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Binding of Src to Na^+/K^+-ATPase Forms a Functional Signaling Complex

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We have shown that ouabain activates Src, resulting in subsequent tyrosine phosphorylation of multiple effectors. Here, we tested if the Na^+/K^+-ATPase and Src can form a functional signaling complex. In LLC-PK1 cells the Na^+/K^+-ATPase and Src colocalized in the plasma membrane. Fluorescence resonance energy transfer analysis indicated that both proteins were in close proximity, suggesting a direct interaction. GST pulldown assay showed a direct, ouabain-regulated, and multifocal interaction between the α1 subunit of Na^+/K^+-ATPase and Src. Although the interaction between the Src kinase domain and the third cytosolic domain (CD3) of α1 is regulated by ouabain, the Src SH3SH2 domain binds to the second cytosolic domain constitutively. Functionally, binding of Src to either the Na^+/K^+-ATPase or GST-CD3 inhibited Src activity. Addition of ouabain, but not vanadate, to the purified Na^+/K^+-ATPase/Src complex freed the kinase domain and restored the Src activity. Consistently, exposure of intact cells to ouabain apparently increased the distance between the Na^+/K^+-ATPase and Src. Concomitantly, it also stimulated tyrosine phosphorylation of the proteins that are associated with the Na^+/K^+-ATPase. These new findings illustrate a novel molecular mechanism of signal transduction involving the interaction of a P-type ATPase and a nonreceptor tyrosine kinase.

INTRODUCTION

Cardiotonic steroids consist of a group of chemicals that specifically bind to the Na^+/K^+-ATPase (Hamlyn *et al.*, 1991; Scheiner-Bobis and Schoner, 2001). They include plant-derived digitalis drugs such as digoxin and ouabain and vertebrate-derived aglycone such as bufalin and marinobufagenin. Recent studies have identified both ouabain and marinobufagenin as endogenous steroids whose production and secretion are regulated by multiple physiological and pathological stimuli including angiotensin II and epinephrine in human. Many laboratories including ours have demonstrated that these steroids can activate protein kinases and regulate cell growth, gene expression, intracellular calcium, and reactive oxygen species (ROS) concentrations (Huang *et al.*, 1997; Kometiani *et al.*, 1998; Xie *et al.*, 1999; Aizman *et al.*, 2001; Aydemir-Koksoy *et al.*, 2001; Tian *et al.*, 2001; Barwe *et al.*, 2005), thus playing an important role in the control of renal and cardiovascular functions (Pierdomenico *et al.*, 2001; Fedorova *et al.*, 2002; Ferrandi *et al.*, 2004).

Src family kinases are 52–62-kDa membrane-associated nonreceptor tyrosine kinases and they participate in several tyrosine phosphorylation-related signaling pathways in response to various extracellular ligands (Brown and Cooper, 1996; Abram and Courtneidge, 2000). Src, for example, contains at least three important protein interaction domains. The SH3 domain binds to polyproline motifs and the SH2 domain interacts with the phosphorylated tyrosine residues. The kinase domain reacts with the nucleotide and phosphorylates the substrate. Binding of protein ligands to the SH3 or SH2 domain can activate Src (Tatosyan and Mizenina, 2000; Young *et al.*, 2001). Proteins that bind with kinase domain of Src were also reported to be capable of regulating Src activity (Ma *et al.*, 2000; Schulte and Sefton, 2003).

Na^+/K^+-ATPase, the molecular machinery of the cellular sodium pump, belongs to a family of evolutionarily ancient enzymes that couple the hydrolysis of ATP to membrane ion translocation (Skou, 1988; Lingrel and Kuntzweiler, 1994; Kaplan, 2002). Recently, we and others have proposed that the Na^+/K^+-ATPase has dual functions. It not only pumps Na^+ and K^+ across cell membranes, but also relays the extracellular ouabain signal to intracellular compartments via activation of different protein kinases (Aizman *et al.*, 2001; Xie, 2001; Barwe *et al.*, 2005). Specifically, we have found that the signaling Na^+/K^+-ATPase resides with its partners in caveolae and that binding of ouabain to the Na^+/K^+-ATPase activates Src that subsequently phosphorylates various effectors, resulting in the assembly and activation of different pathways including the Ras/Raf/ERK1/2 cascade and mitochondrial ROS production (Haas *et al.*, 2000; Liu *et al.*, 2000; Haas *et al.*, 2002). Importantly, the effects of ouabain on Src and subsequent tyrosine phosphor-

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Abbreviations used: BRET, bioluminescence resonance energy transfer; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; GST, glutathione-*S*-transferase; IB, immunoblotting; IP, immunoprecipitation; PKE, purified pig kidney enzyme (Na^+/K^+-ATPase); PP2, 4-amino-5-[4-chlorophenyl]-7-[t-butyl]pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4]pyromidine; RIPA, radioimmunoprecipitation.

ylation of other proteins are independent of changes in intracellular ion concentration. These observations led us to propose that the Na^+/K^+-ATPase may interact with Src to form a functional signaling complex. To test this hypothesis, we performed the following studies.

MATERIALS AND METHODS

Materials

PP2, a Src kinase inhibitor, was obtained from Calbiochem (San Diego, CA). [γ-^{32}P]ATP was obtained from New England Nuclear (Boston, MA). The antibodies used and their sources were as follows: The monoclonal anti-phosphotyrosine antibody (PY99), the monoclonal anti-Src antibody (B12), the goat anti-rabbit and the goat anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-Src pY418 antibody and anti-Src pY529 were from Biosource International (Camarillo, CA). The monoclonal anti-His antibody was from Invitrogen (Carlsbad, CA). Purified recombinant Src and the assay kit for Src kinase activity, anti-phosphotyrosine antibody, and protein G Agarose were obtained from Upstate Biotechnology (Lake Placid, NY). Plasmids pGFP^2-C, pRluc-N, and DeepBlueC were purchased from Biosignal Packard (Montreal, Canada). Plasmids pEYFP-C1 and pECFP-N1 were purchased from Clontech (Palo Alto, CA), and pGEX-4T-1 and pTrc-His were from Invitrogen. All secondary antibodies were conjugated to horseradish peroxidase; therefore, the immunoreactive bands were developed using chemiluminescence (Pierce, Rockford, IL). Glutathione beads were from Amersham Bioscience (Uppsala, Sweden). The Optitran nitrocellulose membranes were obtained from Schleicher & Schuell (Keene, NH).

Plasmid Constructs

The preparation of chicken c-Src lacking the SH4 domain and GST-Src mutants were done as described (Ma *et al.*, 2000). GST-NT (amino acid residue 6–90), GST-CD2 (amino acid residue 152–288), and GST-CD3 (amino acid residue 350–785) expression vectors were constructed based on the sequence of pig kidney Na^+/K^+-ATPase α1 subunit (see Figure 3A). GST-H^+/K^+-CD3 and GST-SERCA-CD3 were constructed based on the rat H^+/K^+-ATPase cDNA and rat cardiac SERCA 2a cDNA, respectively. His-tagged Src constructs were generated by excising the corresponding Src cDNA from the GST-Src vectors and then inserting them into pTrc-His A vector. Src-ECFP and Src-Rluc for fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) assays were constructed by cloning the full-length c-Src in frame into pECFP-N1 or pRluc vector. The rat Na^+/K^+-ATPase α1 cDNA was excised from the expression vector provided by Dr. Pressley (Texas Tech University) and inserted in frame into pEYFP-C1, and the canine Na^+/K^+-ATPase α1 cDNA was cloned into pGFP^2 vectors. All constructs were verified by DNA sequencing.

