large intracellular loop is more than half of the length of the protein (about 550 amino acids), TM6 is in the proximity of TMs 1 and 2 (Ren et al., 2006). The α-1 repeat consists of portions of TM2, TM3, and the loop connecting them. The α-2 repeat consists of part of TM7 and the neighbouring reentrant loop. The α-1 and α-2 repeats are oppositely oriented with respect to the plasma membrane (Nicoll et al., 1999; Iwamoto et al., 1999). The α-repeats are conserved among NCX proteins and mutagenesis studies demonstrate their importance in ion binding and transport activity (Nicoll et al., 1996a; Iwamoto et al., 2000; Kinjo et al., 2005; Kang et al., 2005). Cysteine cross-linking shows that TM2 and TM3 adjoin TM7 and TM8, suggesting the two functionally important domains in the exchanger, the α -1 and α -2 repeats, are close to each other in the folded protein (Qiu et al., 2001). The large cytosolic loop is not essential for transport function, since a mutant lacking a considerable portion of the loop region still retains exchange activity (Matsuoka et al., 1993). Instead, it is involved in various regulatory events such as Na^+ dependent inactivation, phosphorylation, and regulatory Ca^{2+} binding, as described in more detail below (Hilgemann, 1990; Matsuoka et al., 1995; Philipson and Nicoll, 2000).

Alternative splicing

NCX1 is alternatively spliced to generate a variety of isoforms in a tissue-specific manner (Barnes et al., 1997; Kofuji et al., 1994; Lee et al., 1994; Quednau et al., 1997). There are six alternatively spliced exons (A to F), which code for a region within the large intracellular loop. Exons A and B are mutually exclusive and the presence of only

one is required to maintain the correct open read frame. Different NCX1 variants contain either exon A or B, together with any combination of exons C, D, E, and F. Theoretically, 32 different isoforms can be produced. So far, at least 12 splice variants have been detected. Interestingly, excitable cells (heart and neurons) usually contain exon A, while non-excitable cells (kidney, smooth muscle, astrocytes) mainly contain exon B (Kofuji et al., 1994; Lee et al., 1994; Quednau et al., 1997). In cardiac myocytes, the combination of ACDEF is the predominant form (NCX1.1). Although no distinctive functional differences have been observed, the isoforms display different regulatory properties. For example, AD isoform (brain splice variant, NCX1.4) can be activated by protein kinase A, whereas BD (kidney splice variant, NCX1.3) isoform cannot (He et al., 1998). Sodium-dependent inactivation of NCX1.3 is considerably more pronounced than that of NCX1.4. High intracellular Ca^{2+} can alleviate intracellular Na^{+}-dependent inactivation for NCX1.4 but not for NCX1.3 (Dyck et al., 1999; Dunn et al., 2002). Expression of NCX1.3 protects against severe Ca^{2+} overload, whereas NCX1.1 promotes Ca^{2+} overload under certain condition (Hurtado et al., 2006). NCX1.1 and NCX1.3 show differential sensitivities to alpha-linolenic acid (Ander et al., 2006). Since calcium handling is dramatically disparate in different tissues, the different isoforms with distinctive regulatory properties may be selectively designed to adapt to the specific cellular requirements.

Regulation of NCX1

Ca^{2+} plays a number of roles in various tissues. As the major Ca^{2+} extrusion protein, NCX1 is highly regulated in many different ways to control intracellular [Ca^{2+}] precisely. Some common and important regulatory mechanisms are described here.

1. Exchanger inhibitory peptide (XIP)

A segment with 20 amino acids at the N-terminal of the large intracellular loop of NCX1 shows interspersed hydrophobic and basic residues similar to a calmodulin binding site. A synthetic peptide with the same amino acid sequence, called exchange inhibitory peptide (XIP), potently inhibits NCX function (Li et al., 1991; DiPolo and Beauge, 1994). The endogenous XIP region plays an autoregulatory role in exchanger function. XIP-region mutants showed altered Na^{+}-dependent inactivation and phosphatidylinositol-4, 5-biphosphate (PIP_2) interaction (He et al., 2000; Matsuoka et al., 1997).

2. Intracellular Ca^{2+}

NCX1 contains a high affinity Ca^{2+} binding site on the large intracellular loop. Regulatory Ca^{2+}, which is different from that used for transport, interacts with this site to regulate NCX activity (Levitsky et al., 1994; Matsuoka et al., 1995). When the exchanger is working under the reverse mode, Ca^{2+} is transported from the outside of the cell to the inside. However, removal of intracellular Ca^{2+} completely blocks NCX activity

(Philipson and Nicoll, 2000). Thus the regulatory Ca^{2+} is absolutely required for full exchanger activity. The interaction with regulatory Ca^{2+} involves a sequence of 135 amino acids containing two groups of aspartate residues (Matsuoka et al., 1995). Ca^{2+} binding induces marked electrophoretic mobility shifts (Levitsky et al., 1994), indicating substantial conformational change of the NCX protein when it binds to Ca^{2+}. The apparent Ca^{2+} affinity for the binding is controversial. In excised patches, the $K_{1/2}$ for regulation is about 0.2–0.6 μM (Collins et al., 1992; Fujioka et al., 2000; Matsuoka et al., 1995). However, indirect measurements using intact cells show a lower $K_{1/2}$ of 20–50 nM (Fang et al., 1998). If the $K_{1/2}$ is between 20-50 nM in native cells, the regulatory sites would always be saturated. Then this regulation may be little of physiological relevance. However, if the $K_{1/2}$ is within submicromolar range, then the regulatory mechanism should be very important since the exchanger would only be activated to extrude Ca^{2+} when the cytosolic Ca^{2+} concentration has been elevated. The different results from excised patches and intact cells are likely due to different conditions in the experiments. Regulation in whole cells with $K_{1/2}$ of ~300 nM has now been observed (Reeves and Condrescu, 2003). The regulatory effect of Ca^{2+} appears slow, not beat-to-beat, but cumulative (Chernysh et al., 2004). It is noted that both forward mode and reverse mode of operation of the exchanger are absolutely dependent on internal Ca^{2+} (Blaustein and Lederer, 1999). Recently, structure studies have defined the architecture of the regulatory Ca^{2+} binding domains (CBD) (Hilge et al., 2006; Nicoll et al., 2006). CBD1 is consistent with the previous identified Ca^{2+} regulatory site, while CBD2 is newly determined. CBD2, which immediately follows CBD1, exhibits about 27% sequence identity with CBD1 and also has two acidic segments. CBD1, which has a high

Ca^{2+} affinity with a K_d value of 120 to 240 nM, is the primary Ca^{2+} sensor responsible for Ca^{2+} activation. CBD2, on the other hand, with a much lower Ca^{2+} affinity (K_d of 820 nM to 8.6 μM), is related to the effect of Ca^{2+} on Na^+ inactivation, as explained in detail below.

3. Intracellular Na^+

In contrast to the activation induced by intracellular Ca^{2+}, intracellular Na^+ exhibits an inhibitory effect. When outward NCX activity is elicited by raising intracellular Na^+, current develops rapidly, rising to a peak that decays to a steady state level. The current decay process is similar to that commonly observed in ion channels, so the process was called Na^+-dependent inactivation (Hilgemann et al., 1992).

The endogenous XIP region is involved in Na^+-dependent inactivation since mutations within the XIP region either change the kinetics or totally abolish inactivation (Matsuoka et al., 1997). Intracellular Ca^{2+} also affects Na^+-dependent inactivation (Matsuoka et al., 1995). Interestingly, NCX1 alternatively spliced isoforms exhibit different ionic regulatory properties. Na^+-dependent inactivation was alleviated by high intracellular Ca^{2+} in exon A-containing isoforms but not in exon B-containing variants (Dyck et al., 1999; Dunn et al., 2002). The difference is due to the variance in CBD2 which contains the region subjected to alternative splicing. An aspartic acid residue in exon A which is critical in coordinating Ca^{2+} ions is substituted by an arginine residue in exon B. This change is sufficient to prevent Ca^{2+}-dependent alleviation of the Na^+ dependent

inactivation (Dunn et al., 2002). Compared to non-excitable cells (containing exon B), excitable cells (containing exon A) experience much larger fluctuations in terms of membrane potential and intracellular concentrations of Na^+ and Ca^{2+}. The different ionic regulation between these NCX1 isoforms may service as a specialized adaptation to variant cellular environments.

4. Intracellular H^+

Besides Na^+, intracellular H^+ can strongly inhibit the activity of NCX (Philipson et al., 1982; Doering and Lederer, 1993). The site of the H^+ effect is within the large intracellular loop (Espinosa-Tanguma et al., 1993). Interestingly, the inhibitory action of Na^+ and H^+ seems cooperative. Without intracellular Na^+, H^+ has little effect. On the other hand, at high pH (low H^+), intracellular Na^+ does not generate inactivation (Doering and Lederer, 1994). The regulatory action of protons may be important under some pathophysiological conditions. For example, in the case of ischemia, the intracellular Na^+ and H^+ concentration increase. The deregulation of these ions may affect the function of NCX protein by inhibiting the reverse NCX activity, thus limiting the Ca^{2+} entry through NCX.

5. Phosphorylation

Phosphorylation stimulates Na^+-Ca^{2+} exchange activity in the squid giant axon (DiPolo and Beauge, 1991). The mechanism underlying this effect involves an increase in the

affinity for intracellular Ca^{2+} and extracellular Na^{+}. Protein kinase PKA and PKC can modulate the activity of NCX1. The effects of PKA on the cardiac Na^{+}/Ca^{2+} exchanger are quite controversial. Iwamoto *et al.* reported PKA activation did not enhance phosphorylation of NCX1 expressed in CCL39 fibroblasts (Iwamoto et al., 1998), while Ruknudin *et al.* showed *in vitro* phosphorylation of NCX1 immunoprecipitated from Xenopus oocytes expressing the exchanger (Ruknudin et al., 2000). There is also debate as to whether exchange activity is affected by PKA activation. Some groups have suggested no effect of PKA on exchanger activity (Ballard and Schaffer, 1996; Collins et al., 1992; He et al., 2003) but other groups have reported stimulation (Perchenet et al., 2000; Wei et al., 2003). It is possible that previous studies using Ni^{2+}- sensitive current to define NCX1 activity may have been confounded by contamination with PKA-sensitive cystic fibrosis transmembrane conductance regulator (CFTR) chloride current (Lin et al., 2006). Agents acting via PKC can modulate phosphorylation and exchanger activity (Iwamoto et al., 1996). However, the effect of PKC on exchanger activity does not require direct phosphorylation of the exchanger protein (Iwamoto et al., 1998).

6. ATP

Although hydrolysis of ATP is not required to power the exchanger, it is observed that ATP can increase the activity of Na^{+}/Ca^{2+} exchanger (Hilgemann, 1990). Several mechanisms may be involved here. First, ATP is required for any phosphorylation processes that might stimulate NCX1 activity. Second, ATP can activate PKA and/or PKC through G-protein coupled receptors. PKA and/or PKC in turn may stimulate the

exchanger activity. Indeed, exchange activities of NCX1 and NCX3 were modestly increased by agents that activate protein kinases A and C (Linck et al., 1998). Third and most importantly, ATP induces the production of the signaling lipid PIP_2. PIP_2 directly interacts with the XIP region of the exchanger to prevent its inhibitory effect, thus activating NCX1 by eliminating Na^+-dependent inactivation (He et al., 2000; Hilgemann and Ball, 1996).

7. NCX interacting proteins

Protein interaction is important in the regulation of protein activity and sub-cellular distribution. In recent years, many proteins have been reported to interact with NCX1 and modulate its function.

Calcineurin depresses exchanger activity by interacting with the Ca^{2+} regulatory site within the large intracellular loop of NCX (Katanosaka et al., 2005). Calcineurin is known to promote pathological hypertrophic responses (Vega et al., 2003; Molkentin and Dorn II, 2001) and association with calcineurin is enhanced in rat heart with cardiomyopathy. Thus depressed NCX activity by calcineurin may play a role in the development of myocardial hypertrophy and the subsequent contractile dysfunction.

14-3-3 proteins, which are widely expressed in eukaryotes, also play an inhibitory role when they interact with the NCX proteins (Pulina et al., 2006). They bind preferentially to phosphorylated NCX protein (Pulina et al., 2006) and the interacting site is within the

large intracellular loop. Since 14-3-3 proteins have a similar inhibitory effect on PMCA (Rimessi et al., 2005), they may be a common means to modulate Ca^{2+} extrusion.

Phospholemman (PLM), a 15-kDa integral sarcolemmal phosphoprotein, functions as another endogenous protein inhibitor of cardiac NCX1 (Ahlers et al., 2005). PLM co-localizes and co-immunoprecipitates with NCX1, both in cardiac myocytes and in transfected HEK293 cells (Ahlers et al., 2005). The interacting site is the cytoplasmic domain of PLM and the N-terminal part of the big intracellular loop of NCX1 (Wang et al., 2006). PLM also inhibits the activity of Na^+/K^+ ATPase (Crambert et al., 2002). Interestingly, phosphorylation at serine 68 in PLM is required for the inhibition of NCX1 (Zhang et al., 2006). In contrast, phosphorylation of PLM results in relief of inhibition of Na^+-K^+-ATPase (Despa et al., 2005).

Caveolin-3, but not caveolin-1 or caveolin-2, interacts with the NCX1 protein in bovine cardiac sarcolemmal vesicles (Bossuyt et al., 2002). Caveolin proteins are important scaffolding proteins of caveolae, the cholesterol-rich invaginations of the plasma membrane. Signaling complexes are concentrated in caveolae microdomains, an arrangement which is thought to increase the efficiency of signal transmission (Schlegel and Lisanti, 2001). Both NCX1 transport activity and caveolin-3 co-precipitation with NCX1 are decreased when the structure of caveolae is disrupted by depletion of cholesterol (Bossuyt et al., 2002). In addition to NCX1, plasma membrane Ca^{2+}-ATPase (Hammes et al., 1998) and L-type Ca^{2+} channel have been localized to caveolae in cardiomyocytes (Balijepalli et al., 2006). These proteins may be involved in caveolar

transmembrane signaling by modulating Ca^{2+} levels, which in turn regulate a variety of caveolar proteins like Ca^{2+}-sensitive isoforms of adenylyl cyclase and endothelial NO synthase (Crossthwaite et al., 2005; Barouch et al., 2002).

The cytoskeletal protein ankyrin binds to NCX1, which provides a possible mechanism to account for the specific NCX1 sub-cellular distribution (Lencesova et al., 2004; Li et al., 1993). It has been demonstrated that the targeting and stability of NCX1 in cardiomyocytes requires direct interaction with the membrane adaptor ankyrin-B (Cunha et al., 2006). In heart, NCX1, Na^+/K^+ ATPase, and IP_3 receptor are mutually co-immunoprecipited and co-localized in a cardiac T-tubule/SR microdomain coordinated by ankyrin B (Mohler et al., 2005; Lencesova et al., 2004; Li et al., 1993). It is proposed that the ankyrin-B-based complex is a special adaptation of cardiomyocytes with a role for cytosolic Ca^{2+} modulation.

C. Physiological role of NCX1

Heart muscle

In cardiac myocytes, NCX1 is most abundant in the T-tubular membrane (Frank et al., 1992; Kawai et al., 1999; Despa et al., 2003) and works as a major player in excitation-contraction (E-C) coupling. Excitation induces a rapid plasma membrane depolarization. This process opens voltage-gated Ca^{2+} channels (VGCC). Ca^{2+} entry through the

channels activates ryanodine receptors located on the SR to release much more Ca^{2+} through calcium-induced calcium release (CICR) mechanism. The released Ca^{2+} greatly increases the local Ca^{2+} concentration, which activates the myofilaments to produce cardiac contraction. During muscle relaxation, the same amount of Ca^{2+} that entered the cytoplasm is exported out of cell by the NCX and PMCA or pumped back into SR by SERCA to maintain Ca^{2+} homeostasis. Compared to Ca^{2+}-ATPases, NCX1 has a relatively low affinity for cytosolic Ca^{2+} [Michaelis-Menten constant (K_m) = 3.8 μM] (Matsuoka et al., 1993) but a high turnover number (~5,000 s^{-1}) (Hilgemann, 1996). Evidence suggests the Na^+/Ca^{2+} exchanger is the predominant pathway for Ca^{2+} extrusion (Philipson and Nicoll, 2000; Shigekawa and Iwamoto, 2001).

Surprisingly, cardiac-specific NCX1 knockout mice can survive and exhibit near normal cardiac function (Henderson et al., 2004). The reduction of NCX1 is not compensated by increased expression of PMCA. Instead, the L-type Ca^{2+} current is reduced to 50% of the wild-type control. Although the expression of the L-type channel is unchanged, knockout of NCX enhances subsarcolemmal [Ca^{2+}], which in turn promotes Ca^{2+}-dependent inactivation of I_{Ca} (Pott et al., 2006b). In addition to the interplay with L-type Ca^{2+} channel, NCX1 may also affect Kv channel expression (Pott et al., 2006a). Cardiac-specific NCX1 knockout mice exhibit an upregulated expression of I_{to} generating potassium channel subunit Kv4.2, an increased I_{to} and a shortened action potential duration, which in turn limits Ca^{2+} entry. The effect of NCX1 on the L-type Ca^{2+} channel and potassium channel may be essential for maintaining Ca^{2+} homeostasis in both healthy and diseased heart.

In hearts of many neonatal mammals, the sarcoplasmic reticulum is poorly developed. In addition, the expression of cardiac NCX1 is highest in newborns, suggesting plasma membrane Ca^{2+} flux and NCX activity are more crucial for neonates (Artman et al., 1995; Vetter et al., 1995; Koban et al., 1998). Homozygous NCX1-deficient mice die between embryonic days 9 and 10. They have no heartbeat and show cardiomyocyte apoptosis, suggesting Na^{+}/Ca^{2+} exchanger is really essential for normal heart function during development (Wakimoto et al., 2000). Surprisingly, transgenic re-expression of NCX1 in cardiac myocytes does not prevent the death of NCX1-deficient mice (Cho et al., 2003) and mice with cardiac-specific NCX1 knockout beginning during embryonic development are viable (Henderson et al., 2004). These results suggest NCX1 may play some important extra-cardiac roles during embryonic development.

Brain

NCX1 protein is expressed in both neurons and glial cells (Yip et al., 1992; Takuma et al., 1994) and plays a crucial role in many physiological processes by shaping the intracellular $[Ca^{2+}]$ (Fontana et al., 1995; Gill et al., 1984; Goldberg et al., 2003; Hoyt et al., 1998; Kim et al., 2005; Sanchez-Armass and Blaustein, 1987; Takuma et al., 1996).

