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Functional Characterization of Src-interacting Na/K-ATPase Using RNA Interference Assay*

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We have shown that the Na/K-ATPase and Src form a signaling receptor complex. Here we determined how alterations in the amount and properties of the Na/K-ATPase affect basal Src activity and ouabain-induced signal transduction. Several α1 subunit knockdown cell lines were generated by transfecting LLC-PK1 cells with a vector expressing α1-specific small interference RNA. Although the α1 knockdown resulted in significant decreases in Na/K-ATPase activity, it increased the basal Src activity and tyrosine phosphorylation of focal adhesion kinase, a Src effector. Concomitantly it also abolished ouabain-induced activation of Src and ERK1/2. When the knockdown cells were rescued by a rat α1, both Na/K-ATPase activity and the basal Src activity were restored. In addition, ouabain was able to stimulate Src and ERK1/2 in the rescued cells at a much higher concentration, consistent with the established differences in ouabain sensitivity between pig and rat α1. Finally both fluorescence resonance energy transfer analysis and co-immunoprecipitation assay indicated that the pumping-null rat α1 (D371E) mutant could also bind Src. Expression of this mutant restored the basal Src activity and focal adhesion kinase tyrosine phosphorylation. Taken together, the new findings suggest that LLC-PK1 cells contain a pool of Src-interacting Na/K-ATPase that not only regulates Src activity but also serves as a receptor for ouabain to activate protein kinases.

Na/K-ATPase was discovered by Skou (1) as the molecular machinery of the cellular sodium pump. It belongs to a family of evolutionarily ancient ATPases that couple the hydrolysis of ATP to membrane ion translocation (2, 3). A major difference between the Na/K-ATPase and other ATPases is its ability to bind cardiotonic steroids such as ouabain. Studies from many laboratories have now established that the binding of ouabain to this enzyme not only inhibits the ATPase activity but also stimulates protein tyrosine kinases such as Src (4, 5). The activated Src in turn transactivates epidermal growth factor receptor, resulting in the assembly and activation of multiple signaling cascades such as the ERK1/2[2] and phospholipase C-γ/protein kinase C pathways (5, 6).

Because several laboratories have demonstrated that the activation of Src is essential for ouabain-induced changes in many cellular activities including the regulation of intracellular calcium, gene expression, and cell growth (6–9), we have recently examined whether the Na/K-ATPase interacts directly with Src to form a functional signaling receptor (10). Using *in vitro* glutathione *S*-transferase pulldown assays we have identified that the second and the third intracellular domains of the Na/K-ATPase α1 subunit interact with the Src SH2 and the kinase domains, respectively. Functionally these interactions keep Src in an inactive state, and binding of ouabain to this inactive Na/K-ATPase·Src complex frees and then activates the associated Src (10). These new findings suggest that the cellular Src-interacting Na/K-ATPase may play an important role in regulation of the basal Src activity and serve as a functional receptor for ouabain to stimulate protein tyrosine phosphorylation in live cells. To test this hypothesis we have developed an siRNA-based assay that allows us to determine the effect of changes in the amount and properties of the Na/K-ATPase on both basal and ouabain-stimulated Src activity.

EXPERIMENTAL PROCEDURES

Materials—Chemicals of the highest purity were purchased from Sigma. The GeneSuppressor vector was purchased from BioCarta (San Diego, CA). Cell culture media, fetal bovine serum, trypsin, Lipofectamine 2000, and restriction enzymes were purchased from Invitrogen. EYFP expression vector (pEYFP) and ECFP expression vector (pECFP) were obtained from Clontech. QuikChange mutagenesis kit was purchased from Stratagene (La Jolla, CA). Optitran nitrocellulose membrane was from Schleicher & Schuell. Enhanced chemiluminescence SuperSignal kit was purchased from Pierce. Image-iT FX signal enhancer, Antifade kit, Alexa Fluor 488-conjugated anti-mouse/rabbit IgG and Alexa Fluor 546-conjugated anti-mouse/rabbit IgG antibodies were obtained from Molecular Probes (Eugene, OR). Anti-Src (clone GD11) monoclonal antibody, anti-Na/K-ATPase α1 polyclonal and monoclonal (clone C464.6) antibodies, anti-phosphotyrosine (clone 4G10) antibody, and protein G-agarose were from Upstate Biotechnology Inc. (Lake Placid, NY). The polyclonal anti-Tyr(P)^{418}-Src and

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[2] The abbreviations used are: ERK, extracellular signal-regulated kinase; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; FRET, fluorescence resonance energy transfer; siRNA, small interference RNA; FAK, focal adhesion kinase; SH2, Src homology 2; pERK, phosphorylated ERK; PBS, phosphate-buffered saline.

anti-Tyr(P)^{529}-Src antibodies were from BIOSOURCE (Camarillo, CA). The polyclonal anti-FAK and anti-Tyr(P)^{925}-FAK antibodies were from Cell Signaling (Danvers, MA). The monoclonal anti-α1 antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Anti-c-Src (B-12) monoclonal antibody, anti-c-Src (SRC2) polyclonal antibody, anti-ERK (C-16) polyclonal antibody, anti-pERK (E-4) monoclonal antibody, and all the secondary horseradish peroxidase-conjugated antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal rat α1-specific antibody (anti-NASE) was provided by Dr. Thomas Pressley (Texas Tech University, Lubbock, TX).

Cell Culture—LLC-PK1 cells and human embryonic kidney 293T cells were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2-humidified incubator as described previously (10, 11).

Construction of the siRNA Expression Vectors—siRNAs were constructed using the GeneSuppressor construction kit as described previously (11). Briefly four pairs of oligonucleotides (A1–A4) were synthesized using the human α1 cDNA (GenBank^{TM} accession number NM_000701) as template (see Table 1 for details), and the inserts were prepared by annealing two complementary oligonucleotides. The annealed inserts were then cloned into pSuppressor^{TM}-U6 vector digested with SalI and XbaI. Positive clones were confirmed by nucleotide sequencing.

Site-directed Mutagenesis—Rat α1 expression vector pRc/CMV-α1AAC was provided by Dr. Pressley (12). To make the expression of rat α1 insensitive to A4 siRNA, the α1 siRNA-targeted sequence was silently mutated from ^{2530}ggtcgtctgatcttt (GenBank^{TM} accession number NM_012504) to ^{2530}ggcaggctaatattc using the QuikChange mutagenesis kit. The SspI (aat/att) restriction site was generated to facilitate the clone screening. The positive mutant (pRc/CMV-α1AACm1 or AAC in short) was verified by DNA sequencing and then used in this study. The pumping-null mutant (D371E) was generated by mutating the ^{1126}gacaag to ^{1126}gagaag using pRc/CMV-α1AACm1 as the template (13).

Generation of Stable α1 Subunit Knockdown and Knock-in Cell Lines—Human embryonic kidney 293T cells were transiently transfected with different siRNA expression vectors along with pEYFP using Lipofectamine 2000 as we described previously (11). After 48 h, cells were first examined for the expression of EYFP for assessing the transfection efficiency and then collected for analysis of endogenous α1 content by Western blot. To generate stable cell lines, one batch of LLC-PK1 cells was transfected with the A4 siRNA expression vector (pSuppressor-A4 siRNA) (Table 1) and a puromycin selection marker (pBade-puro). Another batch of cells was co-transfected with pEYFP together with the pSuppressor-A4 siRNA and pBade-puro so that the co-expressed EYFP could be used as a marker to pick clones. Empty vector (pSuppressor) or A1 siRNA-transfected cells were co-selected and used as a control. The cells were selected with puromycin (1 μg/ml) 24 h post-transfection. Puromycin-resistant colonies were cloned and expanded. To rescue the Na/K-ATPase knockdown cells, cells were transfected with the pRc/CMV-α1AACm1. Selection was initiated with 3 μM ouabain because untransfected cells were very sensitive to ouabain. After about 1 week, ouabain-resistant colonies were isolated and expanded into stable cell lines in the absence of ouabain. G418 was not used because these cells are resistant to it, requiring more than 3 mg/ml to kill the untransfected cells. The knockdown cells were also sensitive to blasticidin (15 μg/ml), and we have recently used this agent for other selections.