Cell Preparation, Culture, and Transient Transfection

Pig kidney proximal LLC-PK1, human embryo kidney 293T cells, and mouse fibroblast SYF and SYF + Src cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM medium containing 10% fetal bovine serum (FBS) and penicillin (100 U/ml)/streptomycin (100 μg/ml). LLC-PK1 cells and 293T cells were serum-starved for 24 h, whereas SYF and SYF + Src cells were cultured in the medium containing 0.5% FBS for 24 h and used for the experiments. Cells were transfected with various plasmids using Lipofectamine 2000 (Wang *et al.*, 2004). Experiments were performed 24 h after transfection unless indicated otherwise.

Preparation of Src, Na^+/K^+-ATPase, GST-fused Proteins, and His-tagged Proteins

Src, without the first 85 amino acid residues, was purified from sf-9 cells as described (Ma *et al.*, 2000) and used in the initial binding assays to ensure that Src binds to the Na^+/K^+-ATPase, but not the lipid composition in the purified Na^+/K^+-ATPase preparation. In subsequent experiments (e.g., phosphorylation and activity assays), purified recombinant full-length Src from Upstate Biotechnology was used. Na^+/K^+-ATPase was purified from pig kidney outer medulla using the Jorgensen method as we previously described (Xie *et al.*, 1996) and the preparations with specific activities between 1200 and 1400 μmol Pi/mg/h were used in this work. Under our experimental conditions either 100 μM vanadate or 10 μM ouabain caused a complete inhibition of the ATPase activity of the purified pig kidney Na^+/K^+-ATPase. GST fusion proteins or His-tagged proteins were expressed in *Escherichia coli BL21* and purified on glutathione beads or nickel column.

Immunoprecipitation and GST Pulldown

Cells were lysed in RIPA buffer containing 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM Tris-HCl (pH 7.4). Cell lysates were cleared by centrifugation at 16,000 $\times$ *g* for 15 min, and the supernatants (1 mg) were either immunoprecipitated with anti-α1 antibody or incubated with different GST-fusion proteins. The complexes were then pulled down by either protein G agarose or glutathione beads as we previously described (Ma *et al.*, 2000; Haas *et al.*, 2002) and analyzed by Western blot.

Src Kinase Activity

The Src kinase activity was assayed using a commercial kit as we previously described (Haas *et al.*, 2000). To determine how Na^+/K^+-ATPase affects Src kinase activity, the purified Src (4.5 U) was incubated with 5 μg of the purified Na^+/K^+-ATPase in the Src assay buffer for 30 min at room temperature. Afterward, both control Src or the Na^+/K^+-ATPase-bound Src were exposed to 10 μM ouabain and Src kinase activity was determined. In other experiments, the Src pY418 was measured by anti-pY418 antibody to indicate Src activation (Ma *et al.*, 2000). To do so, the purified Src (4.5 U) was incubated with different amount of the purified Na^+/K^+-ATPase or GST-Na^+/K^+-ATPase constructs in phosphate-buffered saline (PBS) for 30 min at 37°C. Afterward, 2 mM ATP/Mg^{2+} was added. The reaction continued for 5 min at 37°C and was stopped by addition of SDS sample buffer.

In Vitro Binding Assay

The purified Na^+/K^+-ATPase was solubilized in 1% Triton X-100 PBS and centrifuged at 100,000 $\times$ *g* for 30 min. The supernatant was collected for the binding assay. GST-fusion proteins (5 μg) were conjugated on glutathione beads and incubated with the solubilized Na^+/K^+-ATPase in 500 μl PBS in the presence of 0.5% Triton X-100 at room temperature for 30 min. The beads were washed with the same buffer for four times. The bound Na^+/K^+-ATPase was resolved on 10% SDS-PAGE and detected by Western blot. Reciprocal binding assay using GST-Na^+/K^+-ATPase constructs (5 μg) and purified Src lacking of first 85 amino acids (200 ng) or His-tagged Src constructs (100 ng) was done similarly. To test if native Na^+/K^+-ATPase binds Src, the above experiments were repeated in the absence of Triton X-100. To make the Na^+/K^+-ATPase/Src complex, 2–5 μg of the purified Na^+/K^+-ATPase was incubated with 4.5 U of Src (~10 ng) in PBS in the absence of Triton X-100 at room temperature for 30 min. The complex was either used for the experiments directly as indicated or collected by centrifugation at 100,000 $\times$ *g* for 30 min. Control experiments showed that the Na^+/K^+-ATPase-bound, but not the free, Src could be copelleted by the centrifugation.

FRET Analysis by Acceptor Photobleaching

Using pECFP-N1 and pEYFP-C1 vectors described above, the enhanced cyan fluorescent protein (ECFP) was fused to the C-terminus of Src, and the enhanced yellow fluorescent protein (EYFP) was fused to the N-terminus of rat Na^+/K^+-ATPase α 1 subunit. Src-ECFP and EYFP-rat α1 plasmids were then cotransfected into LLC-PK1 cells. Cells transfected with either ECFP/EYFP or ECFP/EYFP-rat α1 were used as a control. After 24 h, cells growing on glass coverslip were fixed with ice-cold methanol for 15 min at −20°C and washed twice with PBS solution. The coverslip was then used for FRET measurement with Leica DMIRE2 confocal microscope (Wetzlar, Germany). The laser lines of 456 nm and 515 nm were used to illuminate fluorescence, and the emission intensities were recorded at 465–509 nm for Src-ECFP and 530–570 nm for EYFP-rat α1. The cell that expresses both Src-ECFP and EYFP-rat α1 was chosen to perform the FRET analysis. A membrane region of interest (ROI 1) was selected and photobleached by applying 100% intensity of 515-nm laser. The emission intensities of Src-ECFP and EYFP-rat α1 before and after the photobleaching process in the selected ROI 1 region were used to calculate the FRET efficiency. The FRET efficiency was also calculated at a nonphotobleached region (ROI 2) and used as a control.

FRET Analysis in Live Cells

LLC-PK1 cells were cotransfected with Src-ECFP and EYFP-rat α1 and grown on a glass coverslip for 24 h. The coverslip was then mounted in a metal chamber and analyzed with a Leica DMIRE2 confocal microscope. The laser lines of 456 nm and 515 nm were used to illuminate fluorescence, and the emission intensities were recorded at 465–509 nm for Src-ECFP and 530–570 nm for EYFP-rat α1. The cell that expresses both Src-ECFP and EYFP- rat α1 was chosen and illuminated by only 456-nm laser. The cells express only Src-ECFP or EYFP-rat α1 were used for correction and determination of the laser intensity as well as the gain and offset settings. The emission intensities for both Src-ECFP and EYFP-rat α1 in selected membrane region was recorded at 465–509 nm (F_{ECFP}) and 530–570 nm (F_{EYFP}), respectively. The FRET efficiency was reflected by the ratio of F_{EYFP}/F_{ECFP}. After 50 s of recording, the same cell was exposed to ouabain and the recording was continued for indicated time.

BRET Analysis

BRET assay was done as described by Lowry *et al.* (2002). Briefly, 24 h after transfection with GFP-Na^+/K^+-ATPase and Src-Rluc or other constructs as indicated, cells were seeded in triplicate in a 96-well microplate. After treatment with indicated concentration of ouabain, cells were exposed to equal

volume of BRET analysis buffer containing 10 μM DeepBlue C, the substrate of Rluc. The emission at 410 nm (for Rluc) and 515 nm (for GFP) was immediately acquired using a Fluoroskan Ascent FL (Labsystems, Franklin, MA) with microplate luminometric detection. The BRET ratio was calculated as follows: (Emission at 515 nm − Background at 515 nm)/(Emission at 410 nm − Background at 410 nm), where Background signal was assessed in each experiment by measuring the signal of a sample of nontransfected cells.