The expression level of NCX1 is very high in neurons, especially at synapses (Juhaszova et al., 1996). NCX activity is responsible for the removal of elevated $[Ca^{2+}]_i$ in presynaptic nerve terminals (Reuter and Porzig, 1995). When the exchanger operated in reverse mode in ouabain-containing, Na^{+} free solution, presynaptic terminal excitability

changed (Doi et al., 2002) and exocytosis of synaptic vesicles were observed (Reuter and Porzig, 1995).

In glial cells, the exchanger is involved in controlling the amplitude of Ca^{2+} transients (Takuma et al., 1996; Matsuda et al., 1996). A recent report suggests activation of the Na^{+}/Ca^{2+} exchanger is required for the release of homocysteic acid (HCA), an endogenous agonist for glutamatergic N-methyl-D-aspartate (NMDA) receptors (Benz et al., 2004).

The role of the exchanger in synaptic plasticity has also been demonstrated. When cytosolic Na^{+} increases following α-amino-3-hydroxy-5-methyl-4-isoxazolone-propionate (AMPA) receptor activation, Ca^{2+} is taken into the cells via NCX reverse mode. The Ca^{2+} in turn can trigger Ca^{2+} release from intracellular stores (Smith et al., 2000; Fink et al., 2002). This role for NCX in Ca^{2+} entry is supported in Purkinje neurons because inhibition of NCX activity depressed Ca^{2+} transients induced by AMPA receptor activation (Kim et al., 2005).

Kidney

In kidney, NCX has been found most abundantly in the basolateral membrane of distal convoluted tubule and connecting tubule where it contributes to active Ca^{2+} reabsorption (Blaustein and Lederer, 1999). Experiments suggest parathyroid hormone (PTH)

stimulates distal nephron Ca^{2+} reabsorption by increasing Na^{+}/Ca^{2+} exchanger activity (Hanai et al., 1986; Bouhtiauy et al., 1991).

III. The Function and Physiological Role of Tyrosine Kinase Src

v-Src, which was found in the viral genome in 1970s (Martin, 2004; Brugge and Erikson, 1977), was the first identified viral oncogene. The cellular counterpart - c-Src - was the first proto-oncogene to be discovered in vertebrates. Src is a non-receptor tyrosine kinase belonging to the non-receptor tyrosine kinase family, which also includes Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck and Lyn (Hubbard and Till, 2000). Since its discovery, c-Src activity has been found in multiple signaling pathways and regulates the function of numerous proteins, including many membrane proteins. Interestingly, tyrosine kinase inhibitors tyrphostin A23 and tyrphostin A25 activated membrane current generated by NCX in cardiac myocytes (Missan and McDonald, 2004). So c-Src is a logical candidate as a potential regulator of NCX1.

A. The structure of Src

Src kinase has a myristoylated N-terminal segment followed by SH3 (Src-homology), SH2 and tyrosine kinase domains, and a short C-terminal tail (Figure 3). The myristoylation site is important for membrane localization. The SH3 domain binds to

proline-rich sequences with a core consensus sequence of PxxP (where "x" can be any amino acid) (Ren et al., 1993). The SH2 domain typically binds to specific motifs containing phosphorylated tyrosine (Moran et al., 1990). The kinase domain contains an autophosphorylation site (Tyr419 in human), whose phosphorylation is required for full activation of the kinase. The C-terminal tail contains a negative-regulatory tyrosine residue (Tyr530 in human). When phosphorylated, Tyr530 interacts with the SH2 domain to inactivate the kinase (Yeatman, 2004). Crystallographic studies have shown that interactions between the C-terminus and the SH2 domain, and between the kinase domain and the SH3 domain, make the c-Src molecule form a closed configuration in which the kinase domain is covered and the ability to interact with substrate is reduced (Boggon and Eck, 2004).

B. Activation mechanisms

c-Src kinase activity can be regulated by extracellular signaling as well by cytoplasmic phosphatases. Activation of ligand-activated receptor tyrosine kinases, such as epidermal growth factor receptor (Tice et al., 1999), platelet-derived growth factor receptor (Bowman et al., 2001) and fibroblast growth factor receptor (Landgren et al., 1995), can augment Src activity, probably by interacting with SH2 and/or SH3 domains of Src, thus disrupting the intramolecular interactions that hold Src in an inactive configuration. The mechanism for tyrosine phosphatase to activate c-Src involves the dephosphorylation of C-terminal P-Tyr530 negative regulatory site (Egan et al., 1999; Roskoski, Jr., 2005).

A:

B:

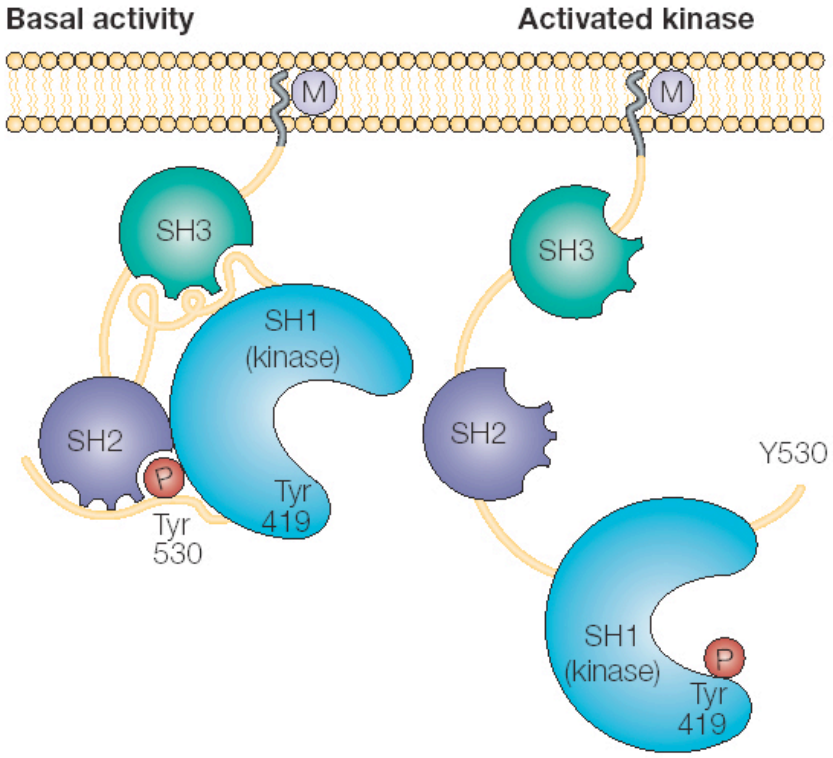


Figure 3: Structure and activation of Src proteins. *A:* The Src protein contains four domains homologous with other proteins (SH1 - SH4). SH1 contains the kinase domain and a conserved tyrosine residue involved in autophosphorylation (Tyr419). Tyr530 can bind to the SH2 domain when phosphorylated. *B:* Inactivation of human c-Src occurs when its phosphorylated C-terminal Tyr530 binds to the SH2 domain and its SH3 domain interacts with its kinase domain. These interactions generate a closed molecular structure with reduced access of substrates to the kinase domain. Conversely, c-Src activation involves dephosphorylation of the C-terminal phosphotyrosine and displacement of inhibitory intramolecular interactions, resulting in an open c-Src molecular structure. Phosphorylation at Tyr419 is required for full activation. M, myristoylation; P, phosphorylation. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Yeatman, 2004)

C. Src and cell signaling

Src plays a significant role in signal transduction. A wide range of substrates have been identified, including focal adhesion proteins, adaptor proteins and transcription factors (Bowman et al., 2001; Courtneidge, 2003). Moreover, Src also regulates several ion channels, e.g., the transient receptor potential (TRP) family of ion channels (Jin et al., 2004; Hisatsune et al., 2004; Xu et al., 2003), KCNQ potassium channels (Gamper et al., 2003), HERG K^+ channel (Cayabyab and Schlichter, 2002), L-type Ca^{2+} channel (Schroder et al., 2004), and NMDA receptor (Wang and Salter, 1994). Usually, Src exerts effects via two mechanisms: by binding to substrate via the SH2 and SH3 domains and /or by phosphorylation of target tyrosines (Martin, 2001). Distinct effects of Src actions by these two mechanisms have been described for Kv1.4 and Kv1.5 potassium channels (Nitabach et al., 2002). In addition, overexpression and/or increased activity of Src may cause changes in the expression level of numerous genes. It's now possible to assess the global effect of Src expression on other genes by using gene-expression profiling techniques, referred to as the Src fingerprint (Irby and Yeatman, 2000).

IV. Protein Glycosylation

Secreted and membrane proteins are frequently modified by glycosylation, a post-translational process of covalent attachment of sugar residues. It is estimated that more

than half of all eukaryotic protein species are glycoproteins (Apweiler et al., 1999). Glycosylation can be divided into four different types mainly depending on the linkage between the amino acid and the sugar. These are N-linked glycosylation, O-linked glycosylation, C-mannosylation, and glycophosphatidlyinositol (GPI) attachment. In N-linked glycosylation, the oligosaccharide is attached to the amino group (NH_2) of an asparagine. Glycosylation is known to be involved in many crucial biochemical events, including intracellular trafficking, proteinase susceptibility, molecular conformation, protein stability, protein-protein interactions, enzymatic activity, etc (Stanley, 1992; Varki, 1993; Hounsell et al., 1996). Oligosaccharide structures are associated with many physiological events (Helenius and Aebi, 2001) while deregulation of glycan synthesis causes a growing number of human genetic diseases, e.g., rheumatoid arthritis and systemic lupus erythrematosus (Malhotra et al., 1995).

The process and regulation of N-linked glycosylation

Asparagine (N)-linked glycosylation is an important modification of proteins and is universally observed in eukaryotic cells (Lowe and Marth, 2003). It is initiated during protein synthesis and the formation of a single complex N-linked oligosaccharide requires more than 30 different enzymes. These enzymes include glycotransferases, which are specific sugar-transferring enzymes and glycosidases which trim specific monosaccharides from precursors to form intermediate structures. The consensus amino acid sequence required for N-linked oligosaccharide attachment is Asn-Xaa-Ser/Thr, in which Xaa may be any amino acid other than proline. Occasionally, such as in the

leukocyte surface protein (CD69), the amino acid sequence of Asn-Xaa-Cys is an acceptable motif. To be glycosylated, the consensus sequence must be found at the surface of a protein and not buried within it. Moreover, because N-linked glycosylation is initiated in the lumen of the ER, target asparagine residues are limited to secretory proteins and in the portions of transmembrane proteins that face the lumen of the ER. Finally, to be effectively glycosylated in polytopic membrane proteins, the target asparagine must be located in an extracellular loop greater than 25 residues in size, more than 12 residues away from the preceding transmembrane segment, and 14 residues away from the following transmembrane segment (Popov et al., 1997). The consensus sequence may occur many times along the polypeptide chain. It is estimated that between 70 to 90% of potential N-glycosylation sites located in an appropriate context are occupied (Grogan et al., 2002).

In the endoplasmic reticulum (ER)

The synthesis of N-linked glycans is initiated in the endoplasmic reticulum and further modified in the Golgi apparatus where most of the structural variations are produced (Figure 4). It occurs in several stages (see Figure 4). First, Dol-p-p-$GlcNAc_2Man_5$ is formed by adding two N-acetylglucosamine (GlcNAc) residues and five mannose (Man) residues to a dolichol (Dol) phosphate on the cytosolic surface of the ER membrane. Then Dol-p-p-$GlcNAc_2Man_5$ is flipped to the luminal side of the ER and further enlarged to form the final dolichol-linked oligosaccharide intermediate (Dol-p-p-$GlcNAc_2$ Man_9Glc_3). $GlcNAc_2$ Man_9Glc_3 is transferred from Dol-p-p to the target asparagine

residue of the newly synthesized polypeptide by oligosaccharyl transferase. Following the transfer, the three glucoses are trimmed by glycosidase I and II, and terminal mannoses by one or more different ER mannosidases.

The ER is important in protein folding and quality control. ER-resident chaperones and folding enzymes help proteins fold and oligomers assemble. Proteins failing to reach the native conformation are retained in the ER and degraded by ER-associated degradation (Klausner and Sitia, 1990; Helenius and Aebi, 2004).

In ER, glycoproteins are of the high mannose-type, meaning five to nine mannose molecules are attached to the innermost GlcNAc residues.

In the Golgi apparatus

When the glycoprotein has folded correctly, it moves into the Golgi apparatus. The oligosaccharide is then further processed variably according to the action of a series of glycosyltransferases and glycosidases as the glycoprotein migrates through the Golgi (Dennis et al., 1999). The oligosaccharide remains high mannose-type before the enzyme N-acetylglucosaminyltransferase I (GlcNAc-T I) acts on the structure. Once this happens, the glycan becomes either a hybrid or complex-type. One-four antennae, which may be found as repeating units of $[GlcNAc(\beta1\rightarrow4)Gal]_n$, attach to the trimannosyl core ($GlcNAc_2Man_3$). Whereas hybrid-type glycans contain both substituted and unsubstituted mannose residues, the complex-type glycans contain only substituted mannose residues.

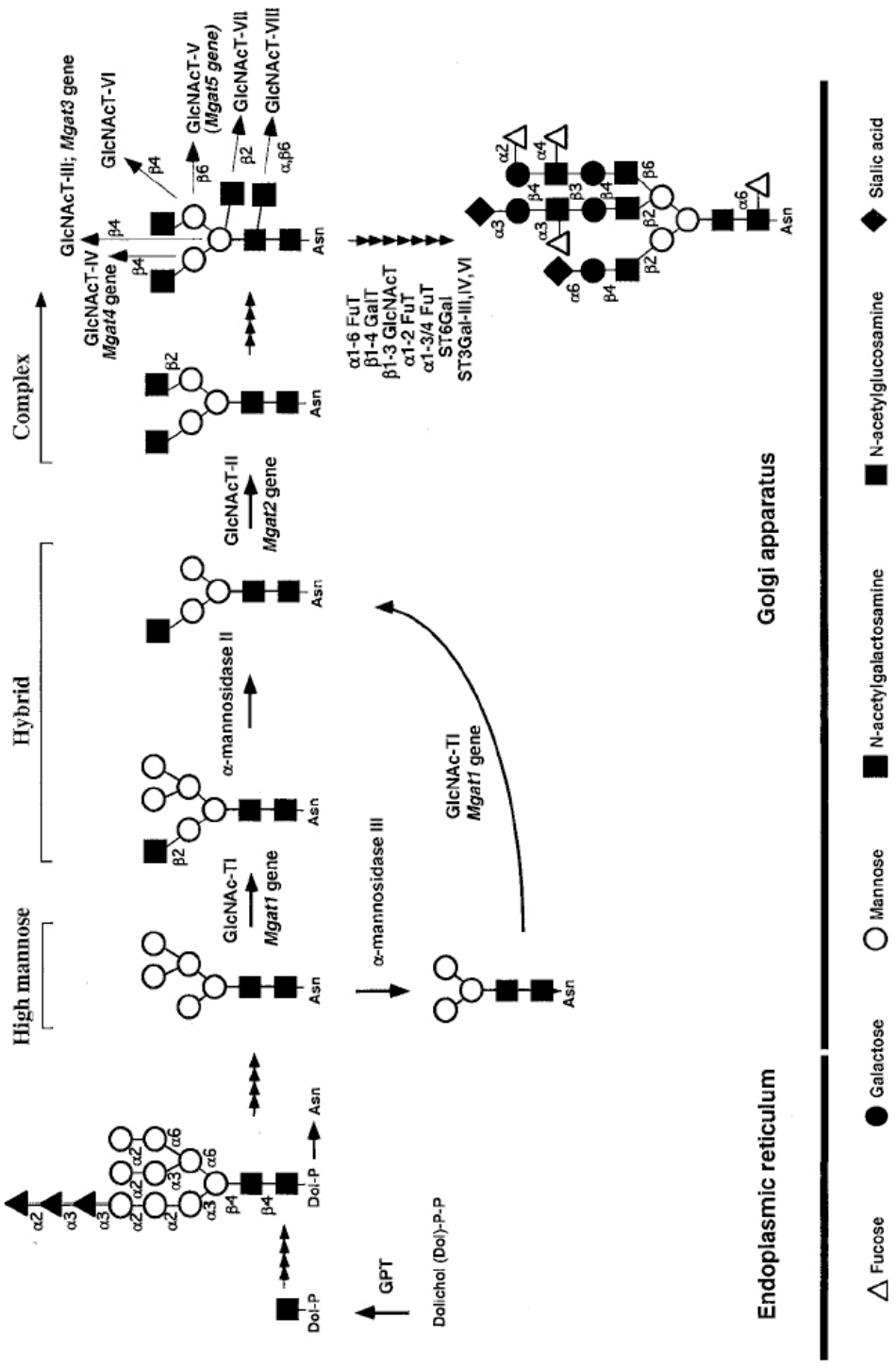


Figure 4: Processing of N-linked glycan (Lowe and Marth, 2003)**.** Reprinted, with permission, from the Annual Review of Biochemistry, Volume 72 (c) 2003 by Annual Reviews www.annualreviews.org

Negatively charged sialic acids are common constituents of complex glycans and are often the outmost residues. Further modification occurs if GlcNAc is attached to the trimannosyl core as a 'bisecting GlcNAc' and/or fucose is attached to the innermost GlcNAc, or on the outer antennae.

In mature glycoproteins, the N-linked glycans are structurally heterogenous. The composition and the size of the glycan branches vary among different proteins, cell types, tissues and species (Helenius and Aebi, 2001).

V. Reasoning and Research Goals

The reasoning that Src kinase is a potential regulator of NCX1 is based on following facts: First, both NCX1 and Src kinase are co-expressed at high level in many tissues and cells such as cardiomyocyte, smooth muscle cells, neurons, etc. Second, NCX1 is an essential participant in Ca^{2+} signaling and its activity is highly regulated by many factors. On the other hand, Src is a regulator of many Ca^{2+} handling proteins like the L-type Ca^{2+} channel. Third, most reports suggest Src activity tends to increase intracellular [Ca^{2+}] either thought plasma membrane proteins like Ca^{2+} channels (Wijetunge et al., 2000), nonselective cation channels (Albert et al., 2001) and store-operated channels (Babnigg et al., 1997), or by modulating intracellular messenger like IP_3 (Tolloczko et al., 2002).

Fourth, both Na^+/Ca^{2+} exchanger and Src kinase play roles in synaptic transmission and plasticity (Jeon et al., 2003; Li et al., 2006; Kalia et al., 2004; Purcell and Carew, 2003). Last but not the least, there is a report that treatment with tyrosine kinase inhibitors tyrophostin A23 and A25 induced a membrane current in guinea-pig ventricular myocytes (Missan and McDonald, 2004). The induced current was sensitive to Ni^{2+}, independent of Cl^- driving force, and suppressed by removal of either external Na^+ or Ca^{2+}, indicating that it was an NCX-related current. Inactive tyrosine kinase inhibitor tyrphostin A1 could not generate the current, suggesting the induced NCX current was indeed due to the inhibition of tyrosine kinases. This direct evidence that tyrosine kinase can modulate the activity of cardiac NCX1 motivated me to investigate the potential interaction between the Na^+/Ca^{2+} exchanger and Src tyrosine kinase.