Immunoprecipitation and Immunoblot Analysis—Cells were washed with PBS, solubilized in modified ice-cold radioimmune precipitation assay buffer, and subjected to immunoprecipitation or Western blot analysis as described previously (11). Protein signal was detected using the enhanced chemiluminescence kit and quantified using a Bio-Rad GS-670 imaging densitometer.

Na/K-ATPase Activity Assay—Na/K-ATPase enzymatic activity was determined as we described previously (14). Briefly cells were collected from the cultures in Tris-EGTA buffer (pH 7.2) and briefly sonicated. The cell lysates were then treated with alamethicin at a concentration of 0.1 mg/mg of protein for 30 min at room temperature. ATPase activity was measured by the determination of the initial release of ^{32}P from [γ-^{32}P]ATP, and the reaction was carried out in a reaction mixture (1 ml) containing 100 mM NaCl, 25 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 5 mM NaN_3, and 50 mM Tris-HCl (pH 7.4). Na/K-ATPase activity was calculated as the difference between the activities measured in the absence of ouabain and in the presence of 1 mM ouabain. To determine the ouabain concentration curve, the alamethicin-treated cell lysates were preincubated with different concentrations of ouabain for 15 min before ATP was added to start the reaction.

Confocal Fluorescence Microscopy—Cells cultured on coverslips were washed twice with PBS and fixed for 15 min with methanol prechilled at −20 °C. The fixed cells were then rinsed with PBS three times and blocked with 200 μl of Image-iT FX signal enhancer for 30 min at room temperature. The cells were washed again and incubated with the primary antibodies in PBS containing 1% bovine serum albumin for 1 h at room temperature. After three washes with PBS, the cells were incubated with corresponding Alexa Fluor-conjugated secondary antibodies. Image visualization was performed using a Leica DMIRE2 confocal microscope (Leica, Mannheim, Germany). Leica confocal software was used for data analysis.

FRET Analysis by Acceptor Photobleaching—ECFP was fused to the C terminus of Src, and EYFP was fused to the N terminus of rat Na/K-ATPase α1 subunit or its mutant. FRET analysis was performed in cells co-transfected with Src-ECFP and EYFP-rat α1 expression vectors using the acceptor photobleaching protocol as we described previously (10). Briefly after 24 h culture, cells on a glass coverslip were fixed with methanol prechilled at −20 °C for 15 min and washed twice with PBS solution. The EYFP-rat α1 was photobleached by applying a high intensity 515 nM laser, and the emission of ECFP excited by 456 nM laser was recorded before (D_{pre}) and after (D_{post}) EYFP photobleaching. The FRET efficiency was then calculated by the ratio of (D_{post} - D_{pre})/D_{pre}. Cells transfected with either Src-ECFP and EYFP or EYFP-α1 and ECFP expression vectors

TABLE 1

Targets and oligo sequences of human Na/K ATPase-α1 subunit-specific siRNAs

The target sequences are marked by bold letters.

siRNA	Target sequence	Oligo inserts
A1	^{467}**agatcatggaatccttcaa**	Sense, 5′-tcgag**agatcatggaatccttcaa**ttcaagaga**ttgaaggattccatgatct**ttttt-3′; anti-sense, 5′-ctagaaaaa**agatcatggaatccttcaa**tctcttgaa**ttgaaggattccatgatct**c-3′
A2	^{1449}**ctccaccaacaagtaccag**	Sense, 5′-tcgag**ctccaccaacaagtaccag**ttcaagaga**ctggtacttgttggtggag**ttttt-3′; anti-sense, 5′ctagaaaaa**ctccaccaacaagtaccag**tctcttgaa**ctggtacttgttggtggag**c-3′
A3	^{1836}**ggtcatcatggtcacagga**	Sense, 5′-tcgag**ggtcatcatggtcacagga**ttcaagaga**tcctgtgaccatgatgacc**ttttt-3′; anti-sense, 5′-ctagaaaaa**ggtcatcatggtcacagga**tctcttgaa**tcctgtgaccatgatgacc**c-3′
A4	^{2293}**ggtcgtctgatctttgata**	Sense, 5′-tcgag**ggtcgtctgatctttgata**ttcaagaga**tatcaaagatcagacgacc**ttttt-3′; anti-sense, 5′-ctagaaaaa**ggtcgtctgatctttgata**tctcttgaa**tatcaaagatcagacgacc**c-3′

TABLE 2

Relative α1 subunit protein content and the composition of DNA constructs used in different cell lines

Cell lines	Relative α1 content (mean ± S.E.)	DNA constructs used in transfection
	%	
P-11	100	pSuppressor, pBade-puro
A1	97.4 ± 2.1	pSuppressor-A1 siRNA, pBade-puro
A4-11	44.1 ± 2.3	pSuppressor-A4 siRNA, pBade-puro
TCN23-19	12.0 ± 4	pSuppressor-A4 siRNA, pBade-puro
PY-17	7.5 ± 3.0	pSuppressor-A4 siRNA, pBade-puro, pEYFP
PY-17-AAC-M1-19 (AAC-19)	93.7 ± 9.9	pSuppressor-A4 siRNA, pBade-puro, pEYFP, pRc/ CMV-α1AACm1 (rat α1)

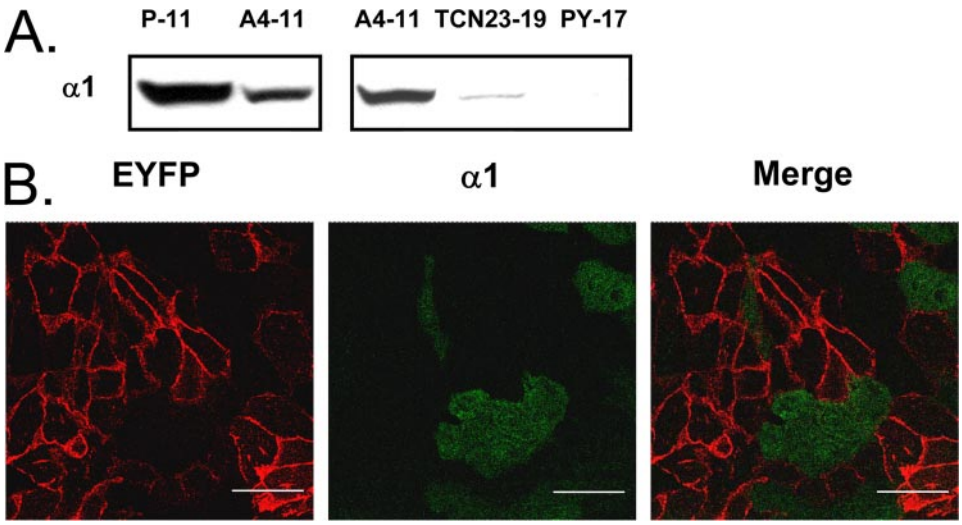


FIGURE 1. **Silencing of the endogenous Na/K-ATPase by siRNA.** *A*, total cell lysates (30 μg/lane) from different cell lines were separated by SDS-PAGE and analyzed by Western blot for the expression of the α1 subunit of the Na/K-ATPase. A representative Western blot is shown (see quantitative data in Table 2). *B*, P-11 and PY-17 cells were mixed, co-cultured for 24 h, and then immunostained with anti-α1 antibody (clone C464.6) as described under "Experimental Procedures." The *scale bar* represents 50 μM.

were used as a control, and no detectable FRET was observed in these control cells.

