Interaction of the α subunit of Na,K-ATPase with cofilin

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The $\alpha 1$ subunit of rat Na,K-ATPase, composed of 1018 amino acids, is arranged in the membrane so that the middle third of the polypeptide forms a large cytoplasmic loop bordered on both sides by multiple transmembrane segments. To identify proteins that might interact with the large cytoplasmic loop of Na,K-ATPase and potentially affect the function and/or the disposition of the pump in the cell, the yeast two-hybrid system was used to screen a rat skeletal muscle cDNA library. Several cDNA clones were isolated, some of which coded for cofilin, an actin-binding protein. Cofilin was co-immunoprecipitated with the α subunit of Na,K-ATPase from extracts of COS-7 cells transiently transfected with haemagglutinin-epitope-tagged cofilin cDNA as well as from yeast extracts. By means of deletion analysis we showed

that the segment of cofilin between residues 45 and 99 is essential for functional association with the large cytoplasmic loop of Na,K-ATPase. Recombinant cofilin was shown to bind to the membrane-bound Na,K-ATPase; the association between the two proteins was demonstrated by confocal microscopy. The increased level of cofilin in transfected COS-7 cells caused an increase in the rate of ouabain-sensitive ⁸⁶Rb⁺ uptake, indicating that cofilin elicits, either directly or indirectly, enhanced Na,K-ATPase activity and that the interaction occurs *in vivo*.

Key words: immunoprecipitation, membrane cytoskeleton, Na⁺ pump, yeast two-hybrid system.

INTRODUCTION

Na,K-ATPase, which is present in the plasma membrane of all animal cells, exchanges intracellular Na⁺ for extracellular K⁺ using the energy of ATP hydrolysis [1]. The enzyme is composed of a catalytic α chain of 112 kDa and a glycosylated β chain of 40–50 kDa [2]. There are four isoforms of the α subunit ($\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$) and three isoforms of the β subunit ($\beta 1$, $\beta 2$ and $\beta 3$) with different tissue distributions [3,4].

The biosynthesis and membrane insertion of the two chains of Na,K-ATPase have been studied [5]: both subunits seem to be synthesized and integrated in the membrane in a co-translational process, which is common for most membrane proteins [6]. It is, of course, relevant that the α chain, like the β chain, lacks a signal sequence; the view is that some of the putative transmembrane segments can act as signal sequences and others as stop transfer sequences [7]. The eventual assembly of the α and β chains into an $\alpha\beta$ complex, a process involving residues 891–916 of the α chain and the extracellular domain of the β chain [8], is required for the appearance of the functional enzyme at the plasma membrane [5]. It is not known whether interactions with cellular proteins are required for its transport and for its stabilization at various regions of the plasma membrane. Nevertheless, it has been found that there are binding sites for ankyrin on the second [9] and third [9,10] cytoplasmic domains and also for adducin on the purified enzyme [11].

The arrangement in the membrane of the α subunit has been deduced by numerous studies that are interpreted to indicate that the N-terminus is on the cytoplasmic side of the membrane, there are four transmembrane segments in the first third of the polypeptide chain, there is a large cytoplasmic loop (LCL) comprising the second third of the chain and there are between four and six transmembrane segments in the C-terminal third of the protein with the C-terminus on the cytoplasmic side of the membrane [12]. Hu and Kaplan [13] recently established a 10-

transmembrane segment model of the α subunit by using Cys-directed surface labelling. The LCL contains the phosphorylation site (Asp-371) and an ATP interaction site (Lys-503) and was proposed to be one of the domains involved in the regulation of Na,K-ATPase activity by insulin [3]. Except for the interaction of regions in this loop with ankyrin [9,10], little is known about the interactions of other cellular proteins with the LCL of Na,K-ATPase.

In the present study we have searched for novel proteins that interact with the LCL of Na,K-ATPase by screening a rat skeletal muscle cDNA library by the yeast two-hybrid system. Using this approach, we isolated cDNA species encoding cofilin, an actin-modulating protein that is present in a variety of mammalian cells. The interaction of cofilin with Na,K-ATPase was confirmed by immunoprecipitation and by direct binding assays. A set of deletions that removed the N-terminus of cofilin were constructed to define a domain that interacted with the LCL of Na,K-ATPase. Na,K-ATPase activity was increased in COS-7 cell transfected with the haemagglutinin (HA)-epitope-tagged cofilin cDNA.