The ***hypothesis*** is that Src plays a role in the modulation of NCX1. The hypothesis will be tested by co-transfecting NCX1 and c-Src into HEK293 cells, and the potential changes of NCX1 will be examined. The objectives of my research are listed as follows:

Objective 1: Characterize the structural change of NCX1 due to Src activity.

Objective 2: Determine the functional consequence of the change.

Objective 3: Explore the mechanism underlying the change.

Objective 4: Determine the specificity of the modulation.

Findings of this study will provide insight into the regulation of the Na^+/Ca^{2+} exchanger and regulation of Ca^{2+} signaling.

CHAPTER TWO

EXPERIMENTAL PROCEDURES

All molecular procedures were conducted according to standard protocols (Autry et al., 2006; Sambrook and Russell, 2001) or the instructions of the manufacturers, unless otherwise indicated. Common chemicals were obtained from Fisher, Sigma, or BDH and were of analytical grade or better. All restriction digests were performed using New England Biolabs (Beverly, MA) restriction enzymes according to the manufacturer's protocol. Nucleic acid and protein amino acid sequence analyses were performed with the MacVector software package (Oxford Molecular Group).

I. Oligonucleotide-directed mutagenesis

The constructs encoding rat heart NCX1.1 and mouse NCKX2 in the mammalian expression vector pcDNA3.1(+) (Invitrogen) were previously prepared in Dr. Jonathan Lytton's laboratory. Different strategies were used to make various mutants. The primers and restriction enzymes used are listed in Table 1.

PCR overlap extension approach was used to generate the glycosylation free mutant NCX1 (N9Q), and the TIG mutant of NCX1 (825TIG827 to 825AAG827). Upstream and downstream primers flanking convenient restriction sites were employed as external primers. Two complementary internal primers, mutation upstream and mutation

downstream, contained mutation to introduce the desired amino acid change. Two fragments were generated by PCR using two paired primers: upstream/mutation upstream and downstream/mutation downstream. The two fragments were combined and amplified again by PCR using the upstream and downstream primers.

For all the experiments, PCRs were conducted under the following conditions: initial denaturation at 94^{o}C for 2 min, followed by 29 cycles of denaturation at 94^{o}C for 20 s, annealing at 54^{o}C for 30 s and extension at 72^{o}C for 1.5 min, followed by a final cycle of extension at 72^{o}C for 5 min. PCR products were digested with appropriate restriction enzymes before cloning the fragments back into the templates digested with the same enzymes.

The deletion mutants of NCKX2 were created using the oligonucleotide linker method. Briefly, two oligos were dissolved in annealing buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.8) at a final concentration of 100 pmol/µl each. The oligos were annealed by heating 20 µl combined solution at 65°C for 15 minutes, and cooling down slowly to room temperature for an hour or so. The annealed oligos worked as a linker to ligate the plasmid that had been digested by the appropriate restriction enzymes without alkali-phosphatase treatment. For a 20 µl ligation system, 1.5 µl annealed solution was used.

All the constructs were confirmed by DNA sequencing using fluorescent dye terminators, which was performed at the University of Calgary Core DNA Services Facility.

Table 1: Summary of oligonucleotide primers used

NCX1 mutants

N9Q mutant
Upstream: AAC GGG ACT TTC CAA AAT GTC
Mutation upstream: AGT GGT TTC TTG TCC TCC TGT
Mutation downstream: ACA GGA GGA CAA GAA ACC ACT
Downstream: GGA CAC AGT CTC ATT CCA GA
Restriction enzymes: Sac I and Age I

TIG mutant
Upstream: GGA GAG CTG GAA TTC CAG AA
Mutation upstream: ATC TTT CAG ACC AGC GGC GCA GCC
Mutation downstream: GGC TGC GCC GCT GGT CTG AAA GAT
Downstream: GCT GAT CAG CGG GTT TAA AC
Restriction enzymes: BamH I and Cla I

NCKX2 loop deletion mutants

Mutant one
Primer 1: ACC AAC TTT A
Primer 2: CCG GTA AAG TTG GT
Restriction enzymes: EcoR V and Age I

Mutant two
Primer 1: CCG GTA GGG AGC
Primer 2: TAG CTC CCT A
Restriction enzymes: Age I and Nde I

Mutant three
Primer 1: TAT GGA AAA C
Primer 2: TGA GTT TTC CA
Restriction enzymes: Nde I and Bpu10 I

II. Cell culture and transfection

Human embryonic kidney cell line 293 (HEK293) transformed with a temperature sensitive SV40 large T antigen were originally from Ron Kopito's lab at Stanford. Chinese hamster ovary (CHO) cells were previously kept in Dr. Jonathan Lytton's lab. Both cell lines were cultured at 37^{o}C under a humidified 5% CO_2 atmosphere using high glucose Dulbecco's modified Eagle's Medium from Gibco (DME) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 1% MEM non-essential amino acids, and 100 U/ml penicillin/100 μg/ml streptomycin (all from Gibco).

Plasmid DNAs (Table 2) used for transfection were prepared using the EndoFree Plasmid Maxi Kit system from Qiagen (Mississauga, Ontario, Canada).

HEK293 cells at 30% ~ 40% confluence in P-100 plates were transfected by using a standard Ca^{2+}-phosphate precipitation protocol essentially as described previously (Tsoi et al., 1998). Briefly, a total of 10 μg DNA (for cotransfection with Src, Src made up 20% of total DNA in all experiments) was diluted to a final volume of 0.45 ml with water followed by addition of 50 μl 2.5 M $CaCl_2$. Then, this solution was added dropwise to 0.5 ml of 2XBES buffer (280 mM NaCl, 50 mM BES, 1.5 mM Na_2HPO_4, pH 7.0), using a 2 ml pipet and pipetting-aid to provide a steady stream of small bubbles from the bottom of the tube for mixing. 1 ml was added to each P-100. The plates were gently swirled and then returned to the incubator.

Table 2: List of constructs used

Rat NCX1.1	Dr. Jonathan Lytton (University of Calgary)
Mouse NCKX2	Dr. Jonathan Lytton (University of Calgary)
Wild-type c-Src, active c-Src and dead c-Src	Dr. Don Fujita (University of Calgary)
Flag-tagged EGFR	Dr. Neil J. Freedman (Duke University)
Flag-tagged mGluR1b	Dr. R.A. Jeffrey McIlhinney (Medical Research Council Anatomical Neuropharmacology Unit)
Flag-tagged mGluR5	Dr. Robert Gereau (Baylor College of Medicine)
Flag-tagged NCKX4	Dr. Jonathan Lytton (University of Calgary)
Myc-tagged Kv1.4	Dr. Don B. Arnold (University of Southern California)
HA-tagged NHE1	Dr. John Orlowski (Mcgill University)
HA-tagged AE1	Dr. Reinhart A. F Reithmeier (University of Toronto)
HA-tagged VSV-G	Dr. Brian Seed (Massachusetts General Hospital)

Lipofectamine 2000 Transfection Reagent (Invitrogen) was used to transfect CHO cells. Briefly, one day before transfection, 5×10^5 CHO cells were plated into a 35-mm dish in 2 ml of growth medium without antibiotics. Cells were about 90% confluent at the time of transfection. 4 μg of DNA was diluted in 250 μl growth medium without antibiotics and serum. 10 μl Lipofectamine 2000 was also diluted in 250 μl growth medium without antibiotics and serum and incubated for 5 minutes at room temperature. Then the diluted DNA and diluted Lipofectamine 2000 were mixed gently and incubated for 20 min at room temperature. 500 μl mixed solution was added to the dish containing cells. After mixing, the dish was returned to the incubator.

III. Post-nuclear protein preparation

Two days following transfection, cells were harvested and post-nuclear extracts were isolated. Briefly, plates with transfected cells were rinsed twice with phosphate-buffered saline (PBS) buffer (137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.2) and then incubated with PBS/5 mM EDTA (2 ml/P-100 plate) at room temperature for 5-10 min. The cells were scraped off with a rubber policeman, collected in a clinical centrifuge tube and spun down at half max speed for 3 min. The supernatant was disposed; the cells were resuspended in 1 ml PBS/EDTA solution and transferred to a 1.5 ml centrifuge tube. The cells were spun down in a low speed centrifuge (~5,000 rpm) for 1 min. The supernatant was discarded and the cells were resuspended in ~100 μl of "RIPA" butter (1% non -ionic detergent NP40, 0.14 M NaCl, 10 mM EDTA, 25 mM Tris-HCl, 3 mg/ml p-nitrophenyphosphate (PnPP), 1 mM Na_3VO_4, 10 mM NaF, 100

units/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4) for 30 min on ice. The lysate was centrifuged at 13,200 rpm for 5 min at 4^{o}C and the supernatants were collected and stored at -70^{o}C. Protein concentration was determined by Bradford dye binding assay (Bradford, 1976) using protein assay kit (Bio-Rad) and bovine gamma-globulin as standard.

IV. Immunoprecipitation

All steps were conducted at 4^{o}C. Briefly, 500 µg of post-nuclear extract from transfected HEK293 cells was used and the volume was adjusted to 1 ml with IP buffer (RIPA buffer plus 0.1 mg/ml ovalbumin as a carrier plus protease inhibitors (complete protease inhibitor cocktail tablets from Roche supplemented with aprotinin, PMSF, and leupeptin) plus 3 mg/ml PnPP, 1 mM Na_3VO_4, 10 mM NaF), mixed together and placed on ice for 30 min. The tube was spun down at 14,000 rpm for 5 min at 4^{o}C. The supernatant was transferred to a new tube and 20 µl pre-washed protein-G beads (Amersham) were added. The tube was rotated for 30 min at 4^{o}C. Then the tube was spun down at 3000 rpm for 2 min at 4^{o}C and the supernatant was transferred to a new tube. 1-5 µg of appropriate antibody was added and the tube was rotated at 4^{o}C overnight. 30 µl pre-washed protein-G beads were added and the tube was rotated for another 2 h at 4^{o}C. Then the beads were spun down and washed 3 times with 0.3% NP40 RIPA buffer. The solution was transferred to a new tube after the third wash. The beads were spun down and al the supernatant was removed. To elute the proteins, 40 µl elution buffer [2× Laemmli sample buffer (20% glycerol, 4% SDS, 65 mM Tris, 0.001% bromophenol blue) plus 2% SDS

plus 5% β-mercaptoethanol] was added to the washed beads and the tube was placed in a 75^{o}C water bath for 10 min. The tube was spun down at 3000 rpm for 2 min and the supernatant was collected for SDS- polyacrylamide (PAGE) gel analysis.

V. Immunoblot

Immunoprecipitated proteins or post-nuclear extracts mixed with 2×Laemmli sample buffer containing 4% β-mercaptoethanol were resolved on 7.5% SDS- PAGE gel and then electrophoretically transferred to nitrocellulose membrane. The membrane was blocked for 30 min in PBS solution supplemented with 5% milk, 0.1% Tween20, followed by 2 h incubation in solution containing primary antibody (the information and working conditions for the antibodies were listed below), followed by 3 washes (5 min each) with PBS/0.1% Tween20, followed by 1 h incubation in solution containing horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-mouse for monoclonal primary antibody and goat anti-rabbit for polyclonal primary antibody; 1:10,000 in PBS/0.1% Tween20; Jackson Immunochemicals), followed by 3 washes (5 min each) with PBS/0.1% Tween20. Then the membrane was incubated in Super Signal enhanced chemiluminescence solutions (Pierce) for 1 min and the signal was visualized using Kodak BioMax film.

The antibodies used were:

a. R3F1 monoclonal antibody against intracellular loop of NCX1 (Porzig et al., 1993) (Dr. Jonathan Lytton's lab): 1: 10,000 in PBS/0.1% Tween

b. anti-Actin polyclonal antibody (Sigma): 1: 2,000 in PBS/0.1% Tween20
c. anti-FLAG monoclonal antibody (Sigma): 1:10,000 in PBS/0.1% Tween20
d. anti- Hemagglutinin (HA) monoclonal antibody (Sigma): 1: 10,000 in PBS/0.1% Tween20
e. Src-327 monoclonal antibody against Src (Dr. Don Fujita's laboratory): 1 μg/ml in PBS/10% FBS/0.02% NaN_3; kept in 4^{o}C and re-used many times
f. anti-phosphotyrosine monoclonal antibody (Dr. Stephen Robbins's laboratory): 1: 2,000 in PBS/0.1% Tween20
g. PA-926 polyclonal antibody (Affinity BioReagents, Inc.) against NCKX2: 1: 1,000 in PBS/0.1% Tween20
h. N1 polyclonal antibody against SERCA: 1:4,000 in PBS/0.1% Tween20

To reprobe with another antibody, the membrane was incubated for 30 min in stripping-solution (Chemicon) at room temperature, followed by milk blocking and the next antibody, as described above.

VI. Generation of stable inducible HEK293 cell line

"Flp-In/T-Rex" system from Invitrogen was utilized to generate cells able to inducibly express NCX1.1. The system is based on methodology developed in Dr. H. Gobind Khorana's laboratory (Reeves et al., 2002). Recombinase Flp mediates integration of the gene of interest, under the control of an inducible promoter, into a single specific site

previously engineered into the genome. Briefly, the NCX1.1 construct was moved from pcDNA3.1(+) vector into the Flp-in vector, pcDNA5/FRT/TO using digestion sites Hind III and Not I. Flp-In T-Rex-HEK293 host cells were cotransfected with Flp recombinase vector pOG44 and pcDNA5/FRT/TO vector containing NCX1.1 cDNA at a 9:1 ratio using the standard Ca^{2+}-phosphate precipitation method described above. The cells were washed 24 h after transfection and allowed to grow in fresh medium for another 48 h. Then the cells were split into new dishes with no more than 25% confluence. After the cells had attached, the medium was replaced with the selective medium (growth medium plus 200 μg/ml hygromycin (Invitrogen)) to select stable integrants. The selective medium was changed every 3-4 days until foci were identified. The hygromycin-resistant cells were pooled, transferred, and grown in a 75 ml culture flask with selective medium. To induce protein expression, tetracycline was added to a final concentration of 1 μg/ml.

VII. Enzymatic deglycosylation analysis

For PNGase F digestion, a 10 μl reaction mixture containing 50 μg post-nuclear extract, 50 mM sodium phosphate pH 8.0, 10 mM EDTA, 1% NP40, 0.1% SDS, 1% β-mercaptoethanol, 2 μl PNGase F (Roche) or 2 μl H_2O (control) was incubated for 2 h at 37^{o}C. For Endo H digestion, a 10 μl reaction mixture containing 50 μg post-nuclear extract. 50 mM sodium citrate pH 5.5, 1% NP40, 0.1% SDS, 1% β-mercaptoethanol, 100 units/ml aprotinin, 0.1 mM PMSF, 2 μl Endo H (Roche) or 2 μl H_2O (control) were

incubated overnight at 37^{o}C. The reactions were stopped by adding 10 µl 4× Laemmli sample buffer.

VIII. Biotinylation analysis of surface expression

HEK293 cells were co-transfected with NCX1 (or N9Q) together with either active or dead Src. Two days after transfection, the intact cells on plates were gently washed three times with ice-cold PBSCM (PBS/0.1 mM $CaCl_2$/0.1 mM $MgCl_2$, pH 8.0) and then incubated with 0.5 mg/ml Sulfo-NHS-LC-biotin (Pierce) in PBSCM (pH 8.0) at 4^{o}C for 1 h with gentle shaking. Cells were then washed four times with ice-cold PBSCM (pH 8.0) containing 100 mM glycine. Post-nuclear proteins were prepared as described above. 500 µg extracted proteins were dissolved in 1 ml buffer containing PBS/1% NP40/0.1% SDS/protease inhibitors (Binding Buffer) for 30 min on ice, followed by a 5-min centrifugation at 14,000 rpm to remove particulate material. The supernatant was collected and streptavidin beads (Pierce) that had been washed twice with Binding Buffer were added. For each reaction, 60 µl of 1:1 bead slurry was used and incubated with rocking overnight at 4^{o}C. Beads were then washed 3 times with Binding Buffer and the bound proteins were eluted by boiling for 5 min in the elution buffer used for immunoprecipitation experiments. 50 µg of total protein and the biotinylated cell surface proteins pulled down with streptavidin were subjected to immunoblot analysis as described above. First, the membranes were probed with anti-NCX1 antibody. Then, the membranes were stripped and re-probed with anti-Actin polyclonal antibody. Finally, the

membranes were stripped again and blotted with HRP-streptavidin (1:20,000 in PBS/0.1% Tween20/5% milk).

IX. Immunofluorescence

Subcellular location of NCX1 was determined using immunofluorescence as described previously (Cai et al., 2002). In short, HEK293 cells grown on glass coverslips that had been pre-coated with 1 mg/ml poly-D-lysine (Sigma) were transfected as described above. Transfected cells, either on coverslips or collected into 1.5 ml tubes, were rinsed with PBSCM and fixed in 3% paraformaldehyde in PBSCM for 15 min. Then they were permeabilized with PBSCM/0.1% Triton and blocked with 0.2% fish gelatin in PBSCM for 15 min each. Cells were incubated for 2 h in PBS/0.2% fish gelatin containing monoclonal antibody R3F1 (diluted 1:800) and polyclonal antibody Src2 (diluted 1:150; Santa Cruz, CA). Cells were washed three times and then incubated for 1 h in PBS/0.2 fish gelatin in which Cy3 conjugated donkey-anti-mouse (Jackson Labs.) and FITC conjugated donkey-anti-rabbit (Jackson Labs.) were diluted 1:500. Following three washes, coverslips or cells were mounted onto slides using Vectashield anti-fade reagent (Vector Labs), sealed with nail polish and viewed with a fluorescent microscope.

X. Protein staining and identification

Immunoprecipited proteins were resolved by 7.5% SDS-PAGE gel. After electrophoresis, the gel was removed from the cassette, placed in a clean tray, and rinsed with ultrapure water. Silver staining was performed using the kit from Invitrogen, following the instructions provided with minor modification. Briefly, the gel was fixed in 100 ml of fixative solution (from the kit) overnight with gentle shaking, followed by wash in 30% ethanol for 10 min. Then the gel was incubated in 100 ml sensitizing solution (from the kit) for 10 min followed by 10-min wash first in 30% ethanol and then in ultrapure water. After washing, the gel was stained in staining solution (from the kit) for 15 min and then washed with 100 ml ultrapure water for 30 s. Then the gel was incubated in 100 ml of developing solution (from the kit) for 4-8 min until bands started to appear and the desired band intensity was reached. Then 10 ml of stopper (from the kit) was added to stop the development. Coomassie blue staining was performed by incubating the gel in 100 ml stain solution (0.25% Coomassie Blue R-250, 50% methanol, 10% glacial acetic acid) for 1 h followed by brief rinse with water. Then the gel was destained (40% methanol, 10% glacial acetic acid) overnight.