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

RESULTS

Manipulation of the Cellular Na/K-ATPase Content by siRNA-based Assays—As shown in Table 1, a total of four pairs of the α1-specific siRNAs was selected based on the strategy we used previously (11). Transient transfection assay in human embryonic kidney 293T cells showed that expression of A4 siRNA resulted in over 40% decreases in the expression of the human α1 subunit, whereas others gave 0 (A1 siRNA) to 20% (A2 and A3 siRNAs) reduction. Because the transfection efficiency was about 50% as indicated by the co-expressed EYFP, we reasoned that A4 siRNA is effective in silencing the expression of endogenous Na/K-ATPase. Therefore, LLC-PK1 cells were transfected with A4 siRNA expression vector (pSuppressor-A4 siRNA) and a puromycin selection marker (pBade-puro) either with or without pEYFP as described under "Experimental Procedures." After two rounds of selection, we collected 20 stable transfectants. Western blot analysis using a monoclonal (α6F) antibody showed that the expression of the α1 subunit in these clones was significantly reduced in comparison with the control P-11 cells that were transfected with empty vector (pSuppressor) and selected. In contrast, cell clones (*e.g.* A1) obtained from the LLC-PK1 cells that were transfected with A1 siRNA expressed α1 at a level comparable to that in P-11 cells (see Table 2). Therefore, we expanded and further characterized three A4 siRNA-expressing clones. As shown in Fig. 1*A*, expression of the α1 subunit was significantly reduced in A4-11, TCN23-19, and PY-17 cells. Of these cell lines, the PY-17 cells, which were cloned by using the co-expressed EYFP as a marker, expressed the lowest level of the Na/K-ATPase. Table 2 shows the quantitative data on the relative amount of the α1 in these and other cell lines we generated. Because control Western blot using purified Na/K-ATPase prepared from pig kidney showed that it was only possible to perform reasonable quantitative assay comparing two samples with less than 6-fold differences in the amount of α1 (data not shown), we measured the relative amount of α1 in these cells by comparing A4-11 with the control P-11 and then TCN23-19 and PY-17 with A4-11. To confirm the above Western blot data, we also probed the blots with a different anti-Na/K-ATPase α1 monoclonal antibody (clone C464.6) and an anti-Na/K-ATPase α1 polyclonal anti-

body, showing essentially the same results as in Fig. 1*A*. In addition, when co-cultured P-11 and PY-17 cells were immunostained using anti-Na/K-ATPase α1 antibody (clone C464.6), we found that the *green* PY-17 cells exhibited no detectable expression of the α1, whereas the plasma membrane of control P-11 cells was clearly labeled by the antibody (Fig. 1*B*). To be sure that knockdown of the α1 subunit does not induce the expression of other isoforms, we analyzed the cell lysates for both α2 and α3 and found no detectable signals in the above cell lines. Finally when ouabain-sensitive ATPase activity was measured in the cell lysates, a significant (80%) reduction was noted in the PY-17 cells in comparison with the control P-11 cells (Table 3).

Because PY-17 cells have very low endogenous Na/K-ATPase, these cells will be very useful for studying the structure-function properties of the Na/K-ATPase if we can rescue the cells by knocking in an exogenous α1. To test this possibility, we first made silent mutations of the rat α1 cDNA to change the siRNA-targeted sequence. We then transfected PY-17 cells with the mutated rat α1 expression vector (pRc/CMV-α1AACm1) and generated several stable transfectants. Further analysis of the clone AAC-19 showed that these cells, unlike both P-11 and PY-17, expressed rat α1 (Fig. 2*A*). When the same blots were analyzed for total α1 using the monoclonal anti-α1 antibody (α6F), we found that AAC-19 cells expressed an amount of α1 comparable to that in control P-11 cells (Fig. 2*A*). This result was further confirmed by immunostaining of the co-cultured P-11 and AAC-19 cells using anti-α1 (clone C464.6) antibody. As depicted in Fig. 2*B*, the *green* AAC-19 and control P-11 cells exhibited similar levels of the Na/K-ATPase in the plasma membrane. Control experiments also demonstrated that the rat α1 was stably expressed in this cell line for at least 20 passages in the absence of ouabain. Functionally knock-in of the rat α1 into PY-17 cells was able to restore Na/K-ATPase activity (Table 3). Most importantly, it shifted the dose-response curve of ouabain on the ATPase activity and made the rescued cells less ouabain-sensitive. In fact, the rescued cells behave as rat cell lines (5) that express only the α1 isoform (Fig. 3). It is important to note that PY-17 cells were as sensitive to ouabain as the control P-11 cells and that 10 μM ouabain caused a complete inhibition of the Na/K-ATPase.

TABLE 3
Na/K-ATPase activity in different cell lines

Cell line	Activity
	%
P-11	100
PY-17	20.8 ± 3.7
AAC-19	92.2 ± 6.4

Regulation of Basal Src Activity by the Na/K-ATPase—Our *in vitro* studies have shown that the Na/K-ATPase directly binds and keeps Src in an inactive state. If this mode of regulation operates in live cells, we would expect that reduction of intracellular Na/K-ATPase will decrease the interaction, resulting in an increase in basal Src activity. To test this, we measured the phosphorylation of Src (Tyr(P)^{418}-Src), indicative of Src activation (4, 10), in the cell lysates from the above cell lines. As depicted in Fig. 4*A*, the expression of total Src was not altered by knockdown of the endogenous Na/K-ATPase. However, the levels of active Src were significantly increased in A4-11, TCN23-19, and PY-17 cells. Interestingly the increase in Src activity appeared to be inversely correlated with the amounts of Na/K-ATPase expressed in these cells (Fig. 4*B*). These findings were further confirmed by immunostaining the cells with anti-Tyr(P)^{418}-Src antibody, showing that TCN23-19 cells contained much more active Src than P-11 cells contained (Fig. 4*C*). It is important to note that there was no difference in the amount of active Src between two control cell lines, P-11 and A1 cells (data not shown).

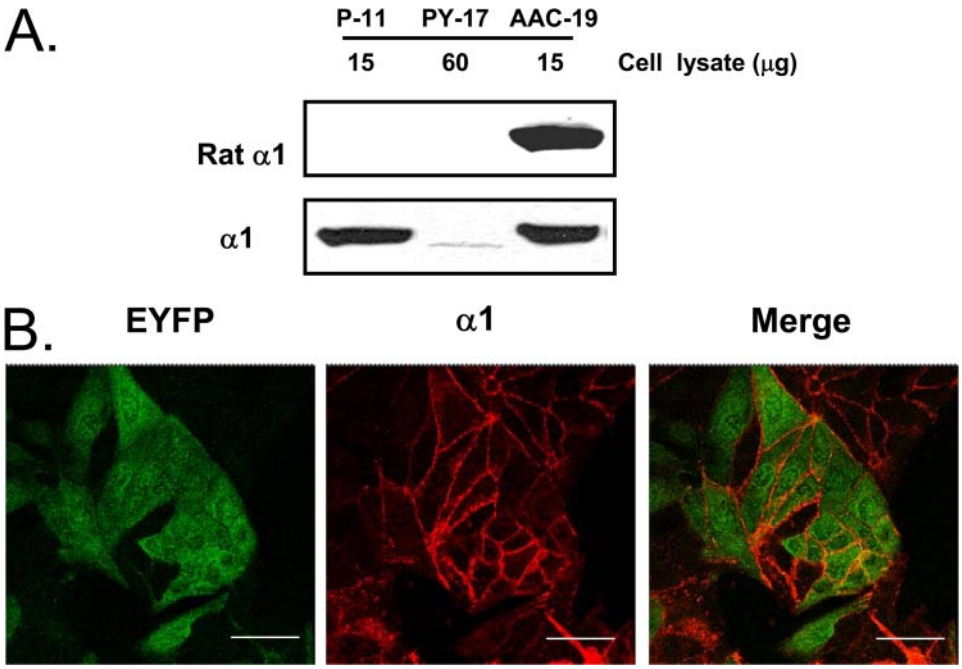


FIGURE 2. **Expression of the Na/K-ATPase in AAC-19 cells.** *A*, clone AAC-19 was generated by transfecting PY-17 cells with a rat α1-expressing vector as described under "Experimental Procedures." Cell lysates (15 μg from P-11 and AAC-19 and 60 μg from PY-17) were separated by SDS-PAGE and analyzed by Western blot. The blot was first probed with antibody α6F that recognizes both pig and rat α1 subunits, then striped, and reprobed with the anti-NASE that specifically reacts with rat α1. *B*, P-11 and AAC-19 cells were mixed, co-cultured for 24 h, and immunostained with anti-α1 antibody (clone C464.6) as described under "Experimental Procedures." The *scale bar* represents 50 μM.

