Identification of a Potential Receptor That Couples Ion Transport to Protein Kinase Activity*

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In our previous studies, we have demonstrated that the Src-coupled α1 Na/K-ATPase works as a receptor for cardiotonic steroids, such as ouabain, to regulate cellular protein kinase cascades. Here, we explore further the structural determinants of the interaction between the α1 Na/K-ATPase and Src and demonstrate that the Src-coupled α1 Na/K-ATPase allows the cell to decode the transmembrane transport activity of the Na/K-ATPase to turn on/off protein kinases. The α1 Na/K-ATPase undergoes E1/E2 conformational transition during an ion pumping cycle. The amount of E1 and E2 Na/K-ATPase is regulated by extracellular K+ and intracellular Na+. Using purified enzyme preparations we find that the E1 Na/K-ATPase can bind both the Src SH2 and kinase domains simultaneously and keep Src in an inactive state. Conversely, the E1 to E2 transition releases the kinase domain and activates the associated Src. Moreover, we demonstrate that changes in E1/E2 Na/K-ATPase by either Na+ or K+ are capable of regulating Src and Src effectors in live cells. Together, the data suggest that the Src-coupled α1 Na/K-ATPase may act as a Na+/K+ receptor, allowing salt to regulate cellular function through Src and Src effectors.

The Na/K-ATPase is ubiquitously expressed in the plasma membrane of almost all eukaryotic cells. It belongs to the type II class of P-type ATPases, hydrolyzing ATP to transport 3 Na+ out and 2 K+ into the cell against their electrochemical gradients. By establishing gradient differences of Na+ and K+ across the plasma membrane, the Na/K-ATPase maintains a steady-state cellular milieu, provides the driving force for Na+-coupled cotransporters on the apical membrane of the epithelium, and reestablishes the electrical potential in excitable tissues. Based on early studies (1), the α1 Na/K-ATPase adopts two major distinct conformations, namely E1 and E2, and the equilibrium between E1 and E2 is determined by the binding of substrates (e.g. Na+). These distinct conformations have been confirmed by x-ray crystallography studies of the α1 Na/K-ATPase and the sarco/endoplasmic reticulum Ca2+-ATPase, another type II class of P-type ATPase (2, 3). Specifically, the crystal structures reveal that the α subunit contains three functionally distinct cytosolic domains. The A3 (actuator) domain consists of the N terminus and the second cytosolic domain (CD2) connected to transmembrane helices M2 and M3. The enzyme also has a highly conserved phosphorylation (P) domain that is close to the membrane and a relatively isolated nucleotide binding (N) domain. It appears that concerted domain movements occur during the E1/E2 transition.

Previous studies show that the α1 Na/K-ATPase contains multiple structural motifs that interact with soluble, membrane and structural proteins including phospholipase C-γ, phosphoinositide 3-kinase, inositol trisphosphate receptor, arrestin, and ankyrin (4–9) and serves as a scaffolding protein to facilitate signal transduction. Moreover, a large fraction of the α1 Na/K-ATPase interacts with Src in cultured epithelial cells as well as in vivo (10, 11). Src kinase is important in several phosphorylation-related signaling pathways (12). It has three important structural domains: Src homology 3 (SH3) domain binds to polyproline motifs; Src homology 2 (SH2) domain can associate with phosphorylated tyrosine residues; and the kinase domain interacts with downstream signaling molecules (13). Functionally, this Src-coupled α1 Na/K-ATPase works as a receptor for cardiotonic steroids such as ouabain. Binding of ouabain stimulates the associated Src, which in turn acts as a signal transducer, converting and amplifying the binding signal to the activation of multiple protein and lipid kinase cascades including the ERK pathway. Because ouabain binding is known to accumulate E2-like Na/K-ATPase, we postulate that ouabain-induced Src activation is dependent on conformational changes of the α1 Na/K-ATPase. Thus, it is likely that other ligands (i.e. Na+ and K+) could use the same receptor to regulate cellular kinase activities because they are known to affect the formation of E1/E2 Na/K-ATPase. Various assessments of α1 Na/K-ATPase conformation and its influence on Src activity presented here provide evidence for a link between ion homeostasis and signal transduction.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal anti-Src antibody (B12), polyclonal anti-ERK1/2 antibody, monoclonal anti-pERK1/2 antibody, goat anti-rabbit and goat anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.).