Bands of interest were cut out from the Coomassie-stained gel and analyzed by mass spectrometry using LC-MS/MS at the Southern Alberta Mass Spectrometry (SAMS) Centre, University of Calgary.

CHAPTER THREE

RESULTS

I. c-Src activity increased the mobility of NCX1

Rat heart NCX1.1 was expressed in HEK293 cells alone, or together with wild-type human c-Src, constitutively active c-Src (Y530F) or catalytically "dead" c-Src (K298M). Post-nuclear proteins were subjected to immunoblot analysis. As shown in Fig. 5A, cotransfection with wild c-Src resulted in a fraction of NCX1 running with decreased apparent size (increased gel mobility). More NCX1 was shifted to the lower position when constitutively active c-Src was used. As shown in Fig. 5B, coexpression with active c-Src in CHO cells also increased the mobility of NCX1. HEK293 cells are of human neuronal origin (Shaw et al., 2002) while CHO cells are derived from mouse ovary cells (Puck et al., 1958). Since the same change in mobility was observed in two totally different cell lines, the effect of Src on NCX1.1 is unlikely to be a cell-specific artifact. The size change was not observed with kinase-dead c-Src, indicating this effect was dependent on the kinase activity of c-Src. Immunoblot using anti-Src monoclonal antibody 327 indicated that both active Src and dead Src were expressed well while endogenous Src expression was very low in both HEK293 cells and CHO cells.

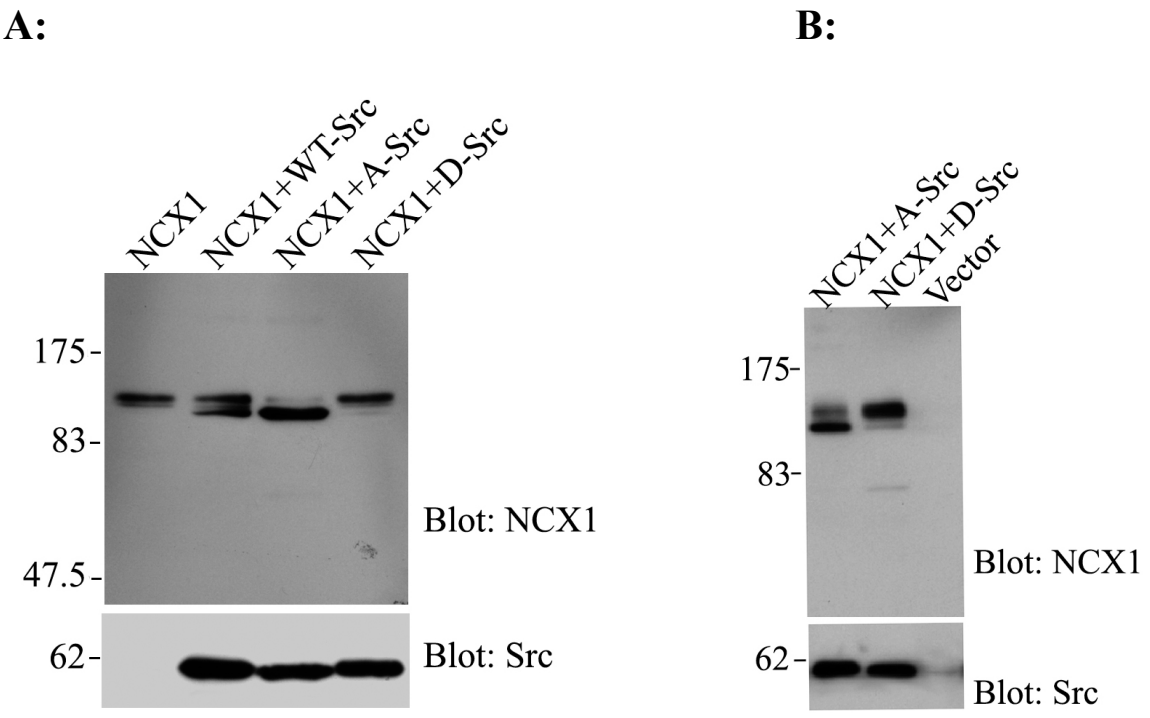


Figure 5: c-Src activity increased the mobility of NCX1.

A: HEK 293 cells were transfected with NCX1, NCX1 plus wild-type Src (WT-Src), NCX1 plus active Src (A-Src), or NCX1 plus catalytically dead Src (D-Src). Lysates from transfected cells were separated by SDS-PAGE and immunoblotted with anti-NCX1 antibody (upper panel) or anti-Src antibody (lower panel). Shown is a typical result of dozens of independent experiments.

B: CHO cells were transfected with NCX1 plus active Src, or NCX1 plus dead Src. Lysates from transfected cells were separated by SDS-PAGE and immunoblotted with anti-NCX1 antibody (upper panel) or anti-Src antibody (lower panel). This blot is representative of two independent experiments.

II. Src did not directly phosphorylate NCX1

To investigate the mechanism of the Src-induced mobility shift, we tested whether c-Src could directly phosphorylate NCX1 in HEK293 cells. NCX1 proteins from different transfection conditions were immunoprecipitated with R3F1 anti-NCX1 monoclonal antibody and separated by SDS-polyacrylamide gel electrophoresis. Immunoblot using monoclonal antibody 4G10 was performed to detect proteins containing phosphorylated tyrosine residues. Under our experimental conditions, we could not detect bands corresponding to the NCX1 protein (Fig. 6). However, strong NCX1 bands appeared when the same membrane was stripped and reprobed with NCX1 antibody R3F1.

To rule out the possibility that the proteins were dephosphorylated during the process of protein preparation and immunoprecipitation, the same protein lysates were immunoprecipitated with anti-Src monoclonal antibody 327, separated by SDS-PAGE and probed with anti-phosphotyrosine antibody 4G10. A band corresponding to phosphorylated Src could clearly be seen, migrating just above the IgG band. The IgG band was visible on the film because the anti-NCX1, anti-Src and anti-phosphotyrosine antibody were all monoclonal antibodies. The Src band detected with 4G10 was not observed after phosphatase treatment, demonstrating the specificity of this antibody (data not shown). Moreover, phosphorylation usually decreases the mobility of a protein, making it exhibit a larger apparent molecular weight (Wegener and Jones, 1984; Peck, 2006). In our case, however, the mobility actually increased. In addition, Src failed to co-

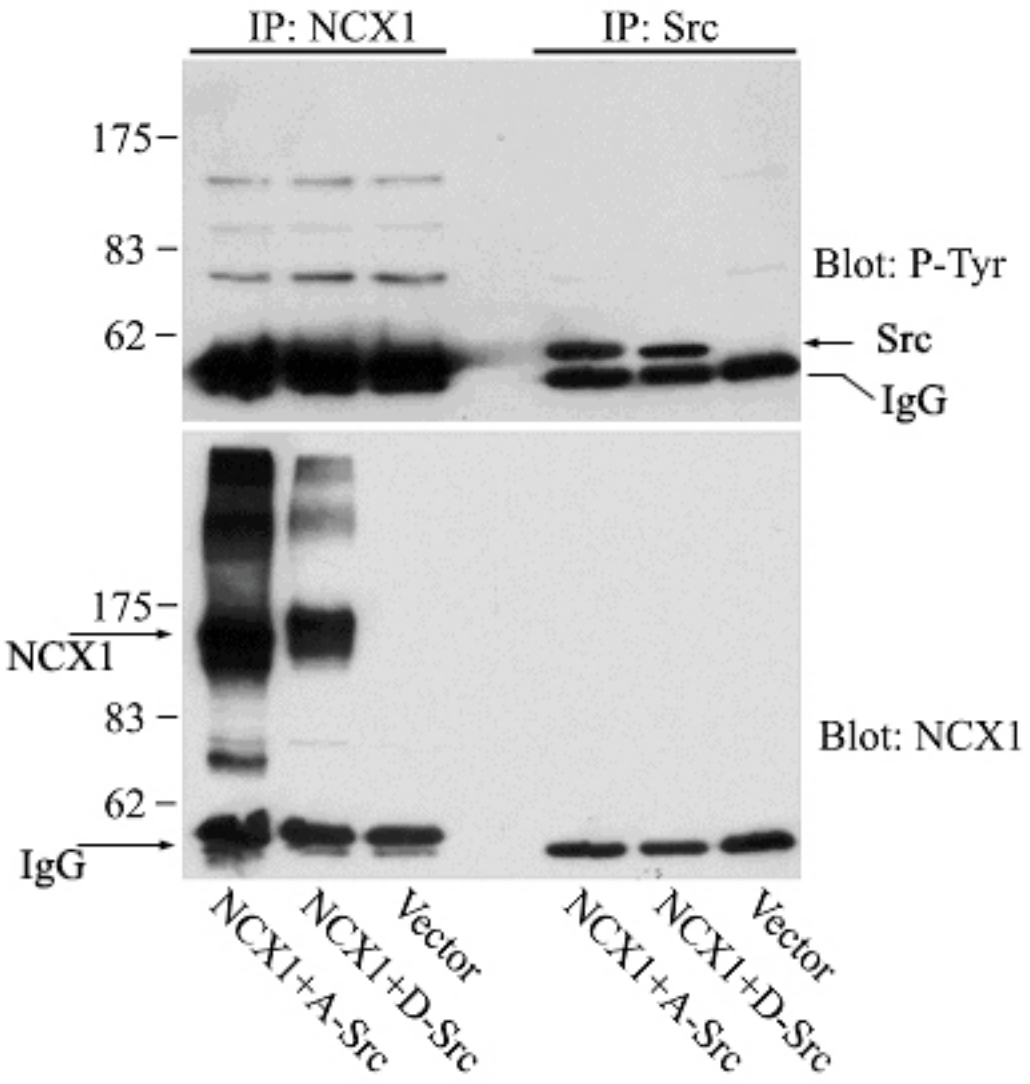


Figure 6: **NCX1 was not directly phosphorylated.**

HEK 293 cells were transfected with NCX1 plus active Src, NCX1 plus dead Src, or vector alone. Lysates from transfected cells were immunoprecipitated (IP) using monoclonal anti-NCX1 antibody or monoclonal anti-Src antibody, separately. The immunoprecipitated proteins were separated by SDS-PAGE and probed with monoclonal anti-phosphotyrosine antibody 4G10 (upper panel). The same membrane was stripped and reprobed with anti-NCX1 antibody (lower panel). This blot is representative of three blots performed from three independent protein preparations.

immunoprecipitate with NCX1, indicating a lack of direct protein-protein interaction between Src kinase and the exchanger (data not shown).

III. The change in gel mobility of NCX1 was caused by alteration of the structure of the N-linked glycan

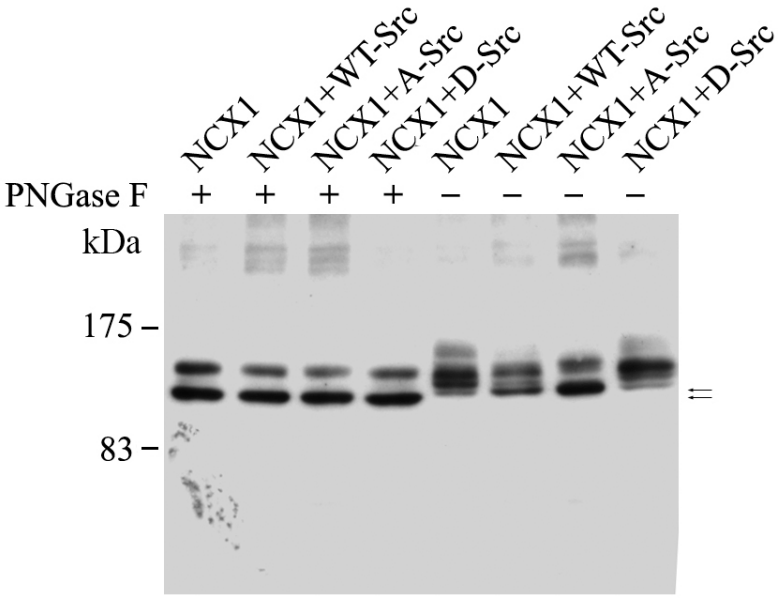
Because NCX1 is an N-linked glycoprotein, and a change in glycosylation may change the mobility of the protein, we examined whether a change in glycosylation was the reason behind the Src-induced NCX1 mobility shift. We carried out PNGase F digestion to test this hypothesis. PNGase F is an enzyme that cleaves the bond between N-acetylglucosamine (GlcNAc) and Asn, therefore removing the entire sugar chain from the protein. As shown in Fig. 7A, after the treatment with PNGase F, the mobility of NCX1 with or without co-transfected Src was the same. To demonstrate further that the changed mobility was due to a change in the structure of the N-linked glycan on NCX1, N-glycosylation free NCX1 was made by mutating the only N-linked glycosylation site Asn9 to Gln. As shown in Fig.7B, the mobility of N-linked glycosylation free NCX1 (N9Q) remained the same regardless whether it was co-transfected with or without Src. It was noteworthy that the mobility of NCX1 cotransfected with active Src was also shifted down after PNGase F treatment (Fig. 7A), suggesting NCX1 remained glycosylated when co-transfected with Src, but that the structure of the N-linked glycan on NCX1 was dramatically changed.

Figure 7: **The Src-induced change in gel mobility of NCX1 was caused by alteration of the N-linked glycan structure.**

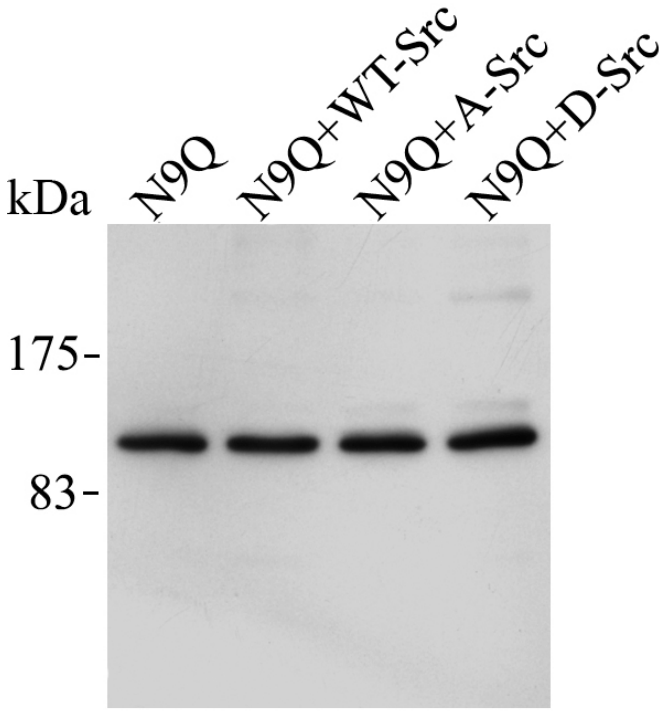
A: Lysates from HEK 293 cells transfected with NCX1, NCX1 plus wild-type Src, NCX1 plus active Src or NCX1 plus dead Src, as indicated, were incubated with (+) or without (-) N-glycosidase F (PNGase F) at 37^{o}C for 3 hours, resolved by SDS-PAGE, and visualized by immunoblotting. The arrows indicate the position of NCX1 proteins co-expressed with active Src with or without PNGase F treatment. This shift in mobility suggests NCX1 protein co-transfected with active Src was still glycosylated. Shown is a typical result from three independent experiments.

B: There was no mobility change for the glycan-free mutant NCX1 (N9Q). Lysates from HEK 293 cells transfected with N9Q, N9Q plus wild-type Src, N9Q plus active Src, or N9Q plus dead Src were immunoblotted with anti-NCX1 antibody. Shown is a typical result from two independent experiments.

A:


NCX1
NCX1+WT-Src
NCX1+A-Src
NCX1+D-Src
NCX1
NCX1+WT-Src
NCX1+A-Src
NCX1+D-Src
PNGase F + + + + − − − −
kDa
175 –
83 –

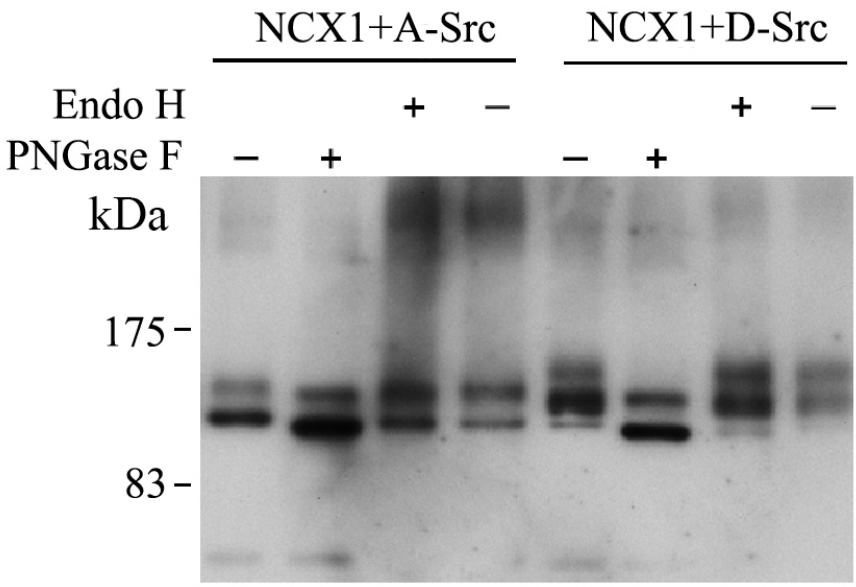
B:


kDa
N9Q
N9Q+WT-Src
N9Q+A-Src
N9Q+D-Src
175-
83-

IV. The Src-altered NCX1 glycan was Endo H insensitive

N-linked glycosylation is initiated in the endoplasmic reticulum and further modified in the Golgi apparatus. There are two major types of *N*-linked oligosaccharides: high-mannose oligosaccharides and complex oligosaccharides. Usually, an oligosaccharide remains in the high-mannose form if it cannot access the saccharide-modifying enzyme GlcNAc-T I located in the Golgi. Since the size of the glycan was reduced when NCX1 was co-transfected with Src and the trafficking of NCX1 was also affected (Fig. 11), we were interested to know whether the NCX1 protein was retained in the ER. If that was the case, the glycan structure should be of the high mannose type, and sensitive to Endo H which only cleaves the high mannose structure. A typical result of three independent experiments from three independent protein preparations is shown in Fig. 8A. When NCX1 was co-transfected with active Src, the NCX1-glycan was resistant to Endo H digestion. When co-transfected with active Src, epidermal growth factor receptor (EGFR) showed a similar phenomenon as NCX1, displaying an increased mobility when analyzed on SDS-PAGE gel (Fig. 13). However, the altered glycan on EGFR was sensitive to Endo H (Fig. 8B), suggesting EGFR might be retained in the ER (Schmidt-Arras et al., 2005). This experiment served as a control to indicate that Endo H digestion was working under our conditions.