Colocalization Analysis

LLC-PK1 cells were cultured for 24 h on glass coverslips, briefly washed twice with PBS, and then fixed with ice-cold methanol for 15 min. The cells were washed again with PBS and blocked using SignalEnhancer (Molecular Probes). Rabbit polyclonal anti-Src antibody and monoclonal anti-Na^+/K^+-ATPase antibody were mixed in 3% BSA and incubated with the coverslip overnight at 4°C. After three washes with PBS, Alexa fluor 546-conjugated anti-mouse antibody and Alexa fluor 488-conjugated anti-rabbit antibody were added and incubated for 1 h at room temperature. The coverslip was washed again with PBS for three times. The Na^+/K^+-ATPase was visualized by excitation at 546 nm and emission at 566–620 nm. Src was visualized by excitation at 488 nm and emission at 505–535 nm. To avoid the crosstalk between the two fluorescence dyes, we used sequential method featured by Leica confocal microscope to measure colocalization of the two proteins, in which, the two laser lines 488 nm and 546 nm were applied to the cells alternatively. Colocalization analysis was performed with Leica Confocal Software, version 2.5 build 1347.

Data Analysis

Data are given as mean ± SE. Statistical analysis was performed using the Student's t test, and significance was accepted at $p < 0.05$.

RESULTS

Interaction of the Na^+/K^+-ATPase with Src

We showed previously that ouabain binding to the Na^+/K^+-ATPase activated Src kinase in several different cell lines. In addition, we found that Src could be coimmunoprecipitated with the Na^+/K^+-ATPase α1 subunit and that ouabain regulated this interaction in a time and dose-dependent manner (Haas *et al.*, 2002). These findings suggest that the signaling Na^+/K^+-ATPase may interact with Src to form a signaling complex. To test this hypothesis, LLC-PK1 cells were fixed and double-stained by a monoclonal anti-α1 and a polyclonal anti-Src antibody. As expected, the Na^+/K^+-ATPase α1 and Src colocalized in the plasma membrane in LLC-PK1 cells (Figure 1A). Pixel analysis indicated that 25.2 ± 1.3% of Na^+/K^+-ATPase in the plasma membrane colocalized with Src. Similar colocalization between these two proteins was also observed in 293T cells that overexpressed Src-ECFP (unpublished data). To test whether the Na^+/K^+-ATPase and Src interact in LLC-PK1 cells, we transfected the cells with Src-ECFP and EYFP-rat α1. Fluorescence resonance energy transfer (FRET) analysis was performed in the transfected cells using acceptor photobleaching protocols. Rat α1 was chosen for the initial FRET experiments because we have a rat α1-specific antibody so that we could confirm the expression of the transfected α1 using Western blot in addition to monitoring YFP fluorescence. The data showed an energy transfer from Src-ECFP to EYFP-rat α1. As shown in Figure 1B, photobleaching of the EYFP-rat α1 resulted in an increase in the Src-ECFP signal. The FRET efficiency measured from a total of 16 cells in six separate experiments ranged from 8.1 to 18.8 (13.2 ± 1.7). In contrast, no FRET was detected in cells transfected with a pair of either ECFP/EYFP or ECFP/EYFP-rat α1. These data show that the Na^+/K^+-ATPase and Src are in close proximity, suggesting a direct interaction between these two proteins in LLC-PK1 cells.

To obtain evidence of direct binding, we first performed in vitro binding assays using the purified pig kidney Na^+/K^+-ATPase (PKE) and GST-Src. It is important to note that the purified Na^+/K^+-ATPase is a membrane-attached preparation in which the α1 and β1 subunits are associated in a 1:1 M ratio and accounts for more than 90% of protein contents in the preparation (Figure 2B and Jorgensen, 1974, 1988). As depicted in Figure 2C, the 1% Triton X-100-solubilized Na^+/K^+-ATPase bound to GST-Src in a concentration-dependent manner. Significant amount of α1 subunit was detected when 0.5 μg of the Na^+/K^+-ATPase was used in the binding assay. To quantitate the binding, experiments as shown in Figure 2D was performed. The data showed that GST-Src pulled down 12 ± 2.4% (n = 3) of the input when 2 μg of the purified Na^+/K^+-ATPase was used. These data suggest a possibility of direct binding between Src and the Na^+/K^+-ATPase. To control that the binding was not induced by solubilization of the Na^+/K^+-ATPase, we repeated the above experiments with the purified Na^+/K^+-ATPase in the absence of detergent, showing the similar interaction between the Na^+/K^+-ATPase and GST-Src. To dissect which domains of Src interact with the Na^+/K^+-ATPase (for domain structures see Figure 3A), we expressed and purified the GST-SH2, GST-SH3, GST-SH3SH2, and GST-kinase domain fusion proteins (Ma *et al.*, 2000). Using the same in vitro binding assay, we observed that the purified Na^+/K^+-ATPase bound to the kinase domain, the SH3SH2, and the SH2 domain, but not the SH3 domain (Figure 3C). Because the GST-SH3SH2 pulled down more Na^+/K^+-ATPase than that of the GST-SH2, this construct was used in subsequent experiments.

Although it is unlikely that Src or its domain constructs pulled down the Na^+/K^+-ATPase via their binding to an intermediate protein component of the purified enzyme preparations, to rule out this possibility and to identify

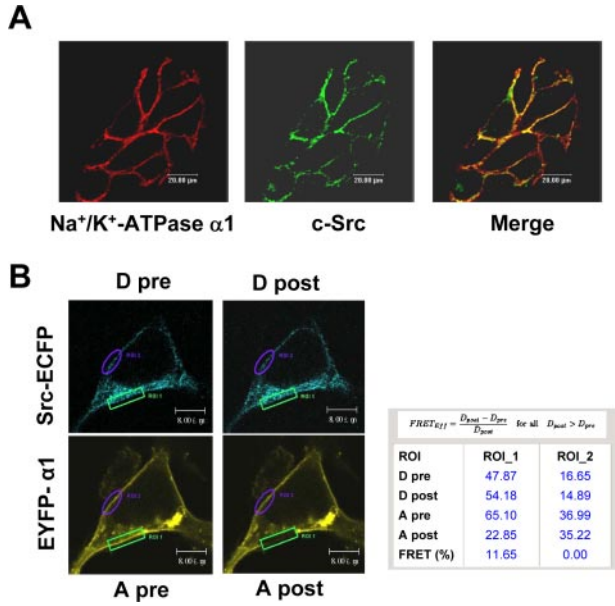

Figure 1. Interaction between Na^+/K^+-ATPase and Src in LLC-PK1 cells. (A) Colocalization of the Na^+/K^+-ATPase (red) and Src (green) in LLC-PK1 cells at a resolution of 1024 × 1024 pixels. Left and center images showed the membrane localization of the Na^+/K^+-ATPase α1 and Src, respectively, and the merged image (right) showed the colocalization of these two proteins. Scale bar, 20 μm. (B) FRET analysis of the interaction between EYFP-rat α1 (yellow) and Src-ECFP (cyan) in LLC-PK1 cells. The boxed area (ROI 1) was photobleached and analyzed for FRET. We also measured FRET at the circled area (ROI 2) that was not photobleached. The same studies were performed in 16 cells from 6 independent experiments. Scale bar, 8 μm.