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CA). The monoclonal anti-His antibody was from GE Healthcare. The polyclonal anti-pY418Src was purchased from Invitrogen. Alexa Fluor 488-conjugated anti-rabbit antibody was from Molecular Probes. Glutathione beads and the Pro-Bond Purification System were obtained from Amersham Biosciences and Invitrogen, respectively. Recombinant human Src was purchased from Upstate. The CMV promoter-driven pEYFP-C1 vector was from Clontech. The pGEX-4T-1 and pTrc-His A vectors were from GE Healthcare and Invitrogen, respectively. Escherichia coli BL21 was obtained from Invitrogen. AG1478 was obtained from Cayman Chemical (Ann Arbor, MI). Other chemicals of the highest purity were all obtained from Sigma–Aldrich.

Cell Culture and Treatment—LLC-PK1 cells were purchased from American Type Culture Collection (Manassas, VA). P-11, PY-17 and AAC-19 cells were generated from LLC-PK1 cells (10). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS as described (11). After cells reached 95–100% confluence, they were serum-starved overnight and then treated. The modified K+ or Na+ medium was prepared as DMEM except that the amount of NaCl, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.4). Cell lysates were centrifuged at 16,000 × g for 30 min to remove remnant glutathione.

Chemical Modifier Treatment of the α1 Na/K-ATPase—To examine the effect of the α1 Na/K-ATPase conformation on Src activity, the enzyme was stabilized in distinct conformation with fluoride compounds (E2), N-ethylmaleimide (NEM)/AMPPNP (E1) and oligomycin (E1). Treatment of the α1 Na/K-ATPase with fluoride compounds was performed according to Danko et al. (18). Purified Na/K-ATPase (18 μg) was exposed to aluminum fluoride in the presence of 2 mM EGTA, 100 mM KCl, 50 mM MES/Tris (pH 6), 0.1 mM MgCl₂, 3 mM KF, and 50 μM AlCl₃ for 1.5 h at 25 °C. The same amount of enzyme was exposed to beryllium fluoride in the presence of 2 mM EGTA, 50 mM LiCl, 50 mM MOPS/Tris (pH 7), 5 mM MgCl₂, 0.5 mM KF, and 20 μM BeSO₄ for 4 h at 25 °C. After treatment, the Na/K-ATPase was centrifuged at 100,000 × g for 45 min to remove unbound chemicals, and the preparations were further subjected to Src autophosphorylation assay (see below) to detect their effect on Src. The α1 Na/K-ATPase was treated with NEM/AMPPNP according to Hegyvary (19). In brief, 8 μg of purified Na/K-ATPase was incubated in the reaction buffer containing 40 mM TES (pH 7.0), 100 mM KCl, 5 mM MgCl₂, 20 mM NaCl, 5 mM NEM, and 1 mM AMPPNP. The reaction continued for 10 min at 30 °C and was stopped by 0.10 volume of 1 mM β-mercaptoethanol. Treating the α1 Na/K-ATPase by oligomycin was according to Hegyvary (19). The indicated amount of oligomycin was incubated with 1 μg of Na/K-ATPase in 50 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl (choline chloride was added to maintain the ionic strength at 155 mM). The preparations were subjected to Src autophosphorylation assay as mentioned below.

Trypsin/Chymotrypsin Cleavage of the α1 Na/K-ATPase—Cleavages of the Na/K-ATPase were obtained by the addition of trypsin and chymotrypsin with established procedures according to Zolotarjova et al. (20) and Huang et al. (21), respectively. Purified Na/K-ATPase (0.5 mg/ml) was exposed to 0.7 μg/ml trypsin in a solution containing 12 mM KCl, 15 mM Tris-HCl (pH 7.4). Similarly, purified enzyme (0.5 mg/ml) was digested by 25 μg/ml chymotrypsin in a solution containing 10 mM NaCl and 15 mM Tris-HCl (pH 7.4) until >75% enzyme activities were lost. The reaction was stopped by the addition of trypsin/chymotrypsin inhibitor (at a dose of 0.7 mg/μg trypsin or 10 μg/μg chymotrypsin; Sigma).