A:



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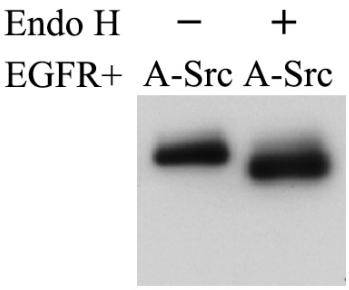


Figure 8: **The Src-altered NCX1 changed glycan was endo H insensitive.**

A: Lysates from HEK 293 cells transfected with NCX1 plus either active Src or dead Src as indicated, were mock treated (-) or digested (+) with PNGase F or Endo H, resolved by SDS-PAGE, and visualized by immunoblotting. Shown is a typical result of three blots performed from three independent protein preparations.

B: Lysates from HEK 293 cells transfected with epidermal growth factor receptor (EGFR) plus active Src were treated with (+) or without (-) Endo H and EGFR was visualized by immunoblotting.

V. The Src-induced alteration of NCX1-glycan happened during biosynthesis

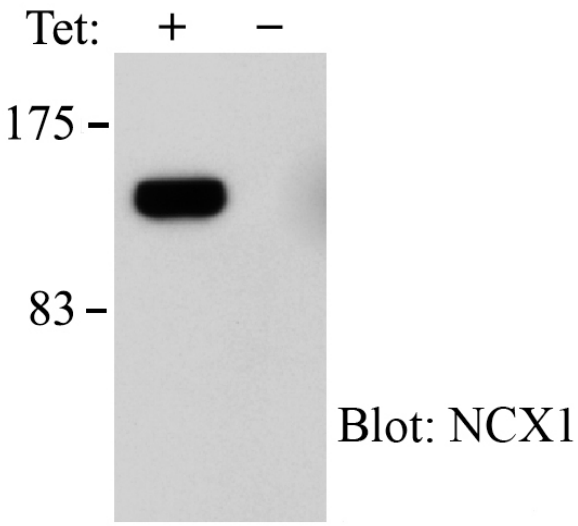
There are two potential ways that the structure of a glycan chain may be changed: (i) the change may happen during the process of glycan chain biosynthesis as a newly synthesized protein migrates from the ER through the Golgi apparatus; (ii) the change may happen after the maturation of the protein and oligosaccharide chain. This latter possibility would require that Src affected the mature NCX1 at the plasma membrane, possibly by inducing recycling (van Koppen, 2001; Tao et al., 2007). To distinguish between these two possibilities, I generated a cell line expressing NCX1 in an inducible fashion under control of the tetracycline operator, as described in the "methods" section. This cell line normally constantly expresses the tetracycline repressor which prevents expression of NCX1 under the control of tetracycline operator. Expression of NCX1 is induced only in the presence of tetracycline (Fig. 9A), which binds to its repressor, inducing a conformational change that removes the repressor from the operator. NCX1 stable cells were transfected with active Src or dead Src inserted in the pcDNA5/FRT/TO vector (Invitrogen) containing the tetracycline operator. 18 hours after transfection, tetracycline was added and both the NCX1 and Src expression were turned on at the same time. Cells were harvested 2 days after the induction. In this experiment, the mobility of NCX1 was shifted partially when cells were transfected with active Src (Fig. 10A). Thus NCX1 expressed in the inducible cells could be affected in the same way as in transiently transfected HEK293 cells. The fraction of NCX1 with a shifted mobility is consistent

Figure 9: Characterization of the tetracycline inducible cells stably expressing NCX1.

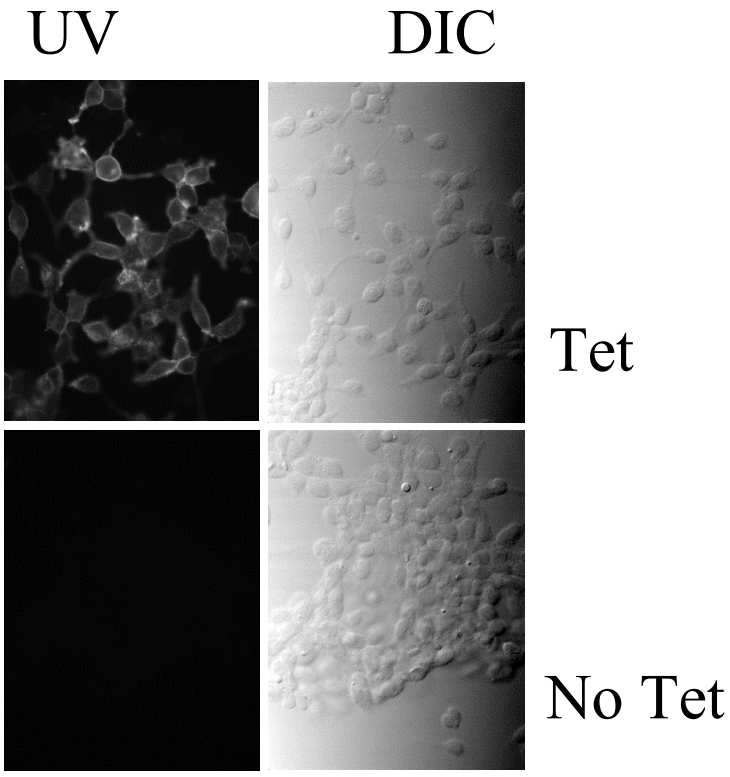
A: NCX1 expression in NCX1-Flp-In/T-Rex cells was analyzed by western blot using anti-NCX1 antibody. NCX1 expression was induced by adding 1μg/ml of tetracycline (Tet) for 24 hours. Lysates from cells with or without induction were separated by SDS-PAGE and immunoblotted with anti-NCX1 antibody.

B: 24 hours following induction with 1ug/ml tetracycline, NCX1 was visualized by indirect fluorescence with R3F1 antibody (left) and cells were imaged by differential interference contrast (DIC) microscopy (right).

A:



B:



with the fraction of cells transfected (see below). In another experiment, NCX1 expression was induced for about 40 hours with the tetracycline. The cells were washed and subcultured into 10-cm culture dishes in medium without tetracycline. 24 hours later, the cells were transfected with active c-Src or dead c-Src inserted in the pcDNA3.1 vector (Invitrogen) and harvested 30 hours or 48 hours following the transfection. In this experiment, there was no mobility change of NCX1 when cells were transfected with active Src (Fig. 10B), suggesting that the matured NCX1 synthesized before expression of Src could not be modified by subsequent Src expression.

Since not all of the induced cells expressed NCX1 and since not all transiently transfected cells expressed Src, we were concerned that the cells expressing NCX1 might not be transfected with Src, thus preventing the mobility shift. To rule out this possibility, stably transfected NCX1 cells were transfected with Src after the induction of NCX1 or were transfected with Src followed by induction as described above. Cells from the plates were collected and immunostained with monoclonal anti-NCX1 antibody and polyclonal anti-Src antibody Src2. Pictures were taken using a 10×objective lens and the total number of cells and the number of cells expressing NCX1 alone, Src alone or both NCX1 and Src were counted. No matter whether Src expression was induced together with NCX1 or following NCX1 induction and maturation, the ratios of the number of cells expressing both NCX1 and Src to cells expressing NCX1, and the number of cells expressing Src to the total number of cells, were similar (Fig. 10C). The result suggested that there was no transfection preference with or without NCX1 induction and a similar and significant fraction of the cells expressed both Src and NCX1 under both conditions. Taken together,

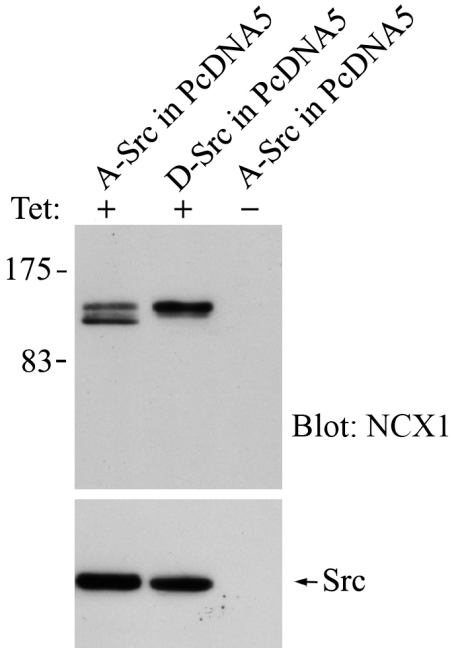
Figure 10: **The Src-induced alteration in NCX1-glycan happened during biosynthesis.**

A: The inducible stable NCX1 cells were transfected with activated Src or dead Src inserted in pcDNA5/TO vector containing the tetracycline operator. 18 hours after transfection, tetracycline was added and both the NCX1 and Src expression were turned on. 30 hours post-addition of tetracycline, cells were collected and lysates were immunoblotted with anti-NCX1 antibody.

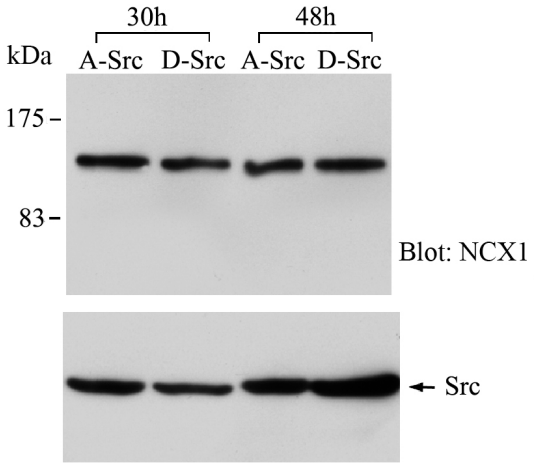
B: NCX1 expression was induced by adding tetracycline for 40 hours, then the cells were washed and splitted to 10cm culture dishes in the medium without tetracycline. The cells were transfected with activated Src or dead Src and harvested 30 hours or 48 hours following the transfection. The lysates were immunoblotted with anti-NCX1 antibody. Shown is a typical result of four independent experiments.

C: Cells from different experiment conditions - active Src transfection following NCX1 induction, dead Src transfection following NCX1 induction, active Src transfection followed by induction of NCX1, and dead Src transfection followed by induction of NCX1 and dead Src – were collected and visualized by indirect fluorescence with monoclonal anti-NCX1 antibody and polyclonal anti-Src antibody. Pictures were taken by using 10×objective lens and the number of cells expressing NCX1, Src, both NCX1 and Src, and the total number of the cells were counted. The ratios of the number of cells expressing both NCX1 and Src to the cells expressing NCX1 and the number of cells expressing Src to the total number of the cells were calculated. The results are expressed as mean ± SEM.

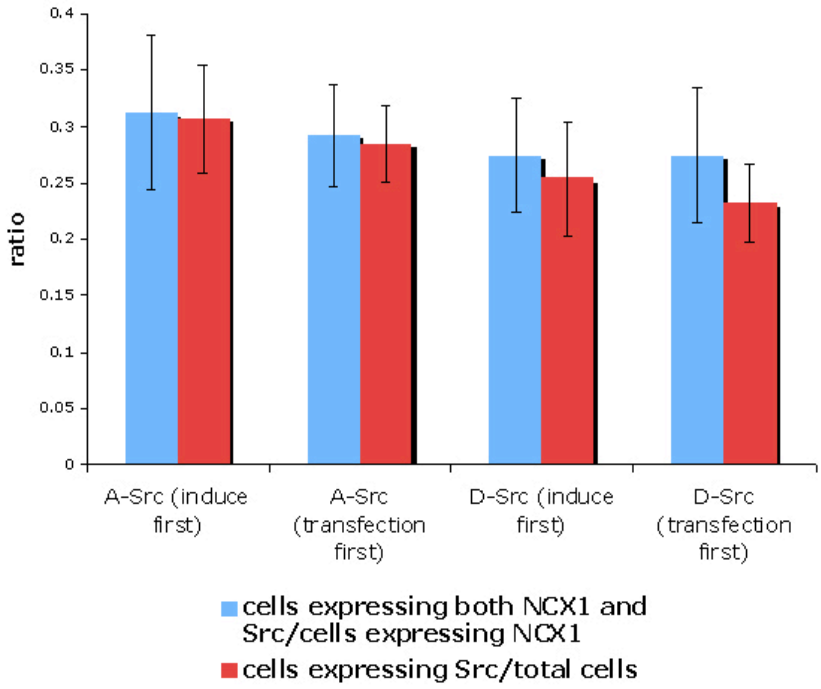
A:


A-Src in PcDNA5
D-Src in PcDNA5
A-Src in PcDNA5
Tet: + + −
175-
83-
Blot: NCX1
←Src

B:


30h
48h
kDa
A-Src D-Src A-Src D-Src
175-
83-
Blot: NCX1
← Src

C:


0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ratio
A-Src (induce first)
A-Src (transfection first)
D-Src (induce first)
D-Src (transfection first)
cells expressing both NCX1 and Src/cells expressing NCX1
cells expressing Src/total cells

I conclude that the Src-induced alteration in the NCX1 glycan happened during the biosynthetic process of glycosylation.

VI. Trafficking of NCX1 was changed by Src activity

The maturation of an oligosaccharide chain occurs in sequential compartments during the biosynthesis and trafficking of surface plasma membrane protein like NCX1. Since Src induced a change in the NCX1 glycan, this alteration may have happened due to a change in trafficking of NCX1. I used surface biotinylation to investigate the potential trafficking changes. HEK293 cells were transiently transfected with NCX1 plus active Src or NCX1 plus dead Src. The transfected cells were biotinylated to label surface proteins, which were isolated from cell extracts using streptavidin beads. Samples of the cell lysate and cell surface fractions were analyzed by immunoblot. The surface expression of NCX1 was dramatically reduced when it was co-transfected with active Src. As shown in Fig. 11A, very little NCX1 was found on the surface of cells coexpressing active Src compared to cells coexpressing dead Src. The membrane was re-probed with anti-actin antibody and streptavidin, separately. Actin, an intracellular protein, served as the control to show that the surface fraction separated by streptavidin beads didn't contain intracellular proteins. The pattern of the streptavidin blot was similar for samples from cells transfected with active Src or dead Src, indicating that overall surface membrane protein expression was not dramatically changed by Src activity and the pull down efficiency was comparable between the two samples.

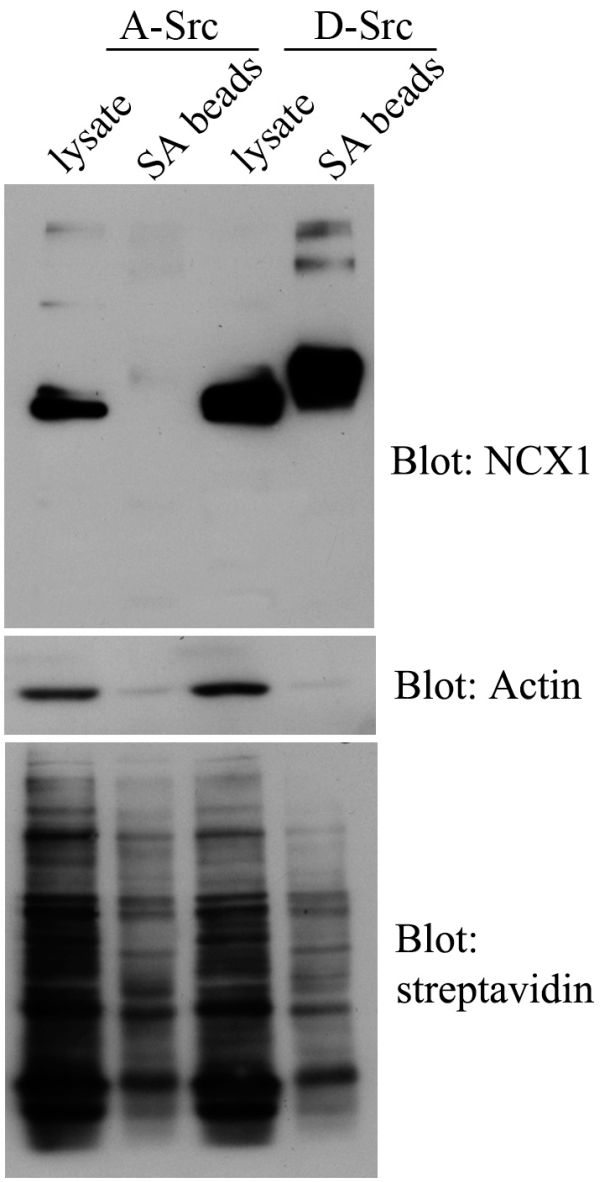


Figure 11: **Trafficking of NCX1 was changed by Src activity.** HEK 293 cells were transfected with NCX1 plus active Src or NCX1 plus dead Src. Transfected cells were biotinylated to label surface proteins, the cells extracted with detergent and the biotinylated proteins separated using streptavidin (SA) beads. Samples of the cell lysate and proteins precipitated by streptavidin beads were analyzed by Western blot. Note that the mobility of NCX1 in the streptavidin lane is slightly different from that in the lysate lanes because the streptavidin beads had to be heated in sample buffer prior to loading the gel to release the bound proteins. Upper panel: probed with anti-NCX1 antibody; middle panel: reprobed with anti-actin antibody; bottom panel: reprobed with HRP-streptavidin. Shown is a typical result of more than five independent experiments.

VII. Src also affected trafficking of the glycosylation defective NCX1 mutant N9Q

N-linked glycosylation is essential for cell surface expression of some proteins (Fan et al., 1997). To investigate whether the Src-induced change of NCX1 glycosylation was the direct cause of the trafficking change, we performed the same biotinylation experiment described above using the N-glycan free mutant NCX1 (N9Q). A typical result from two independent experiments is shown in Fig. 12. First, the N-linked glycosylation at Asn9 was not required for the correct trafficking of NCX1 in HEK293 cells since N-glycan free N9Q coexpressing with dead Src exhibited similar surface expression as wild-type NCX1. Second, the glycan-free NCX1 mutant N9Q showed a similar trafficking problem as wild-type NCX1 when co-transfected with active Src. Taken together, it seemed that the NCX1 glycosylation change induced by Src activity was not the direct trigger of the trafficking changes; rather the trafficking change was likely to have led to the alteration of glycosylation. The glycosylation process could not be completed due to the deficient trafficking and the protein was only partially glycosylated. Thus, Src seems to induce a dramatic change in trafficking of NCX1 during its biosynthesis such that it is not exposed to those enzymes that complete the modification of the glycan chain, and is not delivered to the plasma membrane.