EXPERIMENTAL

Construction of LexA fusion proteins

Dr Roger Brent (Massachusetts General Hospital, Boston, MA, U.S.A.) generously provided us with the reagents for the two-hybrid system [14,15]. The coding sequences of the cytoplasmic domains between transmembrane segments 4 and 5 were PCR-amplified from rat Na,K-ATPase $\alpha 1$ and $\alpha 2$ cDNA species, a gift from Dr Jerry Lingrel (University of Cincinnati, Cincinnati, OH, U.S.A.), with the use of the following forward and reverse oligonucleotide primers containing the sequences for restriction sites (underlined) to facilitate cloning into pEG202, a yeast expression vector: 5'-GGAATTCAAGAACTGGTGAAGAA-

Abbreviations used: ADH, alcohol dehydrogenase; HA, haemagglutinin; LCL, large cytoplasmic loop; X-Gal, 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside; YLB, yeast lysis buffer.

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C-3' (containing an *EcoRI* site) and 5'-CCGCTCGAGCACA-ATGGAGGCAAAGTTGTC-3' (containing an *XhoI* site). The PCR fragments ($\alpha 1$, 1230 bp; $\alpha 2$, 1227 bp) encoding residues 349–754 of Na,K-ATPase were cloned into a LexA-fusion plasmid, pEG202, at the *EcoRI*–*XhoI* site to create pEG $\alpha 1$ LCL and pEG $\alpha 2$ LCL respectively. Because the 1230 bp PCR fragment from the $\alpha 1$ cDNA contained an internal *XhoI* site, two fragments, a 731 bp *EcoRI* to *XhoI* and a 499 bp *XhoI* to *XhoI* fragment, were ligated into pEG202 to yield pEG $\alpha 1$ LCL (see Figure 1A) and plasmids with the cDNA in the correct orientation were identified by using additional internal restriction sites to discern the orientation. The resulting constructs contained the LCL cDNA species in a precise reading frame, confirmed by DNA sequencing, and expressed the LCL of the $\alpha 1$ and $\alpha 2$ chains as fusion proteins with the DNA-binding domain of LexA under the control of the alcohol dehydrogenase (ADH) promoter.

cDNA library construction

Total cytoplasmic RNA from rat skeletal muscle was prepared by the acid guanidinium thiocyanate/phenol/chloroform extraction method [16]. Poly(A)⁺ mRNA was purified with the poly(A) tract mRNA isolation system from Promega (Madison, WI, U.S.A.). cDNA was made with the Zap-cDNA synthesis kit (Stratagene, La Jolla, CA, U.S.A.). Size-fractionated cDNA was ligated into *EcoRI*- and *XhoI*-cut pJG4-5 library vector, which contains a Gal1 promoter, the simian virus 40 T-antigen nuclear localization sequence, the B42 activation domain and the influenza virus HA epitope at the 5' end of the restriction site. The ligated DNA was transformed into Sure cells (Stratagene) by electroporation, using an electroporator from Bio-Rad (Hercules, CA, U.S.A.) in accordance with the manufacturer's instructions. Transformed colonies (9.7×10^6) were scraped from the plates and plasmid DNA was prepared by standard methods.

Screening of activation domain libraries

The yeast strain EGY48/pSH18-34/pEG $\alpha 2$ LCL carrying *LexAop-Leu2*, *LexAop-LacZ* reporters and the LexA- $\alpha 2$ LCL bait was transformed with the library by using the high-efficiency method of Gietz et al. [17]; 2.9×10^6 transformants were recovered and 119 galactose-dependent Leu⁺ colonies and galactose-dependent blue colonies on a 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside (X-Gal) plate were obtained as described [14,15]. Library plasmid DNA species were rescued as described [14,15] and then individually reintroduced into EGY48/pSH18-34, either alone or with the LexA fusion plasmids to eliminate false positives. Plasmids encoding potential interacting proteins from 37 clones were identified by DNA sequencing with a primer, 5'-TACCCTTATGATGTGCCA-3', corresponding to the HA epitope tag sequences.