To test whether the increase in Src activity due to the decreased expression of the Na/K-ATPase is reversible upon repletion of the Na/K-ATPase, we determined the total Src and the active Src in AAC-19 cells. As depicted in Fig. 2, AAC-19 cells were derived from the rat α1-transfected PY-17 cells and expressed an amount of the Na/K-ATPase comparable to that in control P-11 cells. Although knock-in of the rat α1 did not change the total Src in AAC-19 cells, it did reduce the level of the active Src to that seen in control P-11 cells (Fig. 5, *A* and *B*). As illustrated in Table 3, the

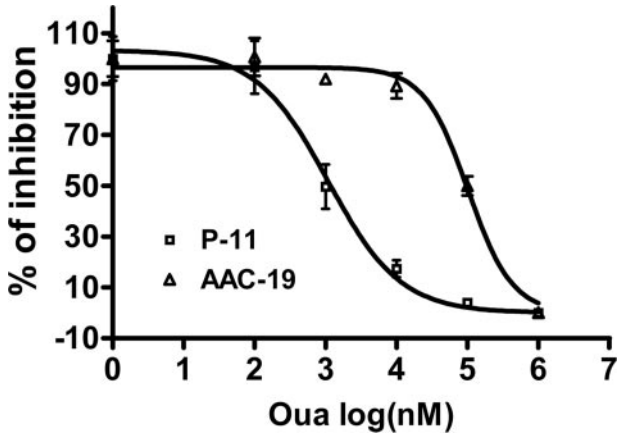


FIGURE 3. **Concentration-dependent effects of ouabain (*oua*) on the Na/K-ATPase activity.** Whole cell lysates from P-11 and AAC-19 cells were prepared and assayed for the Na/K-ATPase activity as described under "Experimental Procedures." Data are shown as percentage of control, and each point is presented as mean ± S.E. of four independent experiments. Curve fit analysis was performed by GraphPad software.

Na/K-ATPase activity was reduced 80% in PY-17 cells. When intracellular Na^+ was measured after the cells were incubated in $^{22}Na^+$ (0.5 μCi/ml) medium for 60 min to fully equilibrate exchangeable intracellular Na^+ with $^{22}Na^+$ (15), we found that the steady state intracellular Na^+ in PY-17 cells was about twice as much as in P-11 cells.[3] To be sure that changes in Src activity observed in AAC-19 cells are not due to the restoration of the functional Na/K-ATPase and subsequent decreases in intracellular Na^+, we tested whether knock-in of a pumping-null mutant of the rat α1 is sufficient for the observed interaction between the Na/K-ATPase and Src. PY-17 cells were transiently transfected with either silently mutated wild-type rat α1 (pRc/CMV-α1AACm1) or the rat α1 pumping-null mutant (D371E). As shown in Fig. 5*C*, expression of either rat α1 or the mutant reduced active Src in PY-17 cells. To further confirm these findings, we also transiently transfected TCN23-19 cells with the EYFP-fused rat α1 mutant expression vector (pEYFP-D371E) and immunostained for active Src. As depicted in Fig. 5*D*, the cells expressing the rat α1 mutant had much less active Src in comparison with the untransfected TCN23-19 cells. These data suggest that the pumping-null Na/K-ATPase mutant is still able to interact and regulate Src. To seek additional support for this notion, we also performed FRET analysis in TCN23-19 cells transiently transfected with EYFP-rat α1 mutant (D371E) and Src-ECFP expression vectors. As depicted in Fig. 6*A*, the pumping-null mutant was targeted to the plasma membrane. When FRET was measured in these transfected cells by acceptor photobleaching protocol, an energy transfer from Src-ECFP to EYFP-D371E was clearly demonstrated (Fig. 6*B*). The FRET efficiency measured from a total of 20 cells in three separate experiments ranged from 10.4 to 15.6 (13.2 ± 1.4). These data indicate that the pumping-null Na/K-ATPase acts like the wild-type α1 (10) and can interact with Src to form a signaling complex. This conclusion is further supported by the co-immunoprecipitation assay showing that the rat α1 mutant could be co-precipitated by anti-Src antibody (Fig. 6*C*).

[3] L. Liu and Z. Xie, unpublished data.

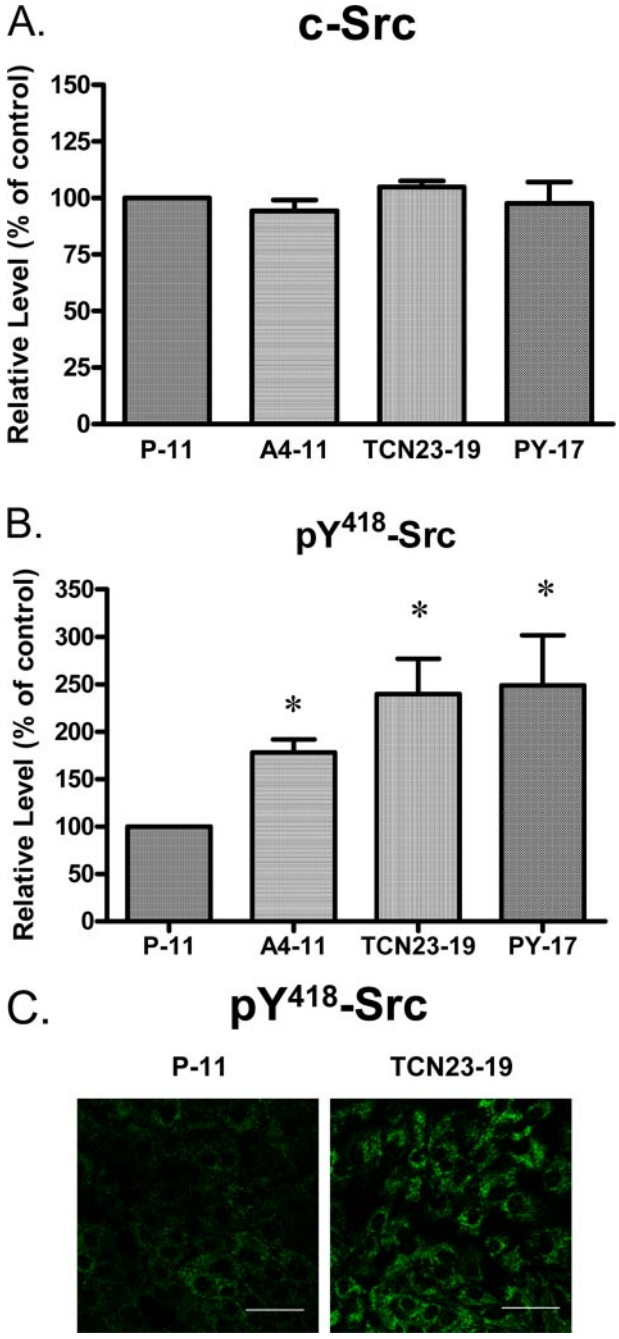


FIGURE 4. **Regulation of Src activity by Na/K-ATPase.** *A* and *B*, cell lysates (30 μg/lane) from different cell lines were separated by SDS-PAGE and analyzed by either anti-c-Src (B-12) or anti-Tyr(P)^{418}-Src antibody. The quantitative data are mean ± S.E. from four separate experiments. *, $p < 0.05$ *versus* P-11. *C*, cultured P-11 and TCN23-19 cells were serum-starved for 12 h and immunostained by anti-Tyr(P)^{418}-Src antibody. The images were collected as described under "Experimental Procedures." The *scale bar* represents 50 μM.