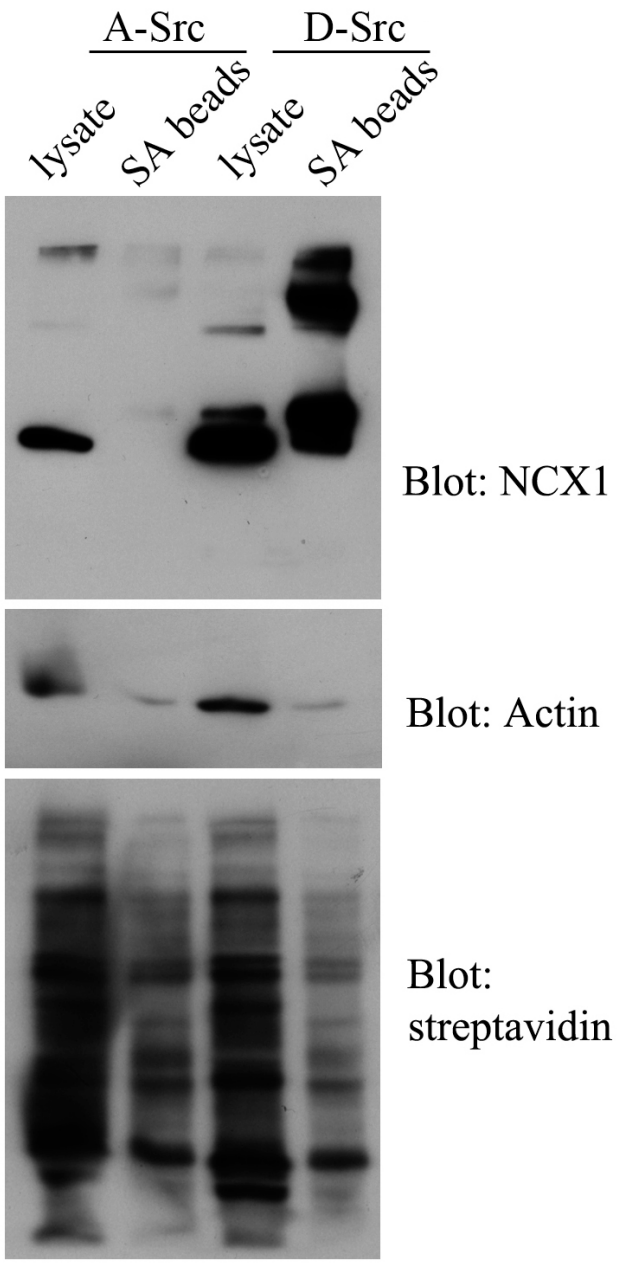


Figure 12: **Src also affected trafficking of the glycosylation defective NCX1 mutant N9Q.** HEK 293 cells were co-transfected with glycosylation free NCX1 mutant N9Q and either active Src or dead Src. Transfected cells were biotinylated to label surface proteins, the cells extracted with detergent and the biotinylated proteins separated using streptavidin (SA) beads. Samples of the cell lysate and proteins precipitated by streptavidin beads were analyzed by Western blot. Upper panel: probed with anti-NCX1 antibody; middle panel: reprobed with anti-actin antibody; bottom panel: reprobed with HRP-streptavidin.

VIII. The Src-induced alteration in glycan structure showed some selectivity to the host protein

Since Src regulated trafficking of NCX1, I next examined whether Src influenced all surface proteins in a similar way, or if the effects on NCX1 were specific. Several other membrane proteins, Na^+/H^+ exchanger (NHE1), Cl^-/HCO_3^- exchanger (AE1), potassium channel Kv1.4, vesicular stomatitis virus membrane glycoprotein (VSV-G), potassium-dependent sodium/calcium exchanger (NCKX2, NCKX4), epithelial growth factor receptor (EGFR), and metabotropic glutamate receptor (mGluR5, mGluR1b), were cotransfected with Src and analyzed by immunoblot. Of all the proteins tested, only EGFR and NCKX2 showed increased mobility when cotransfected with Src (Fig. 13A). However, the mechanism causing the mobility shift of EGFR was different from that of NCX1. The glycan structure of EGFR generated by Src activity was sensitive to Endo H (Fig. 8B). This change was probably due to retention of EGFR in the ER, similar to Fms-like tyrosine kinase 3 (FLT-3) (Schmidt-Arras et al., 2005). The altered glycan structure of NCX1 was resistant to Endo H and thus the mechanism for the Src-induced change was likely to be different for EGFR compared to NCX1. After treatment of PNGase F, the mobility of NCKX2 with or without co-transfected Src was the same (Fig. 13C), suggesting a similar mechanism as NCX1.

Both mGluR5 and mGluR1b expressed in HEK293 cells demonstrated mobility shifts when treated with PNGase F (Fig. 13B and Fig. 14B). The two immunoreactive bands of

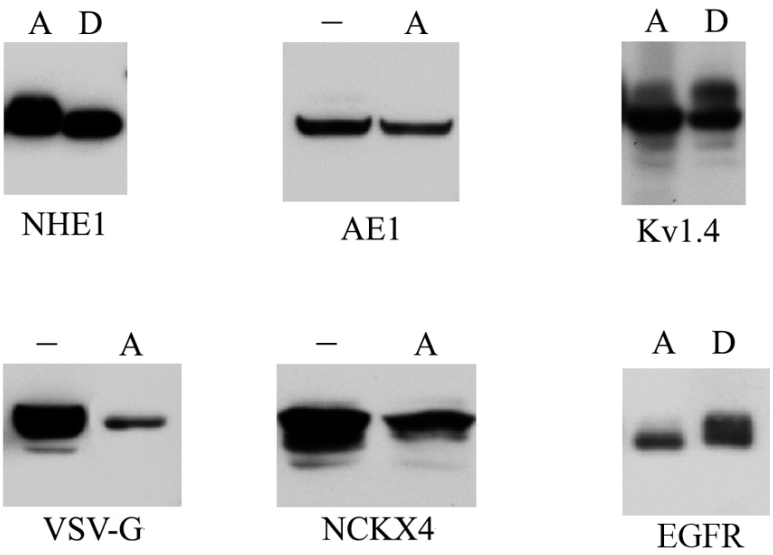
Figure 13: **The Src-induced alteration of glycan chain showed some selectivity to the host protein.**

A: HEK 293 cells were transfected with Na^+/H^+ exchanger (NHE1) plus active Src, NHE plus dead Src, with Cl^-/HCO_3^- exchanger (AE1), AE1 plus active Src, with potassium channel Kv1.4 plus active Src, Kv1.4 plus dead Src, with vesicular stomatitis virus membrane glycoprotein (VSV-G), VSV-G plus active Src, with potassium-dependent sodium/calcium exchanger (NCKX4), NCKX4 plus active Src, with epithelial growth factor receptor (EGFR) plus active Src, EGFR plus dead Src. Proteins were visualized by immunoblotting.

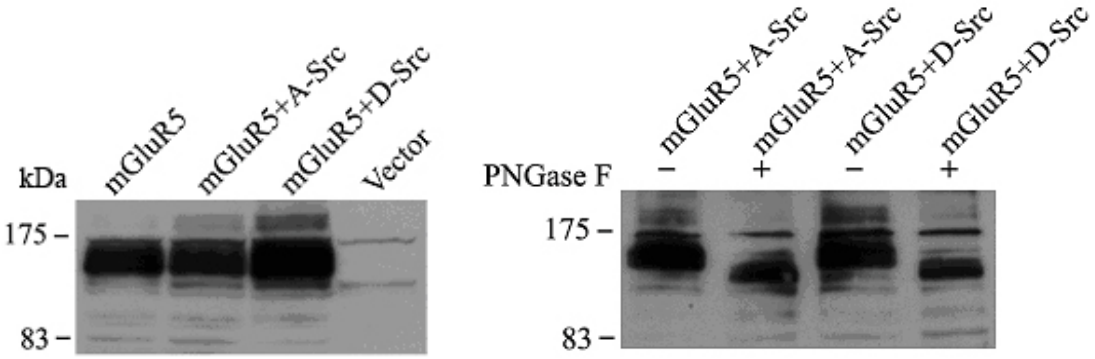
B: HEK 293 cells were transfected with metabotropic glutamate receptor (mGluR5), mGluR5 plus active Src, mGluR5 plus dead Src, or vector alone. mGluR5 proteins were visualized by immunoblotting (left panel). Lysates from transfected cells, as indicated, were mock treated (-) or digested (+) with PNGase F and visualized by immunoblotting (right panel).

C: HEK 293 cells were transfected with potassium-dependent sodium/calcium exchanger (NCKX2) plus active Src or NCKX2 plus dead Src. NCKX2 proteins were visualized by immunoblotting (left panel). Lysates from transfected cells, as indicated, were treated with (+) or without (-) PNGase F and NCKX2 proteins were visualized by immunoblotting (right panel).

A:


A D
NHE1
– A
AE1
A D
Kv1.4
– A
VSV-G
– A
NCKX4
A D
EGFR

B:


kDa
mGluR5
mGluR5+A-Src
mGluR5+D-Src
Vector
175 –
83 –
PNGase F
mGluR5+A-Src
mGluR5+A-Src
mGluR5+D-Src
mGluR5+D-Src
– + – +
175 –
83 –

C:

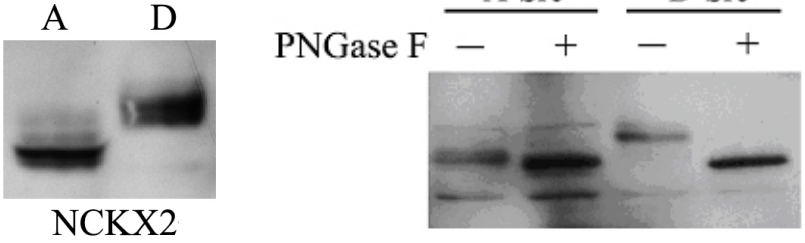

A D
NCKX2
NCKX2+ A-Src
NCKX2+ D-Src
PNGase F — + — +

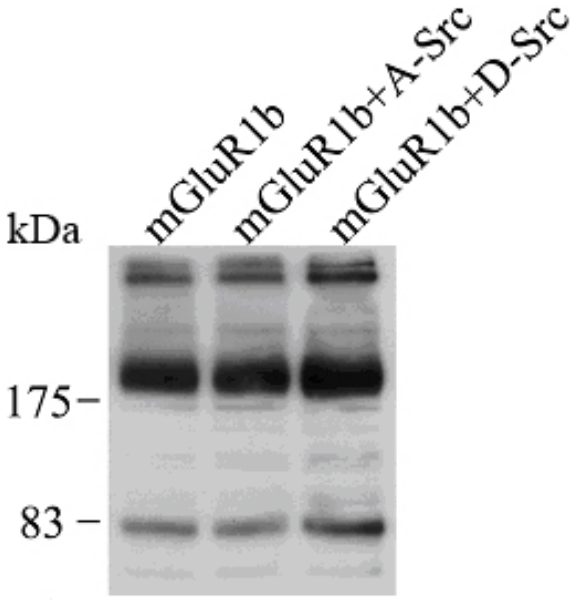
Figure 14: Neither glycan nor trafficking of mGluR1b was changed by Src activity.

A: HEK 293 cells were transfected with mGluR1b, mGluR1b plus active Src or mGluR1b plus dead Src. mGluR1b proteins were detected by immunoblotting using anti-FLAG antibody.

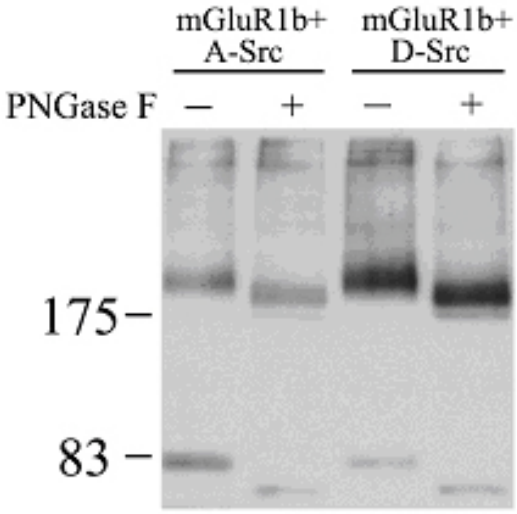
B: Lysates from HEK 293 cells transfected with mGluR1b plus active Src or mGluR1b plus dead Src were treated with (+) or without (-) PNGase F and mGluR1b proteins were visualized by immunoblotting.

C: HEK 293 cells were transfected with mGluR1b plus active Src or mGluR1b plus dead Src. Transfected cells were biotinylated to label surface proteins. Samples of the cell lysates and proteins precipitated by streptavidin (SA) beads were analyzed by Western blot. Upper panel: probed with anti-FLAG antibody; bottom panel: reprobed with anti-actin antibody. The monomer mGluR1b (lower band) was not present in the samples of proteins precipitated by streptavidin beads, in good agreement with a previous report (Ciruela et al., 2000). Shown is a typical result of three independent experiments.

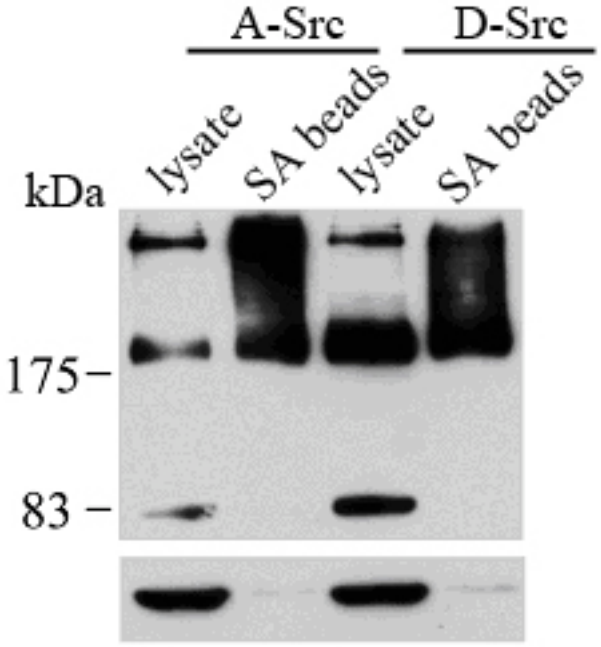
A:


mGluR1b
mGluR1b+A-Src
mGluR1b+D-Src
kDa
175–
83 –

B:


mGluR1b+
A-Src
mGluR1b+
D-Src
PNGase F – + – +
175–
83 –

C:


A-Src
D-Src
lysate
SA beads
lysate
SA beads
kDa
175–
83 –

mGluR1b correspond to mGluR1b receptor monomer and dimer (Ciruela et al., 2000). However, unlike NCX1 and NCKX2, co-expression with active Src did not cause a mobility shift in the mGluRs. The effect of Src expression on mGluR1b surface delivery was tested as described above for NCX1. As shown in Fig. 14C, there was no difference in the fraction of mGluR1b precipitated by streptavidin following surface biotinylation in cells coexpressing active Src compared to dead Src. These data demonstrated the selectivity of Src induced glycan and trafficking changes of NCX1.

IX. Analysis of NCX1 and NCKX2 mutants

Since the Src-induced trafficking and glycosylation changes showed some selectivity for NCX1 and NCKX2, I reasoned that there might be a motif common to both NCX1 and NCKX2 responsible for Src effect on these proteins. I focused on the extracellular loops since they are located within the organelle lumen during the protein migration through the Golgi complex. Alignment of rat NCX1 and mouse NCKX2 protein sequences is shown in Fig. 15. Based on the protein structure and sequence similarity between NCX1 and NCKX2, 825TIG827 located at the extracellular loop connecting TM6 and TM7 of NCX1 was mutated to 825AAG827. This TIG mutant of NCX1 exhibited similar Src induced mobility shift to that seen for wild-type NCX1 (Fig. 16A), suggesting 825TIG827 was not responsible for the Src related regulation. The large intracellular loop is responsible for most of the regulation of NCX1 and NCKX2, so I tested whether it is involved in Src effect on these proteins. The large intracellular loop of NCKX2 was

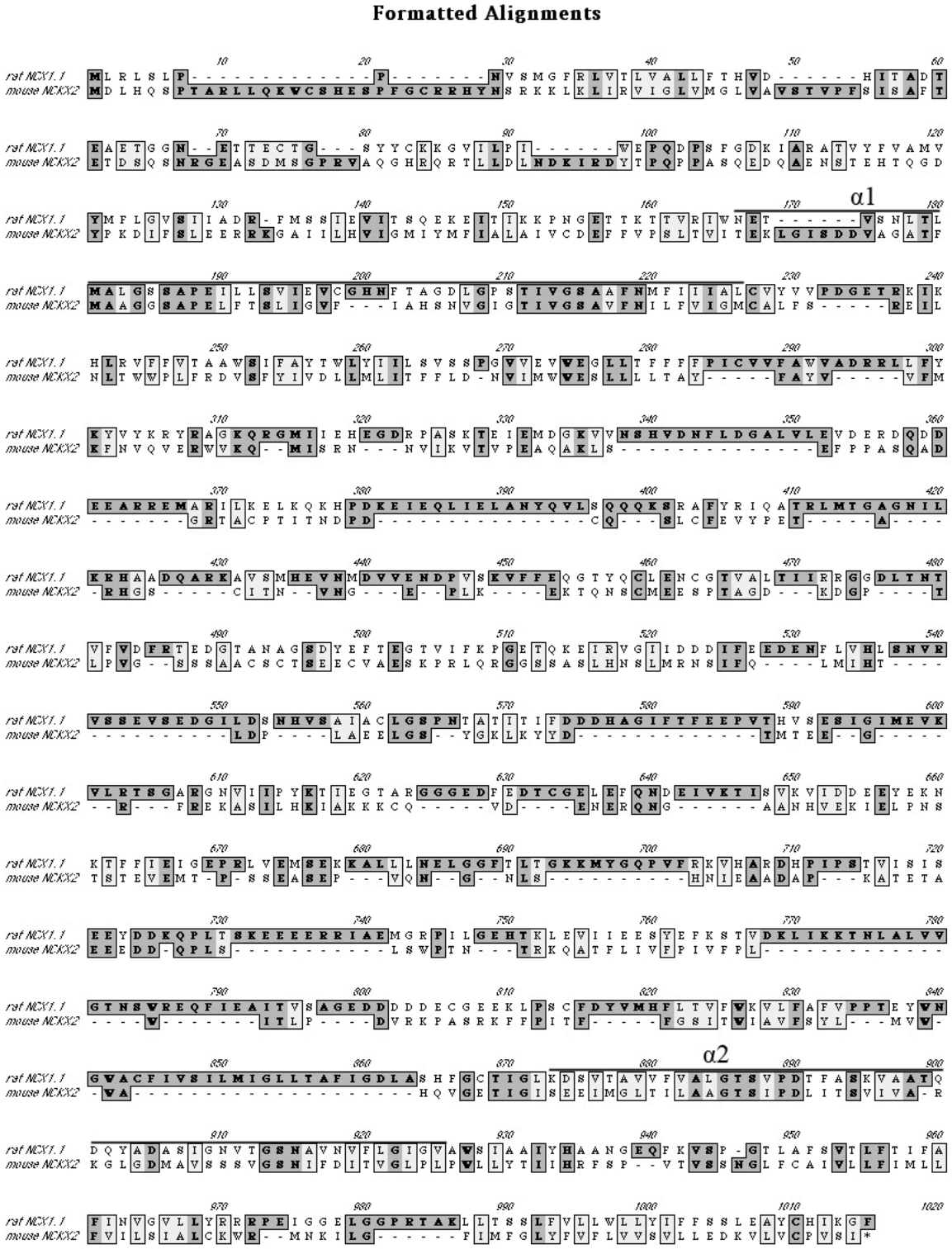


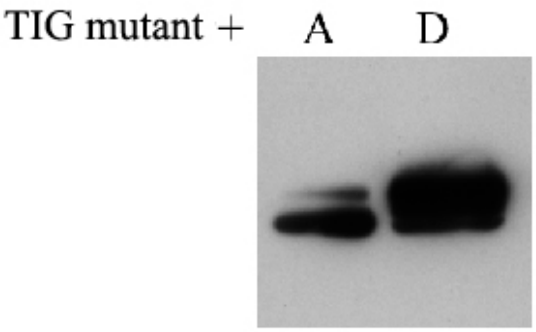
Figure 15: Alignment of rat NCX1 and mouse NCKX2 protein sequences.