Deletion analysis of clone 267 of cofilin

Deletion was done by removing the sequences from the N-terminus of clone 267 by using PCR primers. The various derivatives of clone 267 were isolated by PCR and fused in-frame to the pJG4-5 library vector to generate the deleted constructs. The four different forward primers and a reverse primer for all constructs are as follows, with the restriction site underlined indicated in parentheses: 5'-GGAATTCACCACTTTTGTCAGATG-3' (*EcoRI*) for clone 267A, which removed the coding region of the N-terminal 29 residues of clone 267; 5'-GGAATTCGATTTCATTTTCTGGGCT-3' (*EcoRI*) for clone 267B, which

removed 60 residues of clone 267; 5'-GGAATTCGGAATC-AAGCACGAATTA-3' (*EcoRI*) for clone 267C, which removed 90 residues of clone 267; 5'-GGAATTCAGCCCCAGACCTGCTCT-3' (*EcoRI*) for clone 267D, which removed 139 residues of clone 267; and 5'-AAGCTTCTCGAGTTTTTT-3' (*XhoI*) for the reverse primer. The N-terminal regions of different constructs were confirmed by DNA sequencing with the forward PCR primers; the expression of fusion proteins of the appropriate size was confirmed by immunoblotting with the 12CA5 monoclonal antibody against the HA epitope. The plasmids were used to transform yeast EGY48/pSH18-34/pEG $\alpha 2$ LCL and tested on glucose Ura⁻His⁻Trp⁻Leu⁻, galactose Ura⁻His⁻Trp⁻Leu⁻, glucose Ura⁻His⁻Trp⁻X-Gal and galactose Ura⁻His⁻Trp⁻X-Gal plates. The measurements of β -galactosidase activity were made as previously reported [18].

Immunoblotting with yeast extracts

To prepare protein extracts, 10 ml of yeast cells in selective medium were grown to a D_{600} of 0.8–1.0. The cells were centrifuged at 3000 g for 5 min, resuspended in 0.5 ml of yeast lysis buffer (YLB) [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/5 mM MgCl₂/50 mM NaF/0.5 mM PMSF/5 μ g/ml aprotinin/1 μ g/ml leupeptin/1 mM benzamidine], centrifuged again and resuspended in 100 μ l of YLB with 100 μ l of glass beads. Yeast cells were vortex-mixed six times each for 30 s at high speed at 4 °C. The protein concentration was routinely determined by the protein assay reagent kit (Bio-Rad Laboratories). Yeast extracts (5 mg) were used for immunoblotting as described previously [12].

Construction of the HA-tagged rat cofilin cDNA

pET-22 b(+)-cof plasmid, containing the full-length rat cofilin cDNA, was used as a template for the amplification of the rat cofilin gene by using as primers oligo 155 (5'-CGGGATCC-CAGCCGTCACCATGGTATACCCATATGACGTCCCGGGACTACGCCGCCTCTGGTGTGGCTGTCTCT-3', the underlines indicating *Bam*HI and *HA* respectively) and oligo 47 (5'-CCGCTCGAGCAAAGGCTTGCCCTCCAG-3', containing a *XhoI* site). The primers were designed to contain the sequence for the HA epitope at the 5' end of the gene and the *Bam*HI and *XhoI* restriction sites for cloning into the eukaryotic expression vector pcDNAneo to generate the plasmid FNcof/cDNAneo.

Immunoprecipitation

Yeast cells in 100 ml of selective media were grown to a D_{600} of 2.0. Cells were collected by centrifugation at 3000 g for 5 min, washed twice in ice-cold YLB and pelleted again by centrifugation. The cells were resuspended in 0.5 ml YLB and 0.5 ml of glass beads, then vortex-mixed six times each for 30 s at 4 °C. After adding 1 ml of 2 $\times$ RIPA buffer [20 mM Tris/HCl (pH 8.0)/200 mM NaCl/2 mM EDTA/2% (v/v) Nonidet P40/1% sodium deoxycholate/0.2% SDS], cells were vortex-mixed again. The extracts were centrifuged at 10000 g for 30 min. IgG Sorb [200 μ l, 10% (v/v)] (The Enzyme Center, Malden, MA, U.S.A.) was added to the supernatants. After incubation for 30 min at 4 °C with continuous agitation, the mixtures were centrifuged at 10000 g for 5 min. Affinity-purified ascitic fluid (20 μ l) containing the anti-HA monoclonal antibody 12CA5 was added to the supernatant. The samples were incubated overnight at 4 °C with continuous agitation. Protein A-agarose [100 μ l,