FAK is a known Src effector that plays an important role in regulation of cell migration and proliferation (16–18). Activation of Src stimulates phosphorylation of FAK Tyr^{925}, which subsequently can lead to the activation of ERK1/2 (19, 20). To examine whether an increase in basal Src activity can result in the activation of Src effectors, we measured tyrosine phosphorylation of FAK in α1 knockdown cells. As depicted in Fig. 7*A*, cell lysates were immunoprecipitated by an anti-phosphotyrosine antibody, and the immunoprecipitates

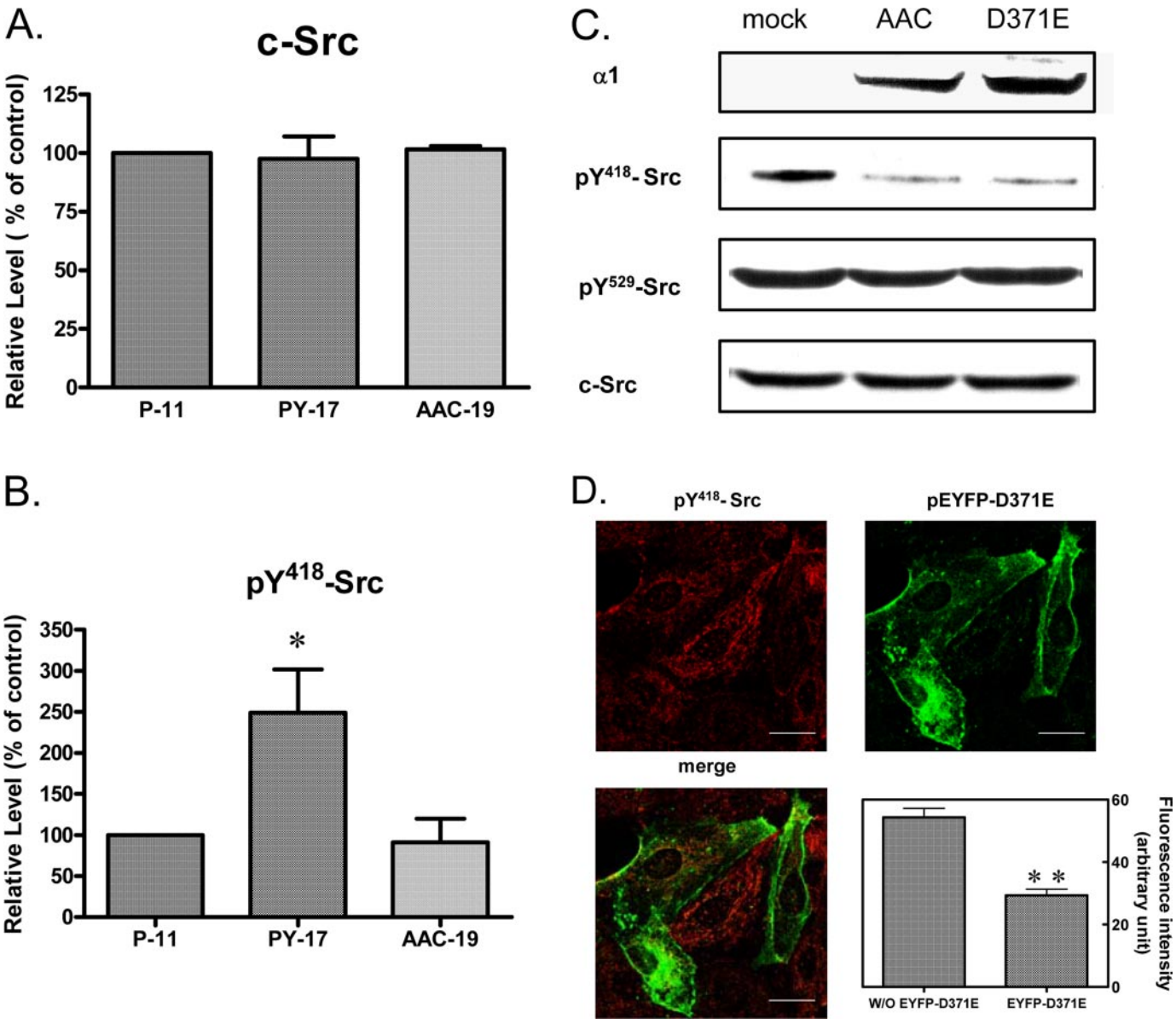
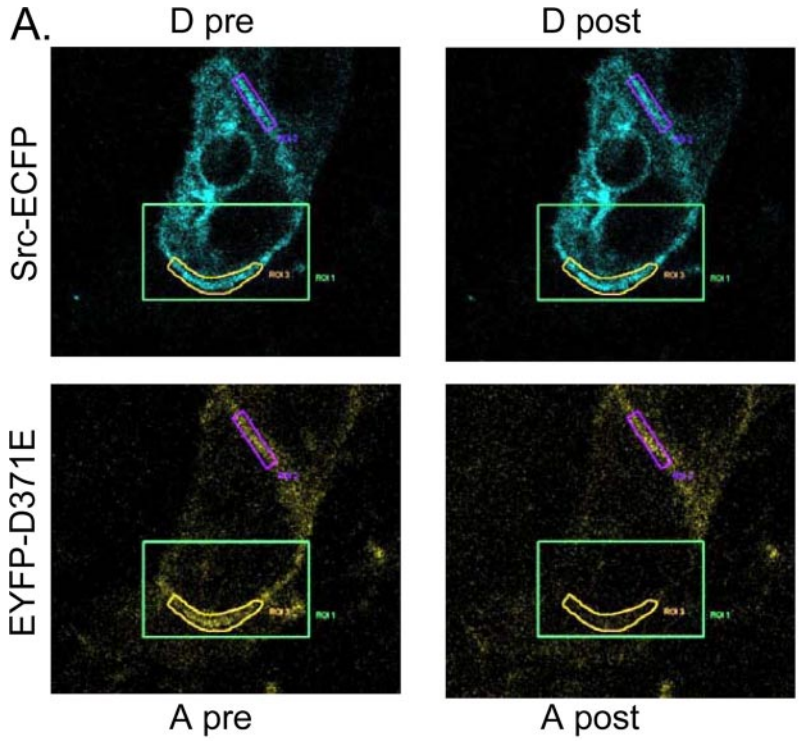


FIGURE 5. **Regulation of Src activity by the pumping-null Na/K-ATPase.** *A* and *B*, cell lysates (30 μg/lane) from different cell lines were separated by SDS-PAGE and analyzed by either anti-c-Src (B-12) or anti-Tyr(P)^{418}-Src antibody. The quantitative data are mean ± S.E. from four separate experiments. *, $p < 0.05$ *versus* P-11. *C*, PY-17 cells were transiently transfected with either an empty vector (*mock*), silently mutated wild-type rat α1 (*AAC*), or the D371E mutant. After 36 h, the transfected cells were lysed and analyzed by Western blot using specific antibodies as indicated. A representative Western blot is shown, and the same experiments were repeated four times. *D*, TCN23-19 cells were transiently transfected with a vector expressing EYFP-fused α1 D371E mutant (*pEYFP-D371E*). After 24 h, cells were serum-starved for 12 h and then immunostained by anti-Tyr(P)^{418}-Src antibody. Images from a representative experiment show that expression of mutant pEYFP-D371E reduced the intensity of *red* (Tyr(P)^{418}-Src) fluorescence (comparing the *green* and nearby *non-green* cells). The quantitative data of Tyr(P)^{418}-Src were collected from 40 different microscope vision fields in four independent experiments and expressed as mean ± S.E. **, $p < 0.01$. The *scale bar* represents 22 μM. *W/O*, without.