Figure 16: Analysis of mutants.

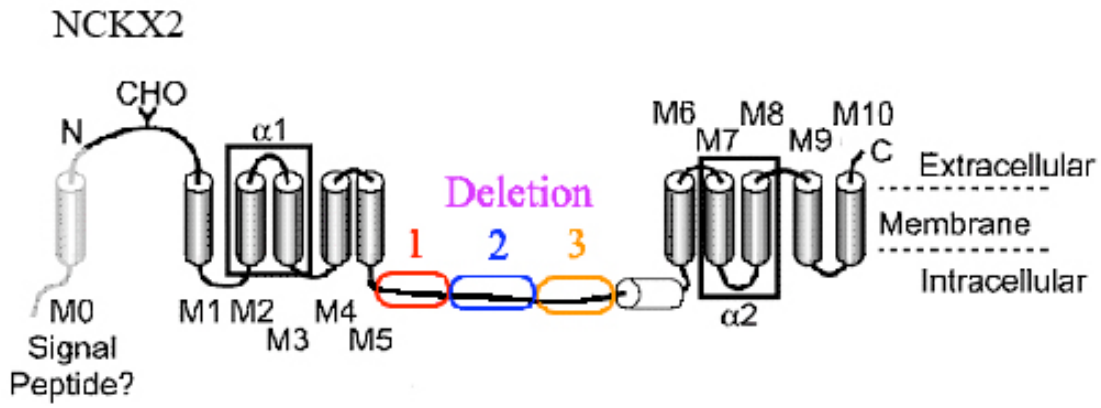
A: NCX1 '825TIG827' mutant was co-transfected with active Src or dead Src. The lysates were separated by SDS-PAGE and immunoblotted with anti-NCX1 antibody.

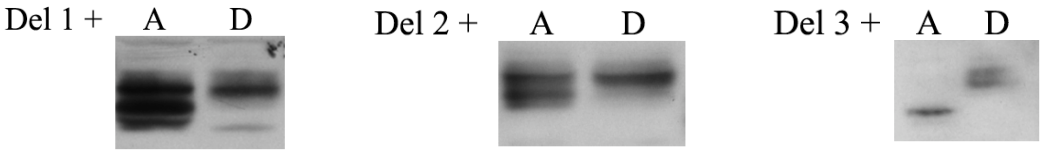
B: The big intracellular loop of NCKX2 was divided into three portions. Each of the three deleted mutants lacked one portion, respectively. The cartoon (upper panel) indicated regions deleted from NCKX2 (Figure modified from Lytton, J. Membrane Transporters: Na^+/Ca^{2+} exchanger. In Lennarz, W.J. & Lane M.D., eds. Encyclopedia of Biological Chemistry Elsevier, Oxford, 2004; Vol. 2; pp. 631-636). Each mutant was co-transfected active Src or dead Src and the lysates were immunoblotted with anti-NCKX2 antibody. Shown is a typical result of two independent experiments.

A:


TIG mutant +
A
D

B:


NCKX2
CHO
N
α1
M6
M8
M10
M7
M9
C
Extracellular
Deletion
Membrane
1
2
3
Intracellular
M0
Signal
Peptide?
M1 M2
M3
M4
M5
α2


Del 1 +
A
D
Del 2 +
A
D
Del 3 +
A
D

divided into three portions. Each of the three deleted mutants lacked one portion, respectively. All these deleted mutants exhibited similar Src induced mobility shifts to that seen for NCKX2 (Fig. 16B), suggesting the large intracellular loop was not critical in terms of Src regulation.

X. NCX1-associated proteins

Since there was no direct tyrosine phosphorylation of NCX1 and no direct interaction between NCX1 and Src, I hypothesized that Src affected the trafficking of NCX1 through a "third" protein in an indirect way. The hypothesis was that some protein/proteins might interact with NCX1, or lose the interaction with NCX1, only in the presence of Src activity. To test this hypothesis, HEK293 cells were transfected with NCX1 alone, NCX1 plus active Src, NCX1 plus dead Src or vector alone. Proteins were immunoprecipitated with antibody R3F1 and resolved by SDS-PAGE gel, followed by silver staining to detect the immunoprecipitated proteins. Specific bands corresponding to NCX1 can be seen by silver staining in the lanes of NCX1, NCX1 plus active Src and NCX1 plus dead Src, but not in the vector lane (Fig. 17A), which correspond to immunoreactive bands seen by immunoblot (Fig. 17B). The size of NCX1 in the lane of NCX1 plus active Src was smaller than that in the lanes of NCX1 and NCX1 plus dead Src, which was consistent with our previous observation that Src activity increased the mobility of NCX1. There was no "missing" band in the lane of NCX1 plus active Src compared to the lane of NCX1 plus dead Src. Interestingly, there was a unique strong band (≈110 kDa) in the lane of NCX1 plus active Src as indicated by an arrow. Although a faint NCX1 band

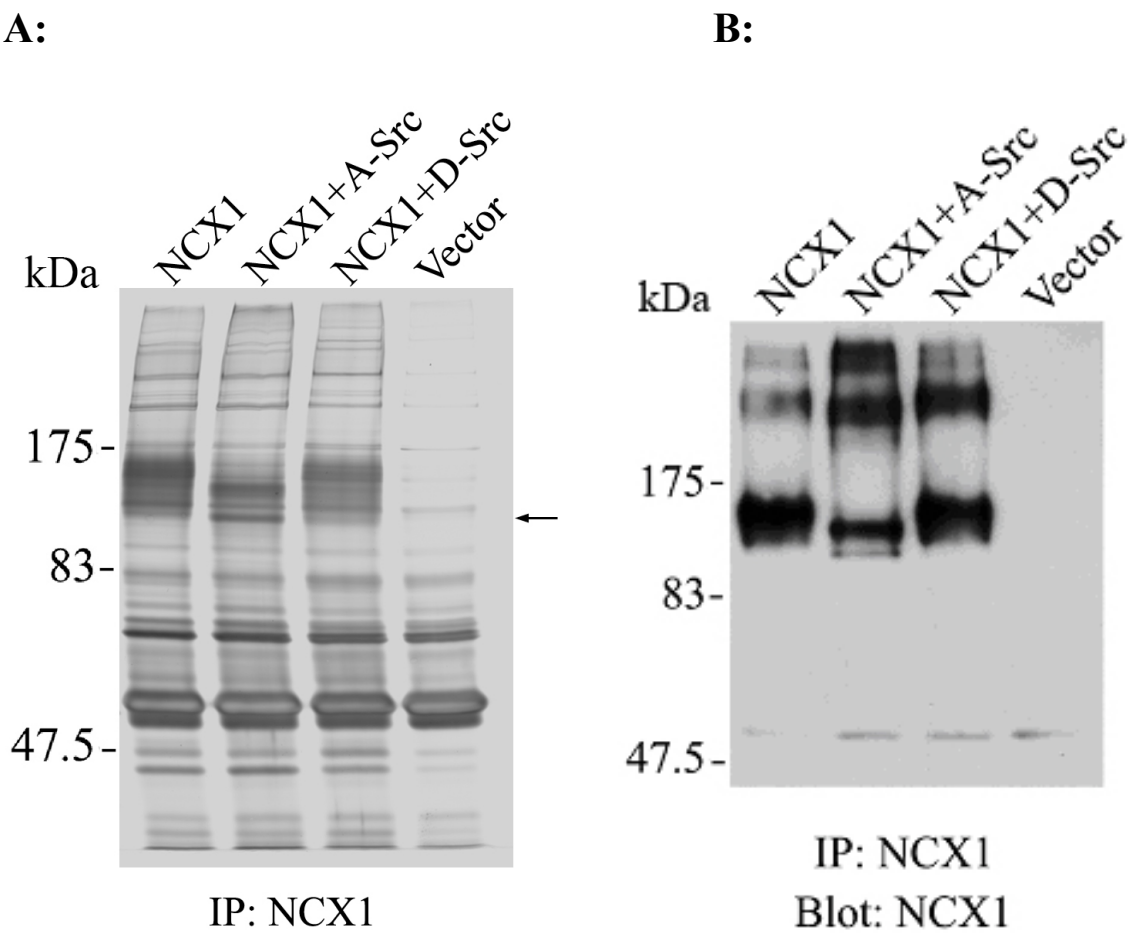


Figure 17: Silver staining of immunoprecipitated NCX1 associated proteins.

A: HEK 293 cells were transfected with NCX1, NCX1 plus active Src, NCX1 plus dead Src, or vector alone. Cell lysates were immunoprecipitated with anti-NCX1 antibody, R3F1, and were resolved on a 7.5% SDS-PAGE gel and visualized by silver staining. The arrow indicates an unusual strong band in the lane of NCX1 plus active Src. Shown is a typical result of three independent experiments.

B: 10% of the same samples were immunoblotted with anti-NCX1 antibody.

Table 3: Summary of the significant hits ($p<0.05$) from samples excised from the Coomassie-stained gel

NCX1 plus active Src

Protein Name	NCBI Match	Nominal Mass	Sequence Coverage
poly(ADP-ribose) synthetase	gi|337424	113882	8%
heat shock protein 70 - human	gi|2135328	79858	15%
ATPase, Ca^{2+} transporting, cardiac muscle, slow twitch 2 isoform 2	gi|4502285	111103	5%
hnRNP U protein	gi|32358	89631	1%
hypothetical protein	gi|4884358	55720	5%
KIAA1008	gi|19923416	109990	2%
T-cell receptor beta chain VJ region	gi|13276288	16112	7%
nuclear corepressor KAP-1	gi|1699027	90247	10%
sarco/endoplasmic reticulum Ca^{2+} -ATPase	gi|3021396	113924	2%
PRSS3 protein	gi|118763987	27040	8%

NCX1 plus dead Src

Protein Name	NCBI Match	Nominal Mass	Sequence Coverage
Poly [ADP-ribose] polymerase 1 (PARP-1)	gi|130781	113811	6%
KIAA1008	gi|19923416	109990	8%
RAX protein	gi|119850798	40268	18%
Heat shock 70 kDa protein 4	gi|6226869	95096	11%
hnRNP U protein	gi|32358	89631	6%
nuclear corepressor KAP-1	gi|1699027	90247	4%
E1B-55kDa-associated protein	gi|3319956	96321	5%
hCG1643231, isoform CRA_b	gi|119567961	35448	7%
ATP citrate lyase isoform 2	gi|38569423	120608	5%
Importin beta-3 (Karyopherin beta-3) (Ran-binding protein 5) (RanBP5)	gi|4033763	125032	7%
GPI-anchored membrane protein 1 (GPI-anchored protein p137) (p137GPI)	gi|2498733	72935	5%
Chain A, Recombinant Human Hexokinase Type I Complexed With Glucose And Phosphate	gi|3891376	103560	4%

The immunoprecipitated proteins from transfected cells were resolved on a 7.5% SDS-PAGE gel and visualized by Coomassie staining. There was an unusual strong band in the lane of NCX1 plus active Src similar to the one indicated by the arrow in Fig.17A. This band and a band of same position from the NCX1 plus dead Src lane (as the control) were cut out from Coomassie-stained gel and analyzed by mass spectrometry.

existed at a similar position revealed by immunoblot analysis, its intensity was too low to explain the prominent band showed by silver staining. The unique band in the NCX1 plus active Src lane could also be observed when the gel was stained with Coomassie blue. This band and a band of same position from NCX1 plus dead Src lane (as the control) were cut out from Coomassie-stained gel and analyzed by mass spectrometry. The significant hits ($p<0.05$) from each sample are listed in Table 3. The potentially interesting proteins should have the following characteristics: (1) the proteins should be present in the sample of NCX1 plus active Src and absent from the sample of NCX1 plus dead Src; (2) the mass of the protein should be around 110 kDa. Based on these criteria, Ca^{2+}-ATPase, which appeared twice from the sample of NCX1 plus active Src, was a potential candidate. However, co-immunoprecipitation using anit-SERCA polyclonal antibody N1 failed to confirm the interaction between SERCA and NCX1 co-transfected with active Src (data not shown). Another candidate is the so-called "hypothetical protein", whose function has not been documented. Although its mass is only 55720 Da, the protein may form a dimmer, thus exhibiting ~110 kDa molecular weight on an SDS-PAGE gel. Further experiments are required to verify the specific interaction between this protein and NCX1 co-transfected with active Src.

CHAPTER

DISCUSSION

I. Overview

The aim of my research was to understand the interaction between the widely expressed Na^+/Ca^{2+} exchanger, NCX1, and non-receptor tyrosine kinase, Src. Previously published studies have revealed the potential effect of tyrosine kinase on NCX1 activity (Missan and McDonald, 2004). In this study, we showed that co-transfection with Src tyrosine kinase led to an increased mobility of NCX1 on SDS-PAGE gel. Direct phosphorylation of NCX1 was not observed. The size mobility change was due to alteration of the N-linked glycan chain. Surface expression of NCX1 was dramatically affected by Src activity, as revealed by biotinylation experiments. The changed glycan was Endo H resistant, suggesting the protein was not retained in the ER. Further experiments suggested the modification happens during the process of biosynthesis process. This phenomenon showed some selectivity to NCX1 although the underlying mechanism was not clear.

My data can be reconciled with the previous finding that tyrosine kinase inhibitors activated the membrane current generated by NCX1 (Missan and McDonald, 2004). Since Src kinase activity reduces the surface expression of NCX1, inhibition of tyrosine kinase may increase the surface density of NCX1, thus increasing NCX1 related current.

In their study, the induced NCX1 current appeared after ~2 min and was relatively stable 5-7 min following addition of the tyrosine kinase inhibitor. Usually, intra-Golgi transport requires several to 30 min, while Golgi-to-cell-surface transport is much faster and may only need 1-2 min (Bonfanti et al., 1998; Losev et al., 2006). If the trafficking of NCX1 is halted by Src activity close to the late cisternae, resulting in a pod of intracellular NCX1 molecules under normal cellular conditions, then inhibition of Src might allow rapid delivering of this pool to the plasma membrane. My data, on the other hand, involve activation of Src, which in this model will reduce surface delivery. However, any observed change in activity will require turnover of existing surface NCX1 molecules, which is a much slower process (Slodzinski and Blaustein, 1998).

II. Src in the regulation of glycosylation

Our data showed the N-linked glycosylation of NCX1 was changed by Src activity. Indeed, the glycosylation of cell surface glycoproteins is a dynamic process that can be regulated by agents such as retinoic acid (Cho et al., 1996), transforming growth factor-β (Miyoshi et al., 1995), tumor necrosis factor-α (Hanasaki et al., 1994), etc. Interestingly, previous data implied that Src could increase the expression of N-acetylglucosaminyltransferase-V (GlcNAcT-V), which in turn might increase the size of the glycoprotein (Buckhaults et al., 1997). However, our observation showed a reduced size of the glycoproteins NCX1 and NCKX2, suggesting the altered expression of GlcNAcT-V could not account for this phenomenon.

Although glycosylation is also related to the metabolism of the cell and Src kinase may affect the metabolism of cells, it is unlikely any change of metabolism induced by Src is the cause of the change in NCX1 glycosylation since other glycosylated proteins, like mGlu1b, showed the same glycosylation status regardless of whether they were co-transfected with constitutively active Src or kinase dead Src. Due to the same reason, it is very unliked that this phenomenon resulted from the altered expression or activity of glycosylation related enzymes. Indeed, although Src can modulate gene expression, results from microarry experiments suggest the expression of glycosylation related genes are not dramatically changed (Malek et al., 2002; Paz et al., 2004).

III. The reason for reduced glycosylation

It is important to distinguish between two different possible mechanisms that could cause a reduction in the NCX1 glycan. First, the reduced size could be due to a change in glycosylation, meaning that the process was incomplete, not as effective as it should be, or altered in some other way. Secondly, the protein and glycan might mature normally and reach the plasma membrane first; then the glycan could have been modified during endocytosis or a recycling process. Indeed, plasma membrane glycoproteins can recycle to the Golgi apparatus and this pathway can be detected by acquired alterations in oligosaccharide structure (Snider and Rogers, 1985; Duncan and Kornfeld, 1988; Reichner et al., 1988; Reichner et al., 1998; Bos et al., 1995; Ghosh et al., 1999). To clarify this question, we generated the inducible NCX1 cell line, which expressed NCX1 only in the presence of tetracycline. We showed that if we turned on the expression of

NCX1 and activated Src at the same time, the glycan struture changed as evidenced by the mobility shift on SDS-PAGE gel. However, when we induced the expression of NCX1 first followed by transfected with activated Src, the glycan chain remained the same. The data strongly supported the first idea that the glycan change happened during the process of biosynthesis.

IV. The reason of reduced surface expression of NCX1

The finding that Src activity dramatically reduced the surface expression of NCX1 provides a mechanism for Src-induced changes in NCX1 activity. The surface expression of a protein is controlled by exocytosis, endocytosis and degration of the protein.

Src can trigger calveolin dependent endocytosis (Cheng et al., 2006; Shajahan et al., 2004) and NCX1 has been shown to associate with calveolin (Bossuyt et al., 2002; Cha et al., 2004b). Of the three calveolin isoforms, NCX1 only interacts with calveolin 3. This mechanism is unlikely to operate in the case of NCX1 though, since caveolin-3 is virtually absent in HEK293 cells (Cha et al., 2004a). Also, as demonstrated and discussed above, Src does not affect mature NCX1, thus degration of NCX1 cannot account for the reduced surface expression.

Based on our data, we concluded that the reduced surface expression of NCX1 is likely due to changed exocytosis.