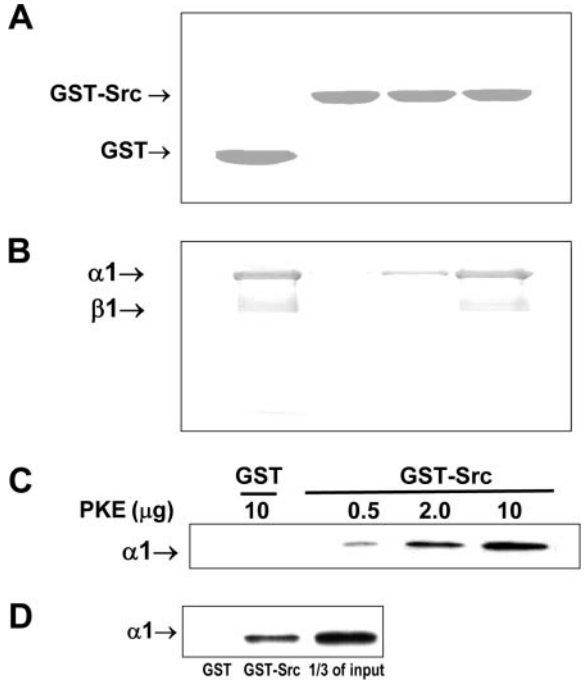

Figure 2. Binding of the purified pig kidney Na^+/K^+-ATPase (PKE) to GST-Src. Purified Na^+/K^+-ATPase was solubilized in 1% Triton X-100. After centrifugation at 100,000 × *g*, indicated amounts of the cleared supernatants were incubated with 5 μg GST-Src in the presence of 0.5% Triton X-100 for 30 min and followed by four washes with the same buffer. (A and B) The Coomassie blue-stained GST-Src and purified Na^+/K^+-ATPase (PKE). (C) A representative Western blot from three independent experiments showing the pulldown products probed with anti-Na^+/K^+-ATPase α1 antibody. (D) The same pulldown assay as in C was performed, and 650 ng (one-third of the total input) of the purified Na^+/K^+-ATPase (PKE) was directly loaded as an input control.

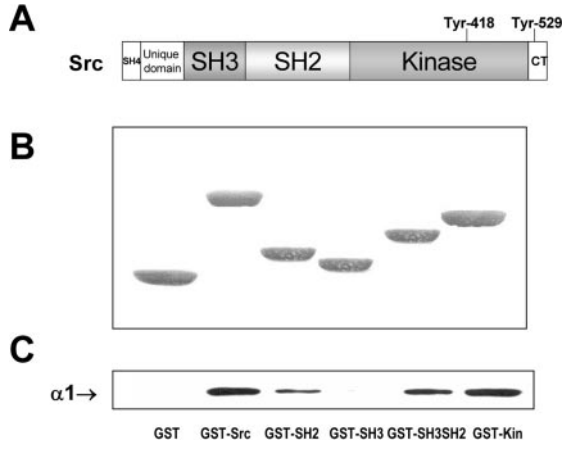

Figure 3. Identification of the Src domains involved in the interaction with the Na^+/K^+-ATPase. (A) Schematic presentation of structures of Src. (B) Coomassie blue staining of GST-Src, GST-SH2, GST-SH3, GST-SH3SH2, and GST-kinase. (C) Binding of GST-Src, GST-SH3SH2, GST-kinase, GST-SH2, but not GST-SH3, domains to the Na^+/K^+-ATPase. An aliquot (2 μg) of the purified Na^+/K^+-ATPase was used for each binding assay. The same experiments were repeated three times.

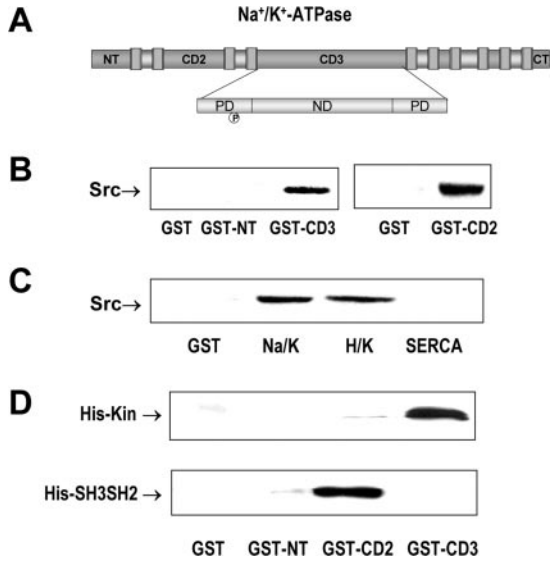

Figure 4. Identification of the Na^+/K^+-ATPase domains involved in the interaction with Src. (A) Schematic presentation of α1 subunit of Na^+/K^+-ATPase. NT, N-terminus; CD2, cytosolic domain 2; CD3, cytosolic domain 3; PD, phosphorylation domain; ND, nucleotide-binding domain; CT, C-terminus. (B) A representative Western blot of four independent experiments shows the binding of purified Src (lacking of first 84 amino acids) to the CD3, but not the NT of the α1 subunit when 200 ng of Src was used. (C) A Western blot showing that Src was pulled down by GST-CD3 of Na^+/K^+-ATPase (Na/K) and H^+/K^+-ATPase (H/K), but not SERCA from 1 mg LLC-PK1 cell lysates. (D) A Western blot showing the domain interaction between the Na^+/K^+-ATPase and Src. Different GST-fused Na^+/K^+-ATPase domain constructs were incubated with either His-tagged SH3SH2 domain or kinase domain of Src, and the pulldown products were analyzed by Western blot.

which domains of the Na^+/K^+-ATPase are involved in its interaction with Src, we prepared GST-fused proteins containing the N-terminus (GST-NT), the second cytosolic loop (GST-CD2), and the large central loop connecting the transmembrane helices M4 and M5 (GST-CD3; Figure 4A) of the α1 subunit of the Na^+/K^+-ATPase because these domains are known to interact with various proteins (Devarajan *et al.*, 1994; Yudowski *et al.*, 2000; Dolgova *et al.*, 2003). As shown in Figure 4B, Src interacted with GST-CD3 and GST-CD2, but not GST-NT. To further test if the binding is specific to the Na^+/K^+-ATPase, we made GST fusion proteins of the CD3 from rat gastric H^+/K^+-ATPase and rat heart sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA). The data showed that the GST-CD3 from the H^+/K^+-ATPase, but not the SERCA, pulled down Src from the LLC-PK1 cell lysates (Figure 4C). To map the specific domain interactions between the Na^+/K^+-ATPase and Src, we prepared His-tagged kinase domain and SH3SH2 domain fusion proteins. Employing the same binding assay, we found that the GST-CD3 interacted with the kinase domain, but not the SH3SH2 domain of Src. In contrast, the CD2 interacted with the SH3SH2 domain, but not the kinase domain (Figure 4D). Taken together, the above results indicate that Na^+/K^+-ATPase can directly interact with Src through the CD2 and CD3 domains of the α1 subunit.