50 % (v/v)] (Boehringer Mannheim, Indianapolis, IN, U.S.A.), was then added; the suspensions were incubated for 2 h at 4 °C, followed by centrifugation for 5 s in a microcentrifuge. Pellets were washed three times with buffer 1 [1 M NaCl/10 mM Tris/HCl (pH 8.0)/0.1 % (v/v) Nonidet P40], buffer 2 [0.1 M NaCl/10 mM Tris/HCl (pH 8.0)/0.1 % (v/v) Nonidet P40/0.1 % SDS], buffer 3 [10 mM Tris/HCl (pH 8.0)/0.1 % (v/v) Nonidet P40] and finally with 1×RIPA. Pellets were resuspended in 40 μ l of 1×SDS sample buffer, heated at 100 °C for 3 min and subjected to SDS/PAGE. Blots were incubated with the rabbit polyclonal LexA antibody, followed by horseradish-peroxidase-linked goat anti-rabbit IgG. Binding of antibodies was detected by enhanced chemiluminescence (ECL[®]; Amersham, Arlington Heights, IL, U.S.A.).

COS-7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin, then transiently transfected with FNcof/cDNAnco with the use of the LIPOFECTAMINE Plus[™] Reagent (Gibco BRL, Gaithersburg, MD, U.S.A.). Approximately 24–48 h later, cells were lysed with ice-cold lysis buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 % (v/v) Nonidet P40, 0.5 % sodium deoxycholate and protein inhibitor cocktail. 12CA5 monoclonal antibody was added to the cell lysate supernatants, after they had been centrifuged for 10 min at 12000 *g* and 4 °C, and incubated overnight at 4 °C on a rocking platform. Protein A–agarose suspension was added to the sample and incubated for 2 h at 4 °C. After the immune complexes had been washed three times with ice-cold PBS, the sample was resolved by SDS/PAGE, transferred to nitrocellulose membrane and probed with a rabbit polyclonal antibody raised against Na,K-ATPase.

Overexpression and purification of cofilin

Cofilin cDNA encoding residues Lys⁴⁵ to Leu¹⁶⁶ was amplified with the PCR primers 5'-GGAATTCCGGCAGGCAAGAACATC-3' (containing an *Eco*RI site) and 5'-CCGCTCGAGCAAAGGCTTGCCCTCCAG-3' (containing an *Xho*I site) with clone 267 as the template. The cofilin construct was cloned into the bacterial expression vector pET-22b(+) (Novagen, Madison, WI, U.S.A.), which contains the sequence for a His₆ tag at the C-terminus of the protein. The His₆ tag facilitates purification through the high affinity of histidine residues for bivalent cations such as Ni²⁺, which are present on the His-Bind resin (Novagen). The cofilin fusion protein was overexpressed and purified with the use of the Novagen protocol. In brief, *Escherichia coli* BL21(DE3)pLysS cells transformed with the expression vector were grown at 37 °C in 50 ml of Luria–Bertani medium containing 50 μ g/ml ampicillin and 34 μ g/ml chloramphenicol until *D*₆₀₀ had reached 0.6. The culture was then induced with 1 mM isopropyl β -D-thiogalactoside and harvested 3 h later by centrifugation at 5500 *g* for 10 min at 4 °C. The cells were washed in 12.5 ml of 50 mM Tris/HCl, pH 8.0, containing 2 mM EDTA, then resuspended in 2 ml of binding buffer [5 mM imidazole/500 mM NaCl/20 mM Tris/HCl (pH 7.9)] and sonicated twice on ice for 10 s at a maximum power setting with a Heat Systems (Farmingdale, CT, U.S.A.) sonicator. The lysed cells were centrifuged at 39000 *g* for 20 min at 4 °C and the supernatants were loaded on a Ni²⁺-charged His-Bind resin. After extensive washing, the retained proteins were eluted with elution buffer [1 M imidazole/500 mM NaCl/20 mM Tris/HCl (pH 7.9)], concentrated with an Ultrafree-4 centrifuge filter (Millipore Corp., Bedford, MA, U.S.A.), analysed by SDS/PAGE [12 % (w/v) gel] and stained with Coomassie Blue.