V. Which happened first: glycosylation change or trafficking change?

Co-expression with Src induced two major alterations of NCX1: (i) change in structure of NCX1 glycan; (ii) change in surface expression of NCX1. What is the relationship between the glycosylation change and the trafficking change? Although N-linked glycosylation is related to the trafficking of some proteins, a change in protein trafficking can also change the glycan structure since the glycosylation process depends upon sequential access to distinct compartments with different enzymes. In the case of NCX1, N-linked glycosylation was not required for correct surface expression, since the N-glycan free mutant N9Q showed normal trafficking. Also, the glycosylation free mutant N9Q exhibited a similar trafficking problem as wild-type NCX1 when co-transfected with activated Src. Taken together, these data strongly suggested that the Src-induced alteration in the NCX1 glycan was not the trigger of the trafficking change. Instead, the trafficking change was more likely the cause of the glycan change.

VI. Where did the interruption in trafficking happen?

Obviously, an interruption in trafficking might happen in the ER or in the Golgi apparatus, two important glycosylation sites through which NCX1 transits before reaching plasma membrane. The mobility of FLT-3 receptor on SDS-PAGE gel increases in the presence of Src activity. However, it is tyrosine phosphorylation that causes the

retention of the receptor protein in the ER (Schmidt-Arras et al., 2005). In our case, however, NCX1 was neither tyrosine phosphorylated nor retained in the ER, since the altered glycan was resistant to Endo H digestion. The acquisition of endo H resistance is due to modification of the glycan by GlcNAcT-I, which is located in the Golgi apparatus. Thus it seems likely that the trafficking change happened in the Golgi apparatus. Indeed, Src kinase had been shown to exist on the Golgi membrane (David-Pfeuty and Nouvian-Dooghe, 1990; Bard et al., 2002) and could affect the trafficking of some proteins (Bard et al., 2003).

VII. Model of trafficking change induced by Src kinase

Based on my results, I propose a model as illustrated in Fig.18. The figure shows that under normal conditions, NCX1 protein travels through the ER, Golgi apparatus to its destination at plasma membrane. The glycan chain is sequentially modified to become a complex type. However, in the presence of Src activity, the journey of NCX1 to cell membrane stops somewhere in Golgi apparatus. Since the trafficking of NCX1 is halted or changed during its move along the Golgi cisternae, it does not access all the glycosylation related enzymes, and consequently the modification of the glycan chain is limited to only the early steps.

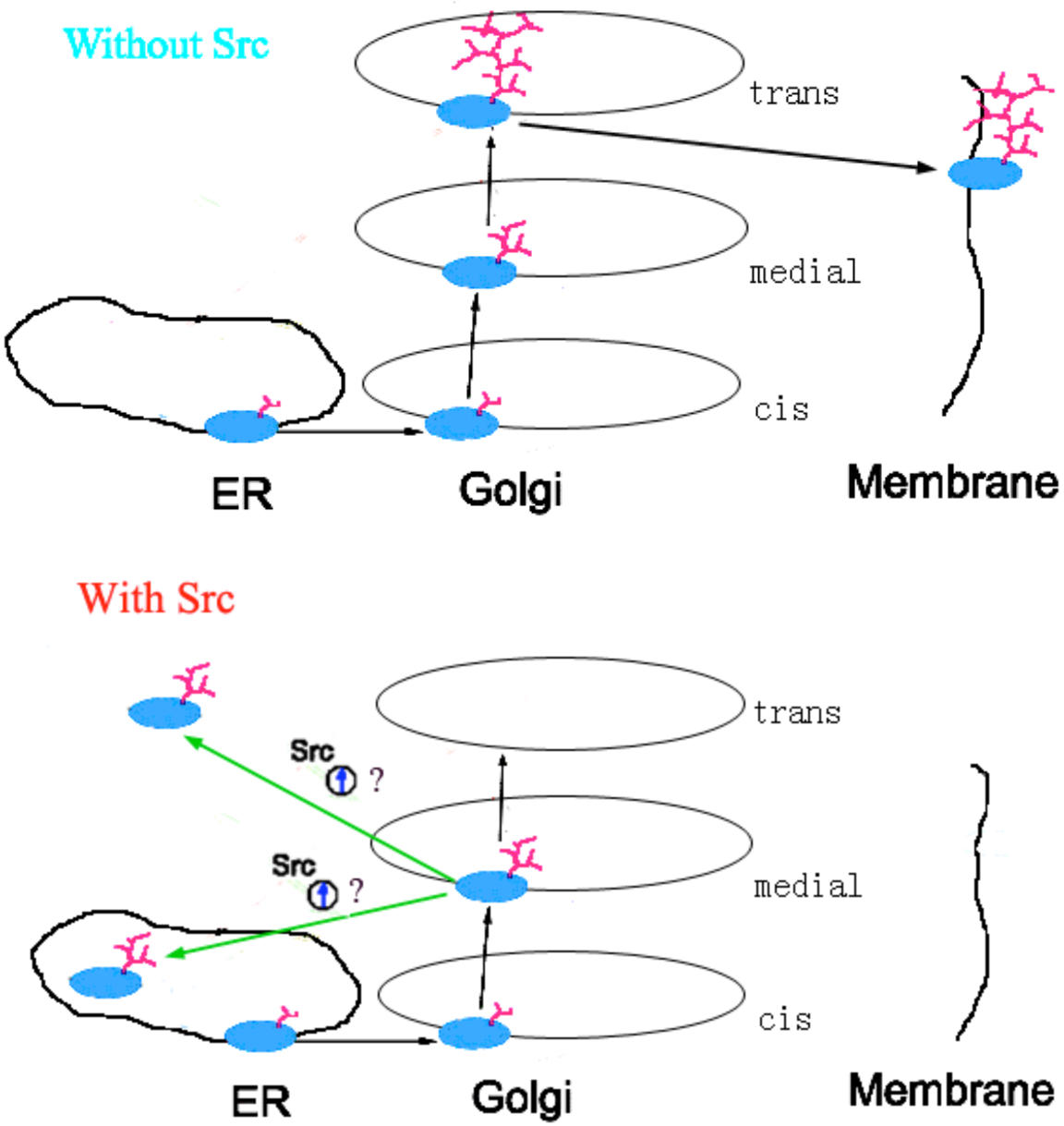


Figure 18: Proposed model. Under normal conditions without significant Src activity, the NCX1 protein travels through the ER, Golgi apparatus and to the plasma membrane. The glycan chain is sequentially modified and becomes complex type in the Golgi during its progress to the plasma membrane. However, when significant Src activity exists, the journey of NCX1 protein to cell membrane is stopped somewhere in Golgi apparatus. Since the trafficking of the protein is halted during its movement along the Golgi cisternae, it does not have the chance to access all the glycosylation modifying enzymes. Thus the protein is only partially glycosylated.

VIII. The possible mechanism - Golgi regulation

Golgi and trafficking

The primary function of the Golgi apparatus is to process incoming proteins and lipids, etc., and sort them within vesicles for export outside of the cell or for transport to other locations in the cell. Thus, it serves as a central junction for membrane traffic. It comprises a stack of distinct compartments or cisternae arranged from *cis* to *trans*. The cis side receives transport vesicles from the endoplasmic reticulum carrying newly made membrane and secreted proteins. This cargo then moves through the stack to the *trans* side, or trans-Golgi network (TGN), where it is sorted into carriers moving to different destinations in the cell, including the plasma membrane, lysosomes and, in some cell types, storage compartments for regulated secretion. The transport from *cis* to *trans* has been proposed to occur either by forward moving vesicles, or by whole cisternae forming at the cis side and then progressing to the TGN to be replaced by more cisternae forming behind them (Altan-Bonnet et al., 2004; Bonifacino and Glick, 2004). The trafficking activities of the various cisternae are very different. Cisternae in different parts of the stack recruit distinct sets of coat proteins to form the various types of vesicles that move to different destinations: returning to the ER, moving within the stack, and mediating transport to other parts of the cell. Many proteins, like the GTPase of the Rab family, Arf family GTPases, coiled-coil proteins, SNAREs, play important roles in membrane trafficking steps (Behnia and Munro, 2005; Munro, 2005).

Quality control in the Golgi complex

The Golgi complex as well as ER, takes part in the protein quality control system. Cycling between the ER and Golgi complex is necessary for the degradation of several misfolded proteins (Yoshida, 2003). Recently, a Golgi complex resident transmembrane ubiquitin ligase, Tul1, was identified. It was required for the ubiquitination of some proteins and might contribute to quality control, by identifying the misfolded membrane proteins and marking them for transport directly from the Golgi to endosomes, rather than via the plasma membrane (Reggiori and Pelham, 2002).

Golgi and Src

Src has been shown to localize to the plasma membrane and other intracellular membranes with particular concentration in the Golgi (David-Pfeuty and Nouvian-Dooghe, 1990; Bard et al., 2002). A recent study even suggested it gets access to the Golgi lumen (Bilodeau et al., 2006). Src may affect the Golgi function directly or indirectly. Direct actions include phosphorylation of the guanine nucleotide exchange factor C3G localized at the Golgi complex (Radha et al., 2004). Indirect actions include Src-dependent activation of phospholipase Cγ1, and subsequently translocation of the Ras guanine nucleotide exchange factor RasGRP1 to the Golgi where it activated Ras protein (Bivona et al., 2003). Src may affect the protein trafficking as well since it has been implicated in retrograde transport by influencing the rate of KDEL receptor recycling between the Golgi complex and the ER (Bard et al., 2003).

Four possibilities are proposed here to account for the Src-induced trafficking change, and altered glycan modification, of NCX1.

(1) Src directly phosphorylated or interacted with some coat proteins specifically involved in NCX1 protein transport through the Golgi apparatus. The surface delivery of NCX1 was impaired due to a change in the function of these proteins.

(2) Src changed the expression of some coat proteins involved in NCX1 transport through the Golgi apparatus, thus affecting the trafficking of NCX1.

(3) Src modulated some molecules and these molecules in turn regulated the coat proteins involved in NCX1 transport through the Golgi apparatus.

(4) Src modulated some molecules and these molecules distracted NCX1 from its normal trafficking pathway.

IX. Specificity

If Src-induced trafficking and glycan change as seen for NCX1 is universal, then all the membrane proteins should be affected in a similar way. However, this idea is unlikely for several reasons. First, the cells transfected with active Src were still viable. If all the membrane proteins cannot be delivered to the membrane surface, cells should not be able to survive. Second, co-transfection with active Src did not change the trafficking and

glycosylation status of the membrane protein mGluR1b, suggesting not all membrane proteins were affected by Src activity in the same way as NCX1. Third, the pattern of the streptavidin blot was similar for samples from cells transfected with active Src or dead Src, indicating that overall surface membrane protein expression was not dramatically changed by Src activity. Fourth, a number of membrane proteins have been studied by co-transfecting with Src, but most of them exhibited normal protein trafficking.

Since Src activity specifically affected NCX1, or possibly a class of several proteins that include NCX1, then what is the determining factor for this selectivity? It seems likely that the Src-induced trafficking change was dependant on some sequence or structural information of NCX1. Based on the amino acid sequence similarity between NCX1 and NCKX2, 825TIG827 located at the extracellular loop connecting TM6 and TM7 of NCX1 was mutated to 825AAG827. Moreover, since the large intracellular loop is involved in most regulations of the exchanger, I examined the potential involvement of this loop by sequentially deleting a portion of the loop. Unfortunately, all of these mutants exhibited similar Src-induced changes, suggesting the regions I tested were not responsible for the Src-induced modification. More mutants might be helpful. However, success is not guaranteed. First, some regions, like the α-repeats, are extremely sensitive to mutation. The mutant may not even get into the Golgi apparatus, where the Src-induced modification is supposed to happen. Second, maybe it is some special structure of NCX1 that defines this Src-induced regulation. However, no detailed structural information is available for NCX1.

X. Physiological implications

Ischemic preconditioning:

The heart possesses the remarkable ability to protect itself from stress by changing its phenotype. A sublethal ischemic stress enhances the tolerance of the myocardium to a subsequent ischemic stress. This process is referred to as ischemic preconditioning, which was first described more than two decades ago by Murray *et al.* (Murry et al., 1986).

The Na^+/Ca^{2+} exchanger can mediate Ca^{2+} influx (reverse mode) as well as Ca^{2+} efflux (forward mode) depending on the membrane potential and the concentrations of Na^+ and Ca^{2+} sensed by the exchanger (Blaustein and Lederer, 1999). During ischemia, the intracellular Na^+ concentration increases and the cell membrane potential depolarizes. These forces combine to induce the "reverse" mode transport that brings Ca^{2+} into the cell, which may in turn contribute to cell injury. Experiments from both pharmacologic study and transgenic mice suggested lowered NCX1 activity facilitated heart protection under ischemic condition. First, drugs that inhibit Na^+/Ca^{2+} exchanger mitigated the severity of ischemic injury (Seki et al., 2002; Iwamoto et al., 2004a). Second, NCX1 knockout hearts were significantly protected against ischemia-reperfusion injury (Imahashi et al., 2005). Third, heterozygous NCX1 knockouts mice had reduced NCX1 protein level as well as reduced susceptibility to ischemic injury (Ohtsuka et al., 2004).

Src activity was decreased by ischemia/reperfusion (Takeishi et al., 1999) but accelerated by ischemic preconditioning (Takeishi et al., 2001b). A significant increase of Src activity in the cytosolic fraction was observed (Ping et al., 1999). Our data suggested Src kinase activity reduced the amount of NCX1 protein on the cell surface. The change was a relatively slow process compared to others involving direct activation or inhibition. The reduced surface amount of NCX1 during preconditioning process may help cell survive during ischemia. Actually, previous data suggested tyrosine kinase activity was required during preconditioning since tyrosine kinase inhibition blocked the protective effect of preconditioning (Baines et al., 1998; Takeishi et al., 2001b). Thus it is possible that the Src induced trafficking change of NCX1 is a possible mechanism underlying the protective effect of preconditioning ischemia. The same mechanism may exist in kidney and brain as well.

Hypertrophy:

Cardiac hypertrophy, a thickening of myocardium resulting from a variety of stimuli, is important in the formation of various heart diseases including heart failure (Frey and Olson, 2003). Perturbations of Ca^{2+} metabolism are crucial in the development of hypertrophy (Berridge, 2006). In many studies, the expression of NCX1 was increased (Wang et al., 2001; Flesch et al., 1996). However, the function of NCX1 appeared downregulated in several studies (Wang et al., 2001; Fowler et al., 2005) although some reported upregulated activity (Sipido et al., 2000). Src activity is increased during hypertrophy (Takeishi et al., 2001a; Kovacic et al., 1998). Our model provides a possible

explanation of the apparent paradox that NCX function is reduced despite the increase of total NCX1 protein because increased Src activity reduces the surface density of NCX1.

Long-term potentiation (LTP):

LTP is an enhancement in synaptic transmission that can last for weeks, which is a potential mechanism for memory formation and learning (Martin and Morris, 2002).

The formation of LTP is critically dependent on the precise control of Ca^{2+} (Lynch, 2004) and exchanger proteins are involved in the regulation of LTP since both *ncx2* and *nckx2* knock-out mice exhibit changes in LTP (Jeon et al., 2003; Li et al., 2006). NCX1 is abundant in pyramidal neurons (Minelli et al., 2006; Thurneysen et al., 2002) and its regulation here may affect Ca^{2+} dynamics, thus influencing LTP. Src is also highly expressed in central nervous system (Brugge et al., 1985; Sugrue et al., 1990) and its activity is required for the induction of LTP in hippocampus (Huang and Hsu, 1999; Lu et al., 1998).

Previous data suggest Src is activated by tetanic stimulation (Lu et al., 1998). As a result, the surface expression of NCX1 and NCKX2 may be reduced, which in turn affects LTP. Actually, although short-term potentiation and early-phase LTP do not require gene expression or new protein synthesis, late-phase LTP does (Bailey et al., 1996). In addition, changed protein surface expression during LTP is not unusual. For example, changed surface expression of NMDA has been documented (Grosshans et al., 2002).

Therefore, Src-induced trafficking change of NCX1 may happen and play a physiological role in the formation of LTP.

XI. Future directions

Our experiments have demonstrated that Src altered the surface expression of NCX1 in HEK293 cells as evidenced by glycan change. However, future work is needed to test this regulation in native tissues and explore the underlying mechanism of this modulation. The proposed experiments are as follows:

(1) Endogenous Src will be activated in cultured cardiomyocytes, and the expression of NCX1 will be examined by immunoblot and immunofluorescence. If there is no obvious change, Src transfection will be conducted.

(2) NCX1 will be co-transfect with activated growth factor receptors to test whether growth factor receptors can modulate NCX1 in a similar way as Src kinase.

(3) More mutants of NCX1 will be made and tested to identify a region responsible for the effect of Src-induced modification.

(4) Since Src-induced modulation of NCX1 was dependent on the kinase activity of Src, cDNA library screening with anti-phosphotyrosine antibody will be conducted to identify Src kinase substrates as described previously (Lock et al., 1998). In addition,

Microarray methodology will be used to find genes with altered expression by Src kinase. Special attention will be paid to Golgi proteins since the modulation probably happens in the Golgi apparatus.

(5) Src is involved in several signaling pathways such as Src-STAT3, Src-Ras pathways (Frame, 2002). The potential involvement of a specific pathway will be investigated by two different means. The first method is to disrupt a specific Src involving pathway. For example, if Src-induced modulation of NCX1 remains in $STAT3^{-/-}$ cells (Debidda et al., 2005), the Src-STAT3 pathway is not critical for this regulation, and vice versa. Alternatively, STAT3 pathway can be selectively inhibited by inhibitor JSI-124 (Blaskovich et al., 2003). The second method is to cotransfect NCX1 and molecules downstream from Src. If a molecule can mimic the effect of Src on NCX1, the pathway involving that specific molecule is responsible for the Src-related regulation of NCX1.

XII. Concluding remarks

Both Na^+/Ca^{2+} exchanger NCX1 and non-receptor tyrosine kinase Src are widely expressed in many tissues. Surprisingly, heterologous expression of NCX1 together with Src led to a reduced N-linked glycan chain. Further evidence suggested the altered glycosylation was the result of deficient protein trafficking. This phenomenon showed some selectivity. However, the exactly mechanism is still unclear. Further work is needed

to elucidate how Src affects the trafficking of NCX1 as well as to demonstrate this regulation in native tissues.

To the best of my knowledge, this is the first study that showed direct regulation of Na^+/Ca^{2+} exchanger proteins by a protein tyrosine kinase. The results shown in this thesis are potentially interesting. NCX1 expression overlaps with Src in many tissues and NCX1 is essential for Ca^{2+} handling. The regulation of NCX1 by Src may play an important role in a variety of physiological and pathological conditions, like LTP and ischemia. The finding provides new fundamental insights into the local control of Ca^{2+} signaling.

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