Regulation of Src by the Na^+/K^+-ATPase

Because binding of SH3SH2 domain to a regulatory protein is sufficient to activate Src (Brown and Cooper, 1996; Boggon

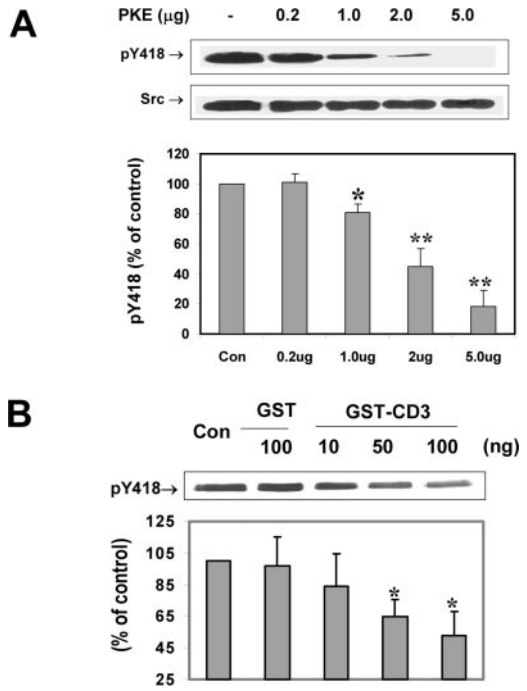

Figure 5. Regulation of Src by the Na^+/K^+-ATPase and GST-CD3. (A) Indicated amount of purified Na^+/K^+-ATPase (PKE) were incubated with recombinant Src (4.5 U) for 30 min in PBS, then 2 mM ATP/Mg^{2+} was added and incubated for another 5 min. After the samples were resolved on SDS-PAGE, the membranes were probed with antibodies as indicated. (B) GST (100 ng) or different amount of GST-CD3 was incubated with recombinant Src (4.5 U) for 30 min in PBS. The phosphorylation of Src was analyzed as in A. Values are mean ± SE of at least four independent experiments. * $p < 0.05$; ** $p < 0.01$ compared with control.

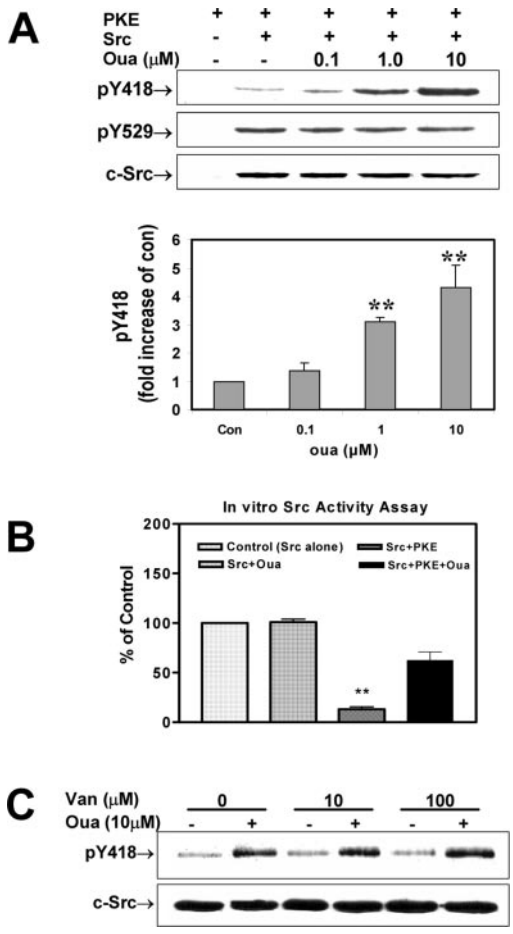

Figure 6. Stimulation of the Na^+/K^+-ATPase/Src complex by ouabain. (A) The preformed Na^+/K^+-ATPase/Src complex was treated with different concentrations of ouabain in the presence of 2 mM ATP/Mg^{2+} for 5 min, and the phosphorylated Src was analyzed using site-specific antibodies as indicated. Values are mean ± SE of at least four independent experiments. (B) Src or Src/Na^+/K^+-ATPase complex were treated with 10 μM ouabain, and the Src activity was measured. (C) A representative Western blot of four experiments showing the effects of ouabain and vanadate on the Na^+/K^+-ATPase/Src complex. A similar experiment as in A was repeated to assess the effects of either vanadate (Van) or vanadate plus ouabain (Oua) on Src phosphorylation. ** $p < 0.01$ compared with control.

and Eck, 2004), the above findings led us to test whether binding of Src to the Na^+/K^+-ATPase results in Src activation. When purified recombinant Src was incubated with different amounts of the purified Na^+/K^+-ATPase in the presence of ATP/Mg^{2+} in detergent-free PBS solution, the autophosphorylation of Src at Tyr418 (pY418), an indication of Src activation, was reduced in a concentration dependent manner (Figure 5A). Because we observed the same results when the experiments were repeated in the presence of 100 μM vanadate that completely inhibited the hydrolysis of ATP by the Na^+/K^+-ATPase, the effect of the Na^+/K^+-ATPase on Src is likely due to the interaction between these two proteins, but not the reduction of ATP. To further test this hypothesis, we determined the effect of CD3 on Src. Because Wiskott-Aldrich syndrome protein is reported to inhibit Src by binding to the kinase domain (Schulte and Sefton, 2003), we reasoned that interaction between the CD3 and the kinase domain might be sufficient to keep Src in an inactive state. Indeed, as shown in Figure 5B, GST-CD3, but not GST, acted as the purified Na^+/K^+-ATPase, caused a dose-dependent inhibition of the Src pY418.

Because the above data suggest that the Na^+/K^+-ATPase may bind Src and keep it in an inactive state, we hypothesized that the Na^+/K^+-ATPase/Src complex may constitute a functional complex for ouabain and act in a manner similar to that of G protein-coupled receptor/G protein complex; namely, binding of ouabain to this complex releases the trapped Src kinase domain, resulting in Src activation and subsequent tyrosine phosphorylation of downstream effectors. To test this hypothesis, we incubated the recombinant Src with the purified Na^+/K^+-ATPase in detergent-free PBS solution in the presence or the absence of ouabain. Western blot analysis indicated that addition of ouabain significantly increased the pY418 in a dose-dependent manner (Figure 6A). To confirm that changes in pY418 correlates with Src activity, we also measured the Src-mediated tyrosine phosphorylation using a commercial available kinase assay kit. As shown in Figure 6B, although the Na^+/K^+-ATPase kept Src in an inactive state, addition of ouabain restored the kinase activity. We also determined if vanadate affected the activity of this Na^+/K^+-ATPase/Src complex. As shown in Figure 6C, although 10–100 μM vanadate completely inhibited the ATPase activity, it showed no effect on Src pY418. More importantly, ouabain was still able to stimulate pY418 of Src in the presence of vanadate.

To test whether ouabain activates Src by dissociating it from the interacting Na^+/K^+-ATPase, we incubated Src