Binding assays of rat kidney Na,K-ATPase and expressed cofilin

Na,K-ATPase was purified from rat kidney [19] and stored frozen at –70 °C until use. In one assay [20], extracts made from uninduced and induced BL21(DE3)pLysS cells harbouring the cofilin construct were fractionated by SDS/PAGE [12 % (w/v) gel] and transferred to nitrocellulose. The blot was incubated for 2 h with blocking buffer [5 % (w/v) BSA and 0.05 % (v/v) Tween 20 dissolved in PBS] and then overnight at 4 °C in binding buffer [7.5 % (w/v) BSA and 4 mM galactose dissolved in blocking buffer] containing 0.4 μ M purified Na,K-ATPase in the absence and the presence of excess cofilin. The blot was washed with blocking buffer and incubated with a rabbit Na,K-ATPase polyclonal antibody. After a thorough wash, the blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000; Bio-Rad), followed by exposure to X-ray film after reaction with chemiluminescence reagent (Amersham).

Determination of binding affinity with the BIAcore system

The CM5 sensor chip was activated by injecting a solution of 50 mM *N*-hydroxysuccinimide and 200 mM *N*-ethyl-*N'*-(3-diethylamino propyl) carbodi-imide. Recombinant cofilin at 10 μ g/ml in 10 mM acetate, pH 4.0, was immobilized on the sensor chip; the remaining *N*-hydroxysuccinimide groups were deactivated with 1 M ethanolamine/1 M Tris/HCl, pH 8.0, after which 0.01 M Hepes (pH 7.4)/0.15 M NaCl/3 mM EDTA/0.005 % surfactant P20 was passed continuously over the sensor chip. Five different concentrations (3.5, 8, 18, 31 and 62.5 nM) of purified Na,K-ATPase from rat kidney were used for the binding assays. The binding curves were analysed with the curve-fitting algorithm of BIAevaluation 3.0 software (Pharmacia Biosensor) [21,22].

Immunofluorescence

COS-7 cells were cultured and stained as described previously [12]. Cells were fixed for 10 min in 3.7 % (v/v) formaldehyde in PBS containing Ca²⁺ and Mg²⁺, then incubated with mouse monoclonal antibody against cofilin (1:100) (provided by the Antibody Center, Ewha Woman's University, Seoul, Korea) and/or rabbit polyclonal antibody against Na,K-ATPase (1:500) in PBS containing 0.1 % saponin for 1 h at room temperature on a tilting platform. Subsequently, cells were incubated with both FITC-labelled anti-mouse second antibody (1:100) and rhodamine-labelled anti-rabbit second antibody (1:100), then mounted in anti-bleaching agent solution and analysed by laser confocal microscopy (Model Leica TCSNT System).

Ouabain-sensitive ⁸⁶Rb uptake

COS-7 cells were transfected with vector or FNcof/cDNAnco and then incubated for 18–24 h. Cells were seeded (8 × 10⁴ cells per well) in a 12-well tissue-culture plate and incubated until the cells were 70–80 % confluent. Rubidium uptake was performed in triplicate [23]. Cells were preincubated in Krebs–Ringer phosphate buffer containing 1 % (w/v) BSA, pH 7.4, for 15 min, and incubated in the presence or absence of 10 μ M ouabain for 15 min at 37 °C. After an additional 20 min incubation at room temperature with isotope, the buffer was removed and washed with ice-cold Krebs–Ringer phosphate buffer (without BSA). The cells were solubilized in 0.1 % SDS and then counted in LSC-cocktail (Insta-Gel; Packard).

RESULTS

LexA fusion proteins

We created pEG α 1LCL and pEG α 2LCL expression plasmids in which the H4–H5 LCL regions of the α 1 and α 2 isoforms of Na,K-ATPase were fused to the LexA DNA-binding domain respectively (Figure 1A). To demonstrate that the fusion proteins were expressed in the correct reading frame, we examined extracts of yeast transformed with pEG α 1LCL and pEG α 2LCL by immunoblotting after SDS/PAGE. Antibody directed against LexA detected 66 kDa and 68 kDa polypeptides in cells expressing pEG α 1LCL and pEG α 2LCL (Figure 1B, lanes 3–6 of Extract) respectively but not in untransformed cells (Figure 1B, lanes 1–2 of Extract). The electrophoretic mobility of the α 2 subunit was slightly, but reproducibly, lower than that of the α 1 subunit, as is that of the intact α 2 polypeptide [24]; evidently the difference in the structures of the LCLs confers this difference in electrophoretic behaviour. The detected sizes agree with the predicted sizes of the LexA- α 1LCL and LexA- α 2LCL fusion proteins. None of these constructs was capable of activating reporter genes independently.