were probed by anti-FAK antibody. The data clearly showed that the α1 knockdown was capable of increasing the amounts of tyrosine-phosphorylated FAK. Specifically when cell lysates were probed for Tyr(P)^{925}-FAK, we found a significant increase in Tyr(P)^{925}-FAK in both A4-11 and PY-17 cells (Fig. 7*B*). Interestingly when total ERK1/2 and pERK1/2 were measured, we found a modest increase in the amount of active ERK1/2 in PY-17 cells (Fig. 7*C*). This is in accordance with the known function of Tyr(P)^{925}-FAK (19, 20). Significantly this increase in Tyr(P)^{925} was sensitive to Src inhibitor PP2 (Fig. 7*D*). It is important to note that the FAK phosphorylation correlated well to the levels of active Src in the PP2-treated knockdown cells. Taken together, these data indicate that the increased Src activity due to the α1 knockdown can stimulate tyrosine phosphorylation of Src effectors. This notion is further supported by the observation that expression of the pumping-null mutant (D371E) not only restored the basal Src activity but also reduced FAK Tyr^{925} phosphorylation in PY-17 cells (Fig. 7*E*).

Knockdown of the Na/K-ATPase Abolishes Ouabain-induced Activation of Src and ERK1/2—Because the Na/K-ATPase·Src complex serves as a functional receptor for ouabain to induce Src activation and subsequent stimulation of ERK1/2 (5, 10), the above findings prompted us to test whether knockdown of



B.

$$FRET_{Eff} = \frac{D_{post} - D_{pre}}{D_{post}} \quad \text{for all} \quad D_{post} > D_{pre}$$

ROI	ROI_2	ROI_3
D pre	50.54	41.16
D post	52.23	49.40
A pre	30.07	27.24
A post	33.79	10.08
FRETeff (%)	3.23	16.67

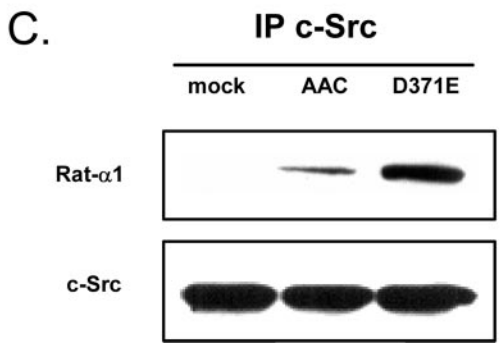


FIGURE 6. **Interaction between Src and the pumping-null Na/K-ATPase.** *A* and *B*, TCN23-19 cells were co-transfected with Src-ECFP and EYFP-rat α1 mutant (D371E) expression vectors. After 24 h, FRET analysis was performed as described under "Experimental Procedures." *Boxed* ROI_1 (*green*) was photobleached, and the ROI_3 (*yellow*) membrane area was analyzed for FRET. The *boxed* ROI_2 (*purple*) was selected and served as a non-bleaching control. The experiments were repeated three times, and a total of 20 cells were analyzed. *C*, TCN23-19 cells were transiently transfected as in *A* with either silently mutated wild-type rat α1 (*AAC*) or rat α1 pumping-null mutant (*D371E*) expression vectors. After 36 h, cell lysates were prepared and subjected to immunoprecipitation using monoclonal anti-Src (clone GD11) antibody. Immunoprecipitates were then analyzed by Western blot using either anti-NASE antibody (for rat α1) or anti-c-Src (SRC2) antibody. The same experiments were repeated three times, and a representative Western blot is shown. *IP*, immunoprecipitate.

the Na/K-ATPase affects ouabain-activated signal transduction. As shown in Fig. 8*A*, although ouabain activated Src in P-11 cells as we reported previously (4, 5), this effect of ouabain was essentially abolished in PY-17 cells, whereas a significant reduction was observed in A4-11 cells. To be sure that this inhibition is not due to nonspecific defects in receptor signal transduction, we also measured the effect of EGF on Src. We found that epidermal growth factor was able to stimulate Src-Tyr(P)^{418} in both P-11 and PY-17 cells (2.5 ± 0.3-fold increase in P-11 *versus* 1.7 ± 0.2-fold increase in PY-17, $n = 3$). Consistent with the findings on Src, we also failed to detect any ouabain-induced change in ERK1/2 phosphorylation in PY-17 cells (Fig. 8*B*). In contrast, epidermal growth factor was able to stimulate ERK1/2 in PY-17 cells (data not shown). These data support the notion that the Na/K-ATPase is indeed the receptor for ouabain-induced signal transduction. This notion is further supported by the findings presented in Fig. 8, *C* and *D*, showing that knock-in of the rat α1 not only restored the ouabain responses but also shifted the dose-response curve to the *right* in AAC-19 cells.

DISCUSSION

In this report we not only introduced an effective and α1-specific RNA interference assay but also provided a protocol for rescuing the Na/K-ATPase-depleted cells. These procedures have made it possible for us to demonstrate that the cellular Na/K-ATPase regulates Src and its effector FAK and that the Na/K-ATPase·Src complex serves as a sole receptor for ouabain to activate Src and subsequently ERK1/2 in live cells.

Manipulation of the Cellular Na/K-ATPase Content by RNA Interference Assays—RNA interference is a cellular mechanism that was first discovered in 1998 in *Caenorhabditis elegans* and refers to the post-transcriptional gene silencing by double-stranded RNA-triggered degradation of a homologous mRNA (21). This has now been developed as a powerful tool for artificially silencing a specific gene in a variety of biological systems including cultured cells and whole organisms. Using the strategy developed by Paul *et al.* (22) and transient transfection assay, we identified that A4 siRNA was effective for silencing the α1 expression. Thus, we transfected pig LLC-PK1 cells with the A4 siRNA expression vector and cloned several stable cell lines. Western blot analysis and immunostaining assay showed that the expression of the α1 in the cloned cell lines was significantly reduced (Figs. 1 and 2 and Table 2). For example, the α1 in PY-17 cells is only about 8% of that in control P-11 cells. Functional analysis revealed that depletion of the α1 resulted in an 80% reduction in ouabain-sensitive ATPase activity in PY-17 cells (Table 3). Clearly we have developed an effective protocol for silencing the expression of endogenous α1 in cultured cells.

To test whether the α1-depleted cells can be used to study the signaling functions of an exogenous/mutant α1, we transfected PY-17 cells with a rat α1 expression vector in which A4 siRNA-targeted sequence was silently mutated. By taking advantage of the availability of an antibody that specifically reacts with rat α1, we

demonstrated that the exogenous rat α1 could be knocked in and that the expression of rat α1 restored not only the total cellular Na/K-ATPase protein but also the Na/K-ATPase activity. Importantly the rat α1-rescued cells (AAC-19) exhibited the same ouabain sensitivity as the rat cell lines that only express the Na/K-ATPase α1 subunit (Fig. 3). Taken together, the data indicate that we have developed an effective protocol for manipulating cellular Na/K-ATPase. It is important to note that this protocol offers additional advantages over the widely used ouabain selection protocol for expression of mutated Na/K-ATPase in ouabain-sensitive cell lines (23–26). First, our protocol makes it possible to deplete endogenous Na/K-ATPase, allowing the investigators to study the effects of decreases in Na/K-ATPase expression on cellular function. Second, it does not require using ouabain to force the expression of the transfected Na/K-ATPase. This is important in view of recent studies showing that ouabain stimulates the signaling function of the Na/K-ATPase and induces the endocytosis of the enzyme (4, 9, 27, 28). Third, this protocol allows us to study the exogenous/mutant Na/K-ATPase in the cells that have very low (less than 10%) endogenous Na/K-ATPase. Fourth, the identified A4 siRNA should be effective in silencing the α1 expression in cells derived from species other than human and pig because the human α1 cDNA sequence (nucleotide 2293 to nucleotide 2312) targeted by A4 siRNA is conserved among all identified α1 subunits (but not other isoforms) from fish to human. Finally rescuing PY-17 cells with different isoforms of the Na/K-ATPase would make it possible for us to uncover the potential isoform-specific signaling functions.