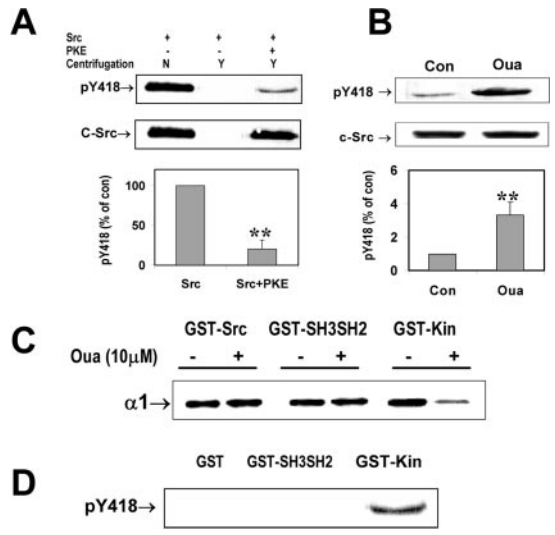

Figure 7. Activation of Src by freeing the kinase domain from the Na^+/K^+-ATPase. (A) A control experiment showing that Src could be cosedimented with Na^+/K^+-ATPase. Src (4.5 U) incubated with or without 5 μg Na^+/K^+-ATPase in 0.5 ml PBS was centrifuged at 100,000 $\times$ *g* for 30 min. The pellets were resuspended in PBS and subjected to phosphorylation assay as described in *Materials and Methods.* As an input control, 4.5 U of Src were directly suspended in PBS and assayed for pY418 phosphorylation. (B) Src (4.5 U) was preincubated with 5 μg of the purified Na^+/K^+-ATPase in PBS and then exposed to 10 μM ouabain for 15 min. Both control and ouabain-treated Na^+/K^+-ATPase/Src complexes were then collected by centrifugation, resuspended in PBS, and subjected to phosphorylation assay as in A. Two representative Western blots are shown in A and B, and the values are mean $\pm$ SE of at least three independent experiments. (C) A representative Western blot of four separate experiments showing that ouabain induced the release of the kinase domain from the Na^+/K^+-ATPase. GST-Src, GST-SH3SH2, or GST-kinase was incubated with 1 μg purified Na^+/K^+-ATPase for 30 min at room temperature in 500 μl PBS. Complexes were then pulled down on glutathione beads, washed three times, resuspended in 500 μl PBS, and exposed to 10 μM ouabain for 15 min. The beads were then washed for three more times using PBS, and the pulled down Na^+/K^+-ATPase was analyzed by Western blot using anti-α1 antibody. (D) A representative Western blot of three independent experiments showing the activation of Src by GST-kinase domain fusion protein. GST, GST-SH3SH2, or GST-kinase (5 μg each) was preincubated with 2 μg of the purified Na^+/K^+-ATPase for 15 min at room temperature. Recombinant Src (4.5 U) was then added to the mixture for additional 30 min. Phosphorylation reaction was started by addition of 2 mM ATP/Mg^{2+} and Src pY418 was measured as in A.

with the purified Na^+/K^+-ATPase. Because the purified Na^+/K^+-ATPase is attached to membrane, it can be pelleted by centrifugation at 100,000 $\times$ *g* for 30 min. As expected, centrifugation was sufficient to sediment Src only when it was bound to the Na^+/K^+-ATPase. Western blot analysis also showed that the cosedimented Src was kept in an inactive state (Figure 7A), which is consistent with the findings presented in Figure 5. Because only the Na^+/K^+-ATPase-bound Src can be pelleted down, we reasoned that the recovered Src would be reduced in ouabain-treated samples if ouabain dissociates Src from the Na^+/K^+-ATPase. To our surprise, when the same analysis was conducted after the samples were treated with ouabain before centrifugation, we found that ouabain had no effect on total Src cosedimented with the Na^+/K^+-ATPase, yet increased the amount of Src pY418 (Figure 7B). Because we have shown that multiple domains are involved in Src interaction with the Na^+/K^+-ATPase, the above findings led us to test if ouabain dissociates only a single (kinase) domain from the interacting Na^+/K^+-ATPase. To do so, 1 μg of the purified Na^+/K^+-ATPase was incubated with GST-Src, GST-SH3SH2, or GST-kinase in detergent-free PBS solution, and the complexes were collected by centrifugation. Afterward, the complexes were exposed to 10 μM ouabain. As depicted in Figure 7C, ouabain showed no effect on the binding of either full-length Src or the SH3SH2 domain to the Na^+/K^+-ATPase, but dissociated the kinase domain from the Na^+/K^+-ATPase, which is in accordance with the findings presented in Figure 5. The fact that ouabain had no effect on the binding of the SH3SH2 domain to the Na^+/K^+-ATPase apparently explains why ouabain did not change the overall binding of Src to the enzyme. To further test if releasing of the kinase domain is sufficient to activate Src, we preincubated the GST-kinase fusion protein with the Na^+/K^+-ATPase before adding full-length Src to compete for the kinase domain binding sites. Western blot analysis showed that GST-kinase, but not GST or GST-SH3SH2, significantly increased Src pY418 (Figure 7D). Taken together, these findings provide strong support for the notion that ouabain activates the Na^+/K^+-ATPase/Src complex by freeing the trapped kinase domain of Src.

Ouabain Activates the Na^+/K^+-ATPase/Src Complex and Stimulate Protein Tyrosine Phosphorylation in Live Cells

If ouabain activates the Na^+/K^+-ATPase/Src complex by releasing the kinase domain in live cells, we would expect ouabain to increase the distance between the kinase domain and the interacting Na^+/K^+-ATPase because the freed kinase domain will bind and phosphorylate its effectors. This could result in the reduction of FRET signal between coexpressed Src-ECFP and EYFP-rat α1. To test this, we performed live cell FRET as well as BRET analysis. As shown in Figure 8A, excitation of ECFP at 456 nm caused emissions in both ECFP spectrum (detected between 465 and 509 nm as F_{ECFP}) and EYFP spectrum (detected between 530 and 570 nm as F_{EYFP}) in control cells, indicating a potential FRET between Src-ECFP and EYFP-rat α1. To test if ouabain stimulates the release of the kinase domain, the same cell was then exposed to ouabain and measured for both ECFP and EYFP intensity. As shown in Figure 8A, once the cells were exposed to 100 μM ouabain, there was a time-dependent decrease in F_{EYFP} and a concomitant increase in F_{ECFP}, indicating that ouabain caused a reduction in FRET between Src-ECFP and EYFP-rat α1. As a control, the same experiments were repeated in cells transfected with ECFP and EYFP, and no detectable FRET was observed (unpublished data).

Because ECFP has to be excited in order to perform FRET analysis, photobleaching and spectral bleedthrough do occur during the experiments, complicating data analysis, especially in live cells. In addition, because ouabain-insensitive rat α1 was used for FRET analysis, we wanted to test if ouabain-sensitive α1 also functions similarly to the rat α1. Therefore, we performed the BRET analysis using GFP-canine α1 and Src-Renilla luciferase (Src-Rluc) to corroborate the above findings. Both constructs were transiently transfected into 293T cells and a construct of GFP-fused Rluc was used as a positive control. Human 293T cells were chosen for BRET analysis because these cells could be more easily transiently transfected under our experimental conditions. As shown in Figure 8B, coexpression of GFP-canine α1 and Src-Rluc yielded a BRET ratio comparable to that of the positive control, indicating that Src interacts with the $Na^+/$

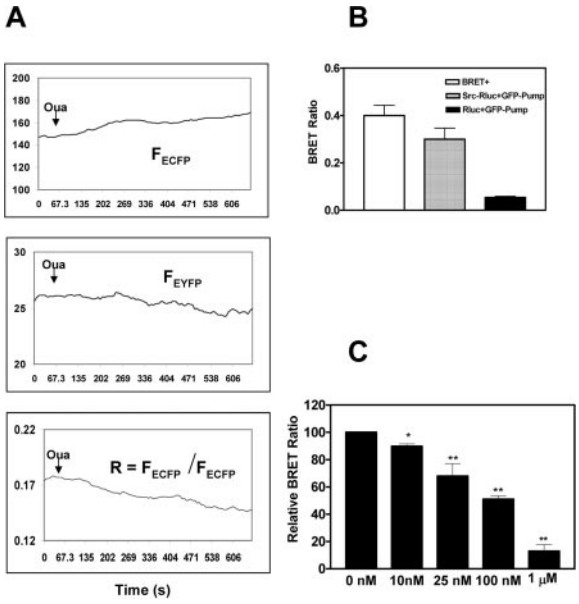

Figure 8. Ouabain dissociates Src kinase domain from the Na^+/K^+-ATPase in live cells. (A) A representative trace of ouabain-induced changes in FRET signal in an LLC-PK1 cell. (B) 293T cells were cotransfected with Src-Rluc and GFP-α1. 293T cells transfected with Rluc-GFP fusion protein were used as a positive control, and cells that cotransfected with Rluc and GFP- Na^+/K^+-ATPase were used as a negative control. (C) Ouabain treatment reduced BRET signal between GFP- Na^+/K^+-ATPase and Src-Rluc in a dose-dependent manner. Values are mean ± SE of at least four experiments. * $p < 0.05$; ** $p < 0.01$.