Assays of Src Autophosphorylation and Na/K-ATPase Activity—Purified Src/K-ATPase after treatment was incubated with 1 unit of purified Src for 30 min at 37 °C in a solution containing 50 mM Tris-HCl (pH 7.4), different ion compositions as indicated in the figure legends (with addition of choline chloride to maintain the ionic strength), and followed by ouabain exposure (10 μM, 5 min) as indicated. The reaction was started by the addition of 2 mM Mg²⁺/ATP. The reaction continued for 10 min and was stopped by adding SDS.
sample buffer. The Src activity was determined by phosphorylation of Src Tyr-418 using immunoblot analysis. Ouabain-sensitive Na/K-ATPase activity was measured as described previously (16). Briefly, purified pig kidney Na/K-ATPase (specific activities between 1,000 and 1,400 μmol of P/mg per h) was incubated in the buffer containing 20 mM Tris (pH 7.4), 1 mM EGTA, 3 mM MgCl₂, 12.5 mM KCl, 100 mM NaCl, and 2 mM ATP. Phosphate generated during the ATP hydrolysis was measured by BIOMOL GREEN reagent (Enzo Life Science). Ouabain-sensitive Na/K-ATPase activities were calculated as the difference between the presence and absence of 1 mM ouabain.

Visualization of Active Src Using Confocal Fluorescence Microscope—Cells were seeded on coverslips. After treatment, the cells were fixed with prechilled (−20 °C) methanol for 15 min. The fixed cells were blocked with Image-iT FX signal enhancer (Invitrogen) and incubated with anti-pY418 Src antibody overnight at 4 °C, followed by incubation with Alexa Fluor 488-conjugated anti-rabbit antibody. The stained cells on coverslips were washed, mounted, and then visualized using a Leica DMIRE2 microscope (Wetzlar, Germany).

RESULTS AND DISCUSSION

Our previous studies have identified the involvement of at least two pairs of protein domains in constituting the Src-coupled α1 Na/K-ATPase receptor complex: the Src SH2 binds to CD2; the Src kinase domain associates with the N domain. Upon ouabain binding, the former interacting pair remains unchanged, whereas the latter interaction is alleviated, leading to Src activation (11). Moreover, we identified a 20-amino acid peptide (NaKtide) from the N domain as the potential binding site and generated cell-permeable NaKtide (pNaKtide) as a specific antagonist for the Src-coupled α1 Na/K-ATPase receptor (22). To explore the roles of these two interacting pairs in the formation of α1 Na/K-ATPase–Src receptor, we performed a GST pulldown assay. As depicted in Fig. 1A, the binding of CD2 to Src occurred at a lower concentration than that of the N domain, suggesting that the interaction between the A and Src SH2 domain exhibits higher affinity than that of the N domain/Src kinase domain.

During the pumping cycle, the α1 Na/K-ATPase undergoes E1 to E2 conformational transition (23) where the N domain closes up and the A domain rotates to dock onto the N and P domains. Structure modeling suggests that the location and the space between the A and N domains in the E2 state are unlikely to allow the α1 subunit to bind both the SH2 and kinase domains simultaneously (Fig. 1B). Because the Src binding, even at the molar ratio of 1:1, had no effect on the Na/K-ATPase activity (Fig. 1C), one of the Src domains must be dissociated from the α1 Na/K-ATPase to allow the required E1 to E2 transition during ATP hydrolysis. Thus, we propose that the α1 Na/K-ATPase regulates the associated Src in a conformation-dependent manner. Specifically, whereas the E1 Na/K-ATPase inhibits Src, the E2 Na/K-ATPase releases the kinase domain, resulting in the activation of α1 Na/K-ATPase-associated Src (Fig. 1B). We further speculate that a coordinated movement of A and N domains may be sufficient and necessary to push the kinase domain off the N domain during the E1 to E2 transition, resulting in the activation of the associated Src.

To assess the above hypotheses, we first utilized different chemical modifiers to stabilize the α1 Na/K-ATPase at distinct conformational states and then assessed the conforma-
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FIGURE 2. Regulation of α1 Na/K-ATPase-associated Src by chemical modifiers. A, to stabilize α1 Na/K-ATPase in the E2P conformation, the purified Na/K-ATPase was treated by beryllium fluoride or aluminum fluoride. The ouabain-treated and nontreated α1 Na/K-ATPase–Src complex was used as a positive and negative control for the experiments, respectively. B and C, similarly, the α1 Na/K-ATPase was stabilized at the E1P state by NEM/AMPPNP or oligomycin. The enzyme preparations were then incubated with purified Src in 50 mM Tris-HCl (pH 7.4) buffer for Src autophosphorylation assay. Src Tyr-418 phosphorylation was probed using Western blotting, and activated Src was represented as a ratio of Tyr-418-phosphorylated Src over total Src. A representative Western blot is shown under each experimental condition, and quantitative data are presented as mean ± S.E. (error bars) of at least three independent experiments. *, p < 0.05; **, p < 0.01 (Student’s t test).