A Pool of Src-interacting Na/K-ATPase—Recently we have shown that the Na/K-ATPase resides in caveolae with Src (11, 29). FRET analysis indicates that the signaling Na/K-ATPase and Src are likely to interact and form a functional receptor complex. *In vitro* binding assay demonstrates that the α1 subunit and

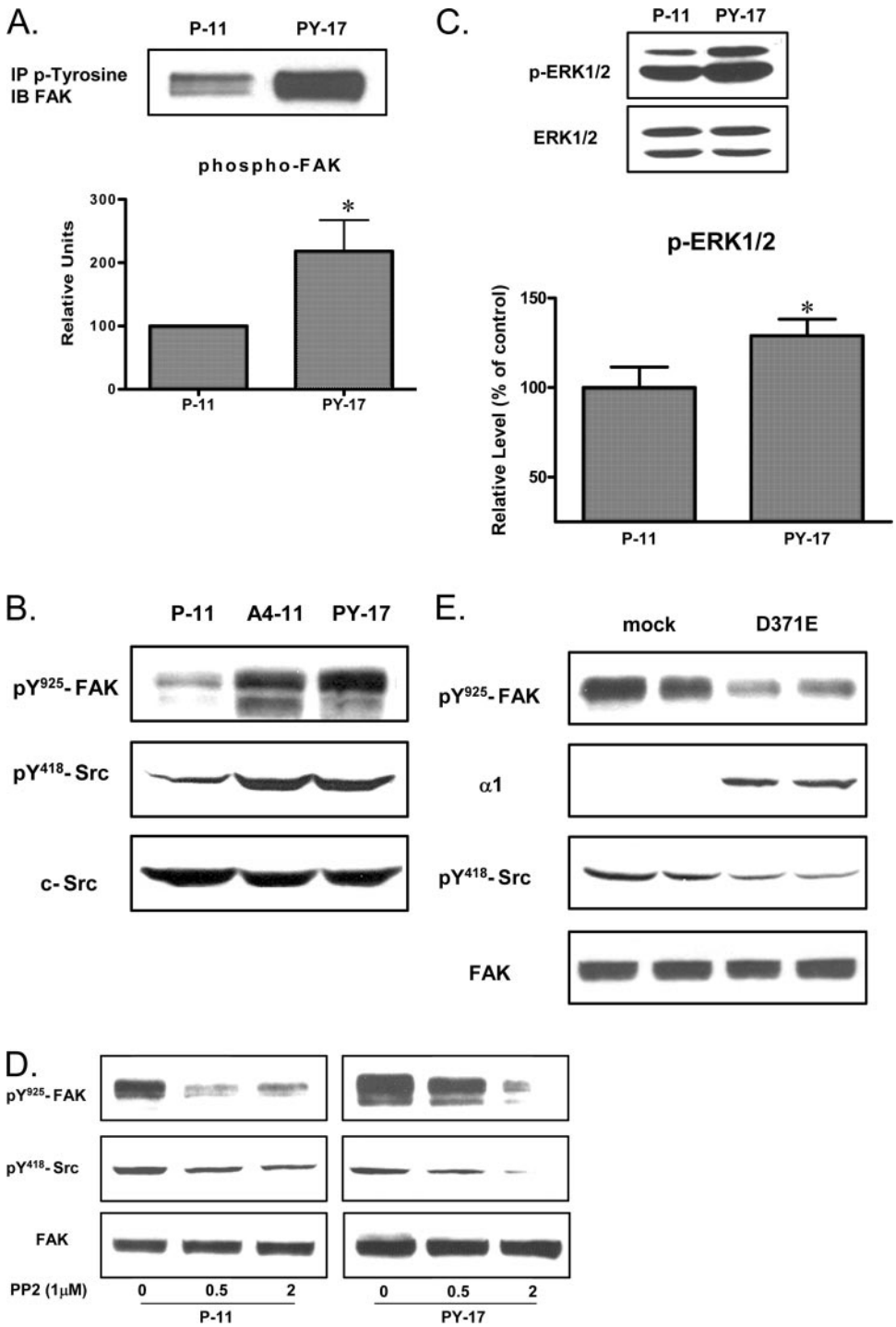


FIGURE 7. **Regulation of FAK phosphorylation by Src-interacting Na/K-ATPase.** *A*, cultured P-11 and PY-17 cells were serum-starved for 12 h. Cell lysates were then immunoprecipitated using anti-phosphotyrosine antibody (4G10), and immunoprecipitates were analyzed by anti-FAK antibody. The combined quantitative data were from three independent experiments. *B*, cell lysates from different cell lines were separated by SDS-PAGE and analyzed by anti-Tyr(P)^{925}-FAK and anti-Tyr(P)^{418}-Src antibodies. The same membrane was striped and reprobed with anti-c-Src (B-12) antibody. A representative blot of three independent experiments is shown. *C*, cell lysates were analyzed by anti-pERK1/2 or anti-ERK1/2 antibody. The quantitative data (mean ± S.E.) were calculated from four separate experiments as relative ratio of pERK/ERK. *D*, P-11 and PY-17 cells were treated with 1 μM PP2 for 0.5 and 2 h. FAK and Src activation was measured by using the specific antibodies. A representative Western blot is shown, and the same experiments were repeated three times. *E*, PY-17 cells were transiently transfected with either an empty vector (*mock*) or the D371E mutant. After 36 h, the transfected cells were lysed and analyzed by Western blot using specific antibodies as indicated. A representative Western blot is shown, and the same experiments were repeated three times. *IP*, immunoprecipitate; *IB*, immunoblot. *, $p < 0.05$ *versus* P-11.