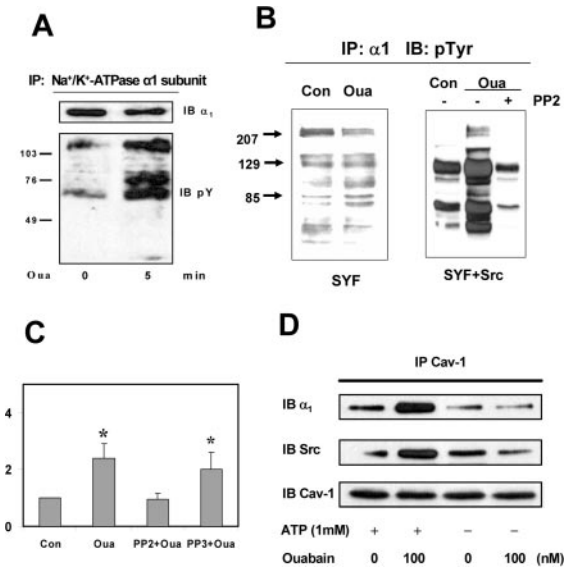

Figure 9. Ouabain-activated Na^+/K^+-ATPase/Src phosphorylates and recruits downstream effectors. (A) LLC-PK1 cells were treated with 1 μM ouabain for 5 min, and cell lysates were immunoprecipitated with anti-α1 antibody and analyzed for tyrosine phosphorylated proteins. (B) Both SYF and SYF + Src cells were treated with 100 μM ouabain for 5 min and analyzed as in A. Representative Western blots of three experiments are shown in both A and B. (C) Inhibition of Src blocks ouabain-induced recruitment of Src to the Na^+/K^+-ATPase signaling complex. LLC-PK1 cells were pretreated with 1 μM PP2 or PP3 for 15 min and then exposed to 1 μM ouabain for 5 min. Cell lysates were immunoprecipitated and analyzed. Values are mean ± SE of at least four independent experiments. (D) Caveolae were isolated and treated with 100 nM ouabain for 5 min in the presence or absence of 2 mM ATP as we previously described (Wang *et al.*, 2004). Afterward, caveolae were lysed in RIPA buffer, and the lysates were cleared by centrifugation and immunoprecipitated with anti-caveolin-1 antibody. Immunoprecipitates were probed for the α1, Src, and caveolin-1 by Western blot. A representative Western blot of three independent experiments is shown.

K^+-ATPase in live cells. Significantly, when the transfected cells were exposed to different concentrations of ouabain, ouabain caused a dose-dependent decrease in the BRET ratio. Significant decrease was detected when 10 nM ouabain was used (Figure 8C). These data are consistent with the known ouabain sensitivity of the canine α1 and support the results of FRET analysis in Figure 8A.

We and others have demonstrated that increases in protein tyrosine phosphorylation are essential for ouabain-induced changes in cellular functions (Haas *et al.*, 2002; Yuan *et al.*, 2005). Although we have shown that activation of Src by ouabain leads to transactivation of the Na^+/K^+-ATPase-associated EGF receptor and PLC-γ (Haas *et al.*, 2002; Yuan *et al.*, 2005), we have not tested whether the activation of the identified Na^+/K^+-ATPase/Src complex is responsible for ouabain-induced tyrosine phosphorylation of other proteins that are associated with the signaling complex. To test this possibility, LLC-PK1 cells were exposed to 1 μM ouabain for 5 min. Cell lysates from both control and treated cells were then immunoprecipitated with anti-α1 antibody. When the immunoprecipitates were resolved on SDS-PAGE and probed for phosphotyrosine with anti-phosphotyrosine antibody, we observed that ouabain indeed stimulated tyrosine phosphorylation of multiple Na^+/K^+-ATPase-associated proteins (Figure 9A). To confirm that Src is required for the initiation of protein tyrosine phosphorylation in response to ouabain, we repeated the same experiments in Src family kinase-knockout SYF cells. As shown in Figure 9B, the effects of ouabain on protein tyrosine phosphorylation were completely abolished in SYF cells. On the other hand, when Src is knocked back into the SYF cells (SYF + Src), ouabain's effects on protein tyrosine phosphorylation were restored, indicating an essential role of Src in initiation of ouabain-activated protein tyrosine phosphorylation. This notion is further supported by the fact that Src inhibitor PP2 was able to block ouabain-induced protein tyrosine phosphorylation in SYF + Src cells.

Because we have shown that ouabain stimulated the recruitment of Src to the Na^+/K^+-ATPase signaling complex (Haas *et al.*, 2002). The above data led us to propose that ouabain first activates the Na^+/K^+-ATPase-bound Src and subsequently results in tyrosine phosphorylation of EGFR, caveolin-1, and other effectors (Haas *et al.*, 2002; Yuan *et al.*, 2005). These effectors in turn provide binding sites for recruiting additional Src and other signaling proteins onto the signaling complex. To test this hypothesis, we treated the LLC-PK1 cells with 1 μM ouabain for 5 min in the presence or absence of 1 μM PP2. Cell lysates were then immunoprecipitated by anti-α1 antibody. Western blot analysis of the immunoprecipitants showed that ouabain increased coprecipitated Src in control cells, but not in cells that were pretreated with PP2 (Figure 9C), supporting the notion that the initial activation of Src is necessary for recruiting additional Src to the complex. Control experiments also showed that pretreatment of LLC-PK1 cells with PP3, an inactive analog of Src inhibitor PP2, failed to block ouabain-induced recruitment of Src to the Na^+/K^+-ATPase (Figure 9C). To corroborate the above findings, we also performed the im-

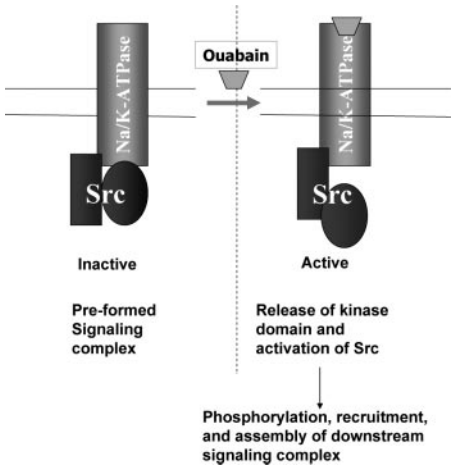

Figure 10. Schematic presentation shows how ouabain regulates the Na^+/K^+-ATPase/Src receptor complex.

munoprecipitation experiment with isolated caveolae preparations from LLC-PK1 cells. We showed previously that ouabain increased tyrosine phosphorylation of proteins in a Src-dependent manner in the isolated caveolae preparations. It also stimulated the formation of the Na^+/K^+-ATPase/caveolin-1/Src complex (Wang *et al.,* 2004). However, because addition of ATP is required for ouabain to activate Src in the isolated caveolae, we expected that ouabain could not stimulate the recruitment of Src to the caveolin-1 complex in the absence of ATP if Src activation and tyrosine phosphorylation of caveolin-1 is required for the additional recruitment of Src. Indeed, this is what we observed when we repeated the above experiments in the absence of ATP (Figure 9D). Taken together, the data clearly show that ouabain signals through the Na^+/K^+-ATPase by first activating Src and then recruiting more effector proteins including Src to the signaling Na^+/K^+-ATPase.

DISCUSSION

In this report, we have mapped the domains that are involved in Na^+/K^+-ATPase/Src interaction. We further demonstrate that the Na^+/K^+-ATPase and Src can assemble into a functional signaling complex via the identified protein domains and that the binding of ouabain to the Na^+/K^+-ATPase activates Src and provokes downstream protein tyrosine phosphorylation. This and other conclusions are summarized in Figure 10 and discussed below.