Figure 2A shows the regulation of α1 Na/K-ATPase-associated Src by chemical modifiers. In the presence of Na+, chymotrypsin cleaves E1 Na/K-ATPase at Leu-266 in the A domain, producing an 83-kDa fragment (Fig. 4A). Whereas the 83-kDa peptide retains the ability to form a ouabain-sensitive phosphoenzyme intermediate (EP), the chymotrypsin cleavage peptide disrupts the coordinated movement of A and N domains, resulting in a complete inhibition of ATPase activity (26–28). Thus, if the coordinated movement of the A and N domain was required for the conformation-dependent activation of the α1 Na/K-ATPase-associated Src as predicted in Fig. 1B, we would expect that the inability of the A domain to push off the Src kinase domain from the N domain would cause a complete inhibition of Src. Indeed, as depicted in Fig. 4B, whereas Na+ (lane 1) and ouabain (lane 2) were able to stimulate the α1 Na/K-ATPase-associated Src, they failed to do so after >75% α1 Na/K-ATPase was digested by chymotrypsin in 80 min. As expected, less digestion of α1 Na/K-ATPase by chymotrypsin (20 min) resulted in a partial inhibition of ouabain-induced Src activation (Fig. 4C). To confirm further the importance of coordinated movement of the A and N domains, the E2 Na/K-ATPase was digested by trypsin in the presence of K+ and subjected to the same assay. Unlike chymotrypsin, trypsin cleaves E2 Na/K-ATPase at Arg-438 in the N domain, producing a 48-kDa fragment that retains the capability of forming EP (28, 29). However, disruption of the coordinated movement of the A and N domains by trypsin was equally effective in inhibiting Na+ and ouabain-induced activation of Src as chymotrypsin digestion (Fig. 4B). It is important to mention that the protease-digested α1 Na/K-ATPase was washed and then incubated with Src in the presence of trypsin/chymotrypsin inhibitor. Under these conditions, no detectable digestion of Src by proteases was observed (Fig. 4D).

The above in vitro studies indicate that the α1 Na/K-ATPase can turn Src on/off through coordinated movements of the A and N domains during E2/E1 conformational transi-
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Because the balance of E1/E2 Na/K-ATPase in live cells is regulated by extracellular K⁺ and intracellular Na⁺, we first measured Src activity after cells were exposed to different concentrations of extracellular K⁺. Lowering extracellular K⁺ increases intracellular Na⁺, and together they accumulate E2 Na/K-ATPase in live cells by accelerating its production and preventing its dephosphorylation (30, 31). As depicted in Fig. 5A, confocal imaging analyses showed that lowering K⁺ from 5 to 1 mM significantly increased active Src in both peripheral and intracellular components in LLC-PK1 cells. To verify these findings, we also conducted Western blot analyses of cell lysates after the cells were exposed to low K⁺ medium. As shown in Fig. 5B, lowering K⁺ from 5 to 1 mM, like addition of ouabain (10), not only stimulated cellular Src activity, but also activated the downstream kinase cascade of ERKs. Moreover, when the pNaKtide, a specific Src-coupled α1 Na/K-ATPase receptor antagonist developed in our laboratory (22), was added to the cultured cells, it abolished the activation of ERKs (Fig. 5C). The epidermal growth factor receptor inhibitor AG1478 was also able to block the ERK activation, indicating that low K⁺, like ouabain, activates ERK cascade via the pathways of Src-mediated transactivation of EGFR through the α1 Na/K-ATPase (32).

To verify further the role of Src-coupled α1 Na/K-ATPase receptor in low K⁺-induced activation of protein kinases, we repeated the above experiments in α1 Na/K-ATPase-knockdown cells. PY-17 cells were derived from LLC-PK1 cells expressing α1-specific siRNA. Compared with P-11 control cells (transfected with empty vector), PY-17 cells express approximately 10% of α1 and have a reduced number of the Src-coupled α1 Na/K-ATPase receptors (10). As shown in Fig. 5D, lowering extracellular K⁺ from 5 to 1 mM failed to increase cellular Src activity in PY-17 cells, indicating that formation of the α1 Na/K-ATPase–Src complex is required for low K⁺ to stimulate cellular Src activity. This notion is further supported by the fact that rescuing PY-17 cells by knock-in a rat α1 (the AAC-19 cells) was sufficient to restore low K⁺-induced Src activation (Fig. 5D).