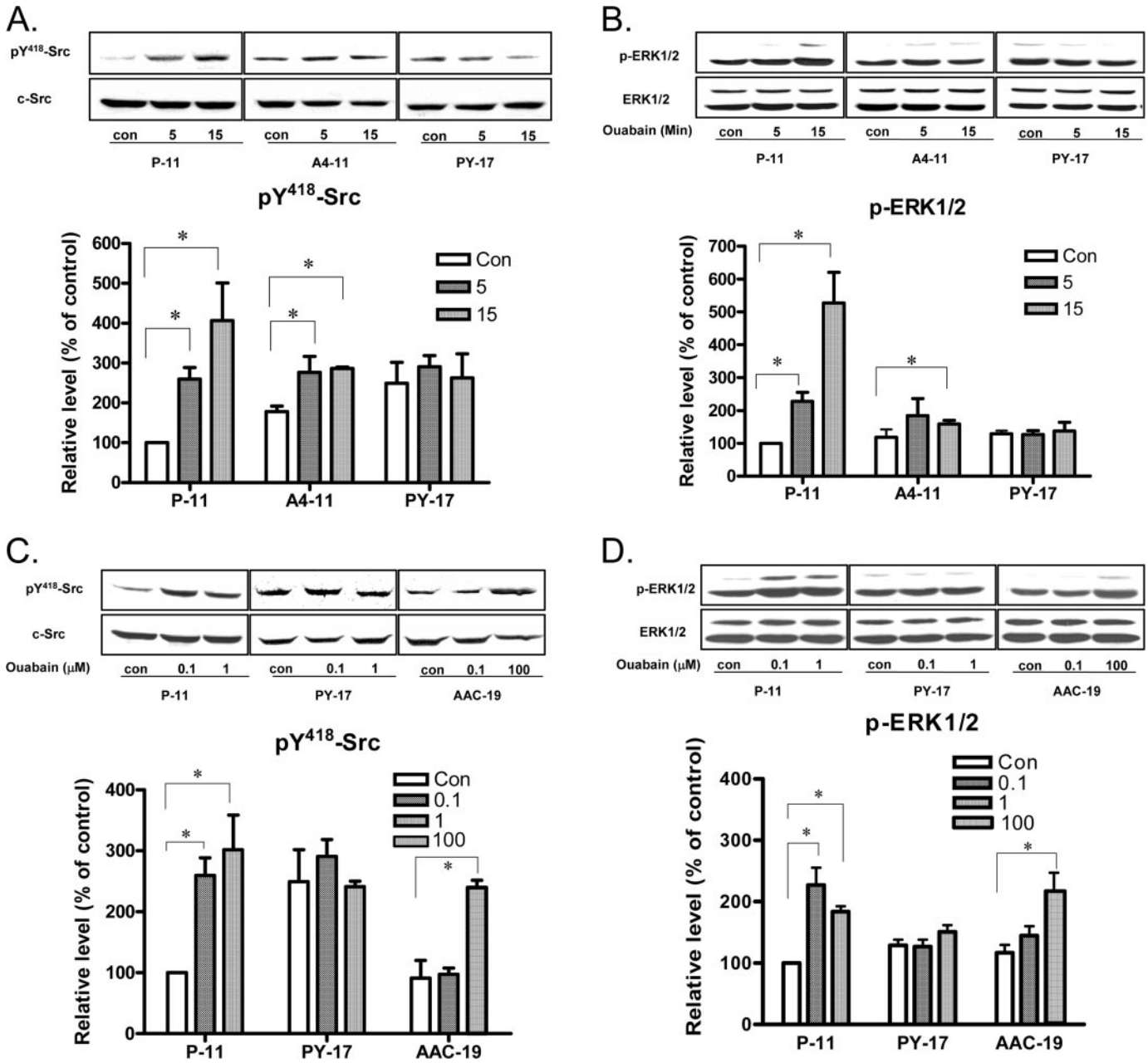


FIGURE 8. **Effects of ouabain on Src and ERK1/2.** *A* and *B*, cells were exposed to 100 nM ouabain for either 5 or 15 min, and the cell lysates (50 μg/lane) were analyzed by Western blot for active Src or active ERK1/2. Blots were probed first with anti-Tyr(P)^{418}-Src or anti-pERK antibody, then stripped, and reprobed for total Src or ERK1/2 to ensure equal loading. *C* and *D*, cells were treated with indicated concentrations of ouabain for 5 min, and total cell lysates were analyzed for Tyr(P)^{418}-Src and total Src or pERK1/2 and total ERK1/2 as in *A* and *B*. A representative Western blot and combined quantitative data are shown. The quantitative data (relative ratio of pSrc/Src or pERK/ERK) from three independent experiments (mean ± S.E.) were calculated relative to the control condition of P-11 cells. *, $p < 0.05$ *versus* the respective control condition of each cell line. *con*, control.

Src can interact directly via multiple domains and that the interaction keeps Src in an inactive state (10). These findings led us to propose that there may be a Src-interacting pool of Na/K-ATPase that not only regulates the basal Src activity but also serves as a receptor for ouabain to stimulate Src-dependent tyrosine phosphorylation of multiple effectors. The data presented here provide further support to this hypothesis. First, because the signaling Na/K-ATPase binds and keeps Src in an inactive state (10), we expected that reduction of the endogenous Na/K-ATPase would deplete the Src-interacting pool of Na/K-ATPase, thus resulting in the Src activation. Indeed as shown in Fig. 4, the α1 knockdown cells contain more active Src than the control P-11 cells. It is important to mention that the α1 knockdown did cause a significant increase in intracellular Na^+ concentration in PY-17 cells. However, when intracellular Ca^{2+} was measured by fura-2 as we described previously (30), the steady state Ca^{2+} in PY-17 cells was comparable to that in P-11 cells.[3] Thus, it is unlikely that increases in Src activity are due to changes in intracellular Na^+ or Ca^{2+}. Second, when the α1 knockdown PY-17 cells were rescued by the rat α1, we observed that the knock-in of the rat α1 was sufficient to replete the pool of Src-interacting Na/K-ATPase, leading to the restoration of basal Src activity. Finally because our *in vitro* bind-

ing assay showed that the third intracellular domain of the α1 interacts and inhibits Src activity (10), we expected that a pumping-null mutant of the rat α1 should be able to bind and inhibit Src in live cells. Indeed we found that knock-in of rat α1 mutant D371E into PY-17 cells was also able to replete this Src-interacting pool of Na/K-ATPase and reduce the amount of active Src (Fig. 5). In addition, both FRET analysis and co-immunoprecipitation assay showed that the pumping-null mutant could interact with Src in live cells (Fig. 6). Because expression of the pumping-null mutant would not reduce intracellular Na^+ concentration in PY-17 cells, these data also indicate that the Na/K-ATPase can interact and regulate Src independently of changes in intracellular Na^+ concentration.

FAK is involved in regulation of cell proliferation, cell survival, and cell migration (16–18). It is also one of the effectors of Src. Binding of active Src to FAK leads to full activation of FAK and tyrosine phosphorylation of FAK Tyr^{925}, which results in the assembly of several downstream signaling modules including the activation of ERK1/2. Interestingly we found that depletion of cellular Na/K-ATPase not only activated Src but also stimulated tyrosine phosphorylation of FAK. Inhibition of Src by either PP2 or knock-in of a pump-null α1 mutant reduced $Tyr(P)^{925}$-FAK in PY-17 cells (Fig. 7). Consistently we have also observed that ouabain stimulated Src and subsequently FAK in the control LLC-PK1 cells.[4] These findings are significant. First, they support the notion that the Na/K-ATPase is an important regulator of protein kinases. Second, the regulatory effects of the Na/K-ATPase on Src and Src effector FAK depend on the ability of the Na/K-ATPase to interact with proteins, but not to pump ions. Third, the α1 depletion-induced Src activation is capable of generating downstream pathways. To this end, it is worth noting that FAK plays a key role in regulation of cell motility. It has been reported that depletion of β1 in epithelial cells affects the formation of tight junctions and cell motility (31, 32). Thus, it will be of interest to further test the role of α1 depletion and subsequent activation of FAK in the regulation of cell migration.

We showed previously that ouabain-induced signal transduction appears to be initiated by the activation of Src (4). Because ouabain uses the Na/K-ATPase·Src complex as a functional receptor, we expected that the ouabain-induced activation of Src should correlate with the size of the pool of Src-interacting Na/K-ATPase. Indeed we found that the effect of ouabain on Src activation correlated inversely with cellular levels of the Na/K-ATPase. Although ouabain induced a modest activation of Src in A4-11 cells, it failed to activate Src in PY-17 cells. Because Src is required to transmit the ouabain signal to many downstream effectors (5, 10, 11), the new findings support the notion that the Na/K-ATPase·Src complex is the sole receptor for ouabain to provoke the protein kinase cascades. This notion is further supported by the following observations. First, rescuing PY-17 cells with the rat α1 restored the effect of ouabain on Src and ERK1/2. Second, because the rescued cells expressed the ouabain-insensitive rat α1, a much higher ouabain concentration was required to stimulate Src and subsequently ERK1/2 in AAC-19 cells (Fig. 8).

[4] M. Liang and Z. Xie, unpublished data.

In short, we have developed a powerful protocol for manipulating the cellular Na/K-ATPase that has allowed us to further characterize the signaling properties of the Na/K-ATPase. In addition, these new findings support the hypothesis that the Na/K-ATPase is an important receptor capable of transmitting ouabain signals via protein kinases (4, 5, 9, 33, 34). Finally because Src is actively involved in control of cell growth (35), our new findings warrant the need for re-examining the issue of whether the Na/K-ATPase-mediated repression of Src and ouabain-provoked activation of Src play a role in cancer biology.

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