The Na^+/K^+-ATPase/Src Complex as a Receptor for Cardiotonic Steroids

Because the α1 subunit of Na^+/K^+-ATPase contains a conserved proline-rich motif in its N-terminus (Yudowski *et al.,* 2000), we initially thought that ouabain might promote the interaction between the SH3 of Src and the Na^+/K^+-ATPase, resulting in the activation of Src. To our surprise, GST pulldown assay showed that the SH3 domain was not involved in direct interaction with the Na^+/K^+-ATPase. Instead, the SH2 and the kinase domains of Src interact with the CD2 and CD3 domains of the Na^+/K^+-ATPase α1 subunit, respectively. In addition, our results showed that both Na^+/K^+-ATPase and GST-CD3 inhibited Src activity (Figures 5). Although we cannot exclude the possibility that other Src regulator copurified with Na^+/K^+-ATPase is involved, the fact that purified CD3 domain alone could mimic the effect of Na^+/K^+-ATPase strongly suggested that Na^+/K^+-ATPase is sufficient to inactivate Src. This function of the Na^+/K^+-ATPase is reminiscent of the inactivation of Src by Wiskott-Aldrich syndrome protein (Schulte and Sefton, 2003).

The fact that the Na^+/K^+-ATPase and Src form an inactive Src complex led us to speculate that this receptor complex may transmit the ouabain signals in a way similar to those of cytokine receptors (Ihle, 1994; Wan *et al.,* 2001). Although these receptors have no intrinsic kinase activity, coupling to Src allows them to activate the downstream protein tyrosine phosphorylation. Several lines of evidence support this notion. First, ouabain-induced changes in the conformation of the Na^+/K^+-ATPase are sufficient to free the kinase domain of Src (Figure 7). Interestingly, a recent study on SERCA shows that thapsigargin, an inhibitor of SERCA, is able to bring the CD3 close to the membrane (Toyoshima and Nomura, 2002). If ouabain can exert similar effect on the CD3, this may explain how ouabain releases the kinase domain from the Na^+/K^+-ATPase. On the other hand, because ouabain has no effect on the binding of the SH3SH2 domain to the CD2, this domain could function as a hinge, keeping the activated Src binding to the signaling Na^+/K^+-ATPase for specific and robust signal transmission. Second, antagonizing the binding of Src kinase domain to the Na^+/K^+-ATPase by addition of GST-kinase domain fusion protein acted as ouabain and stimulated Src pY418. Third, the observed effect of ouabain on Src (Figure 6) is not due to the inhibition of the ATPase activity because vanadate showed no effect on Src at the concentration that completely inhibited the ATPase activity. Furthermore, the GST-CD3, which does not hydrolyze ATP, can also inhibit Src activation. Similarly, the findings also argue against the involvement of changes in ion concentrations in ouabain-induced activation of Src because these experiments were performed in the test tubes under the same ionic conditions. Finally, both FRET and BRET analyses indicated that ouabain did release the kinase domain in live cells (Figure 8). It is important to note that the effects of ouabain on the Na^+/K^+-ATPase/Src-kinase domain interaction were dose-dependent and correlated well with the known dose-response curve of ouabain binding to the Na^+/K^+-ATPase (Haas *et al.,* 2002).

In short, we have demonstrated a novel mechanism of ouabain-provoked signal transduction. However, several issues remain to be resolved. Because Src family kinases are highly conserved, we speculate that the signaling Na^+/K^+-ATPase may interact with other members of Src family. In addition, mammalian cells express at least four different types of α subunits in a tissue-specific manner, it is quite possible that different isoforms may also interact with Src in a tissue-specific manner. To this end, it is of interest to note that Src also interacts with the CD3 domain of H^+/K^+-ATPase (Figure 4C), suggesting a potential signaling function of the H^+/K^+-ATPase in regulation of Src activity. It is of interest to note that these P-ATPases may also serve as Src effectors because recent studies have suggested a Src-mediated tyrosine phosphorylation of these P-ATPases. (Kanagawa *et al.,* 2000; Masaki *et al.,* 2000; Ferrandi *et al.,* 2004). Finally, because the Na^+/K^+-ATPase contains both α and β subunits, it remains to be determined whether the β subunit contributes to the Src interaction with the enzyme.

Implications

Na^+/K^+-ATPase is well known for its essential function in maintaining the Na^+ and K^+ ion concentrations across cell

membrane in mammalian cells. The fact that the binding site for cardiotonic steroids is so conserved throughout the phylogeny of eukaryotes indicates that these steroids must play an important role in regulation of the Na^+/K^+-ATPase function. Because the ion pumping was the only known function of the Na^+/K^+-ATPase until a few years ago, it is well accepted by the field that cardiotonic steroids must signal by inhibition of the ATPase activity although there is no hormonal precedent for such signal transduction. This mode of action has led many in the field to question the significance of endogenous cardiotonic steroids because they circulate at subnanomolar concentrations under normal physiological conditions, and can only bind to 1–2% of cell surface Na^+/K^+-ATPase. Because most mammalian cells contain ~1 million Na^+/K^+-ATPase molecules per cell (Baker and Willis, 1969, 1970; McCall, 1979), it is highly inefficient for cardiotonic steroids to purely function as an inhibitor to the pumping function of Na^+/K^+-ATPase because they have to work against the large pumping capacity of the cells. On the other hand, if the binding site is conserved for regulating the signaling function of the Na^+/K^+-ATPase, cardiotonic steroids will function as true agonists. As estimated by our colocalization analysis, ~25% of the Na^+/K^+-ATPase has the potential to interact with Src. Activation of 1–2% of these receptors by ouabain will produce a few thousand active molecules per cell. Based on the findings of EGF signaling in HeLa cells (Berkers *et al.*, 1991) and the principle of signal amplification, this will be sufficient to generate strong signals via kinase cascades, especially if the signaling event occurs in a membrane microdomain such as caveolae (Wang *et al.*, 2004). Consistent with this, recent studies have demonstrated in both cultured cells and animal models that physiological concentrations of ouabain (e.g., 0.1–1 nM) were able to activate Src and ERKs (Aydemir-Koksoy *et al.*, 2001; Ferrandi *et al.*, 2004).

Pharmacologically, we have demonstrated that ouabain-induced inotropy is accompanied by the activation of Src and ERKs in the isolated heart preparations as well as in the cultured myocytes (Mohammadi *et al.*, 2003). Furthermore, inhibition of Src and ERKs blocked ouabain-induced increases in intracellular Ca^{2+} in cardiac myocytes (Tian *et al.*, 2001). Thus, the new findings further revealed the possible molecular mechanism of digitalis-induced inotropy in the heart. They also point to a possibility of developing chemicals or peptides that can stimulate the signaling function of the Na^+/K^+-ATPase without affecting the ion pumping function. Finally, this is the first report (to our knowledge) to provide the insight into the molecular mechanism, by which a membrane transporter uses Src to form a functional signaling complex. Because many membrane transporters and ion channels undergo either substrate- or ligand-dependent conformational changes as the Na^+/K^+-ATPase, these findings brought about an important biological question as to whether other membrane transporters are also involved in signal transduction, thus constituting another group of important receptors and signal transducers. To this end, it is of interest to note the following. First, the CD3 of Na^+/K^+-ATPase is highly conserved among many different P-type ATPases. Thus, it is quite possible that other P-type ATPases are (e.g., H^+/K^+-ATPase shown in Figure 4C) also involved in regulation of Src. Second, several recent reports have demonstrated that Src interacts with and regulates many other membrane ion channels (Yu *et al.*, 1997; Sobko *et al.*, 1998; Tiran *et al.*, 2003). It will be worth testing if these ion channels regulate Src activity as the signaling Na^+/K^+-ATPase.

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