We also exposed the cells to low extracellular Na⁺ to confirm ion-induced regulation of the Src-coupled α1 Na/K-ATPase receptor. It is known that lowering the extracellular Na⁺ would favor the formation of E1 Na/K-ATPase in live cells. Indeed, we observed that decreasing Na⁺ from 150 to 15 mM caused further inhibition of cellular Src activity as depicted in Fig. 5E.

To complement the above studies in cultured cells, we incubated the purified Na/K-ATPase–Src complex in the presence of different ions that are known to alter the formation of E1/E2 Na/K-ATPase and assessed their effects on Src activity. Changes of Na⁺ or K⁺ concentration in reaction buffer had no effect on Src autophosphorylation (data not shown). However, these changes had significant effects on the α1 Na/K-ATPase-associated Src. For example, we know that the cellular Na/K-ATPase is exposed to about 15 mM intracellular Na⁺ and 5 mM extracellular K⁺. Under these ionic conditions, we saw that most Src was inactive. Lowering K⁺ from 5 to 1 mM produced a robust stimulation of Src (Fig. 6).
In short, the above findings suggest that the formation of α1 Na/K-ATPase–Src complex allows Na⁺ and K⁺ to regulate Src and its effectors through the E1/E2 conformational transition of the α1 Na/K-ATPase. Several unique properties of this novel cellular signaling mechanism are worthy of note. First, our studies reveal a unique model of receptor formation. A differential binding affinity of the two pairs of the interactions that connect Src to the A and N domains of the α1 Na/K-ATPase appears to be responsible for a conformation-dependent activation and inactivation of the receptor. To this end, it would be of interest to examine whether similar interactions occur between the α1 Na/K-ATPase and other proteins such as PI3K and inositol trisphosphate receptor. Moreover, membrane transporters all share the same capability of ligand-induced conformational change. Thus, the new findings would suggest the possibility of other transporters being important signal transducers in a way similar to that of the α1 Na/K-ATPase (4, 8, 33–37).

Second, the α1 Na/K-ATPase–Src complex is not simply another receptor being responsive to a class of hormone (i.e., CTS) as we described previously (38, 39). It is actually capable of...
of sensing ouabain, intracellular (e.g. Na\(^+\)) and extracellular (e.g. K\(^+\)) cues. Functionally, because the activation/inhibition of protein kinases is essential for regulating the activity and trafficking of many membrane transporters (40), the Src-coupled α1 Na/K-ATPase receptor could be responsible for the coordination of the transporter activities (pump-leak coupling) across the cell membrane. The importance of pump-leak coupling was recognized even before the identification of the Na/K-ATPase (41). Hoffman and Tosteson explained this concept using the red blood cell as a model (42). It is now well documented in epithelial cells as well as in other types of cells (40). For example, expression of α1 Na/K-ATPase and apical Na/H exchanger 3 is coordinated in renal epithelial cells through a Src-dependent pathway (43). Changes in the expression or activity of Na/K-ATPase are closely associated with parallel changes in the expression and activity of apical and basolateral K\(^+\) channels (44, 45). Moreover, K\(^+\) deficiency stimulates renal Src activity in vivo, resulting in a decrease in surface expression of renal outer medulla K channels (45, 46).

Third, this new receptor mechanism would explain the complexity and the physiological interaction among endogenous CTS, extracellular K\(^+\), and intracellular Na\(^+\). Specifically, we suggest that any deviation from the steady state of E1/E2 balance could affect the receptor function, which can be triggered by changes in any of these ligands and enhanced or antagonized by the other ligands. For example, low extracellular K\(^+\) would work in concert with the increase in intracellular Na\(^+\), favoring the formation of E2 Na/K-ATPase, which in turn could enhance ouabain binding, further stabilizing the E2 conformation and consequently maximizing the activation of Src. Similarly, this type of feedforward regulation could be initiated by the binding of CTS to the Src-coupled α1 Na/K-ATPase.

Finally, several uncertainties are recognized. For example, our data are insufficient for us to describe kinetic properties of this regulation. The apparent turnover rate of Src is ranged from 26 to 744/min (47), much slower than that of the α1 Na/K-ATPase, which is 8,000–10,000 cycles/min for purified enzyme and 1,500–5,000 cycles/minute in cultured cells or in isolated renal tubules. Thus, we speculate that only if the α1 Na/K-ATPase stays long enough in an E2-like conformation, it is then possible to stimulate Src. Apparently, this happens when extracellular K\(^+\) is lowered (Fig. 5A). Needless to say, it also remains to be determined whether this newly described signaling mechanism occurs under physiological conditions in vivo in response to dietary salt loading or depletion.

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