Screening for α 2LCL-binding proteins in yeast

The yeast reporter strain EGY48 (*MAT α* , *his3*, *trp1*, *ura3-52*, *leu2::pLEU2-LexAop6*) containing the pSH18-34 plasmid with the *LexAop-lacZ* reporter construct was transformed with pEG α 2LCL bait. To identify proteins interacting specifically with the H4–H5 LCL region of the α 2 subunit, cDNA species made from rat skeletal muscle mRNA were inserted C-terminal to the B42 transcription activation domain and the HA epitope tag in a library vector, pJG4-5, that allows galactose-inducible expression of the resulting fusion proteins [14]. This library was introduced into EGY48. Transcription of the reporter genes was activated by interaction of the LCL of the α 2 subunit fused to the LexA-DNA-binding domain (bait) with a polypeptide fused to the B42 transcriptional activation domain.

Potential activation domain hybrids encoded on *Trp*-containing plasmids were isolated from these cells and transformed back into two different reporter strains, one with and one without the bait. After restriction mapping and sorting out, 37 clones were chosen for analysis by DNA sequencing. Of these, four (clones 50, 261, 267 and 277) had DNA sequences that corresponded to the gene for rat cofilin according to a computer search of the database with the use of BLAST [25]. In all cases the insert began at residue 45 of rat cofilin and continued through the entire open reading frame until residue 166, followed by a 3' untranslated region with two more stop codons at residues 178 and 230 (Figure 2A). Activation required the α 2LCL region of the LexA- α 2LCL hybrid and no activation was observed when the library plasmid encoding cofilin was transformed into a strain expressing pEG202 alone. Therefore the cofilin clones seemed to encode a protein that interacted with the H4–H5 loop region of the Na⁺ pump but did not activate transcription of the reporter gene by direct binding to the DNA sequence in the reporter genes.

Determination of complex by immunoprecipitation

To show that the LCL of Na,K-ATPase associates directly with cofilin, we used the 12CA5 monoclonal antibody against the HA-epitope-tagged cofilin to co-precipitate the LCL. Figure 1(B) (immunoprecipitation lanes) shows the results obtained by examination of the immunoprecipitates with LexA polyclonal antibodies after SDS/PAGE. Affinity-purified 12CA5 monoclonal antibodies precipitated LexA fusion proteins from yeast

Figure 1 Schematic diagram of the α 1LCL and α 2LCL insert (A) and immunoprecipitations of Na⁺ pump from yeast extracts (B) and from COS-7 cell extracts (C) with anti-HA antibody against HA-tagged cofilin

(A) A cDNA fragment obtained by PCR coding for the H4–H5 cytoplasmic loop region of the α 1 and α 2 isoforms of Na,K-ATPase was fused to the DNA binding domain of LexA at the *EcoRI*–*XhoI* site. The expression of LexA fusion proteins is controlled by the ADH promoter and the ADH terminator of the yeast ADH gene. The numbers refer to the amino acid residues in the native proteins. (B) Extracts were prepared from yeast cells EGY48/pSH18-34/clone 267 that had been transformed with one of the following plasmids in glucose (lanes 1, 3 and 5) or galactose (lanes 2, 4 and 6) medium: pRFHM1 expressing bicoid (lanes 1 and 2), pEG α 1LCL (lanes 3 and 4) and pEG α 2LCL (lanes 5 and 6). Lanes 1–6 headed 'Immunoprecipitation': after removal of a portion of each sample as a control, the extracts were subjected to immunoprecipitation with anti-HA antibody, followed by SDS/PAGE [10% (w/v) gel], transferred to nitrocellulose membranes and detected by hybridization with anti-LexA antibody. Exposure of the filter showed the immunoprecipitated 66 and 68 kDa proteins (marked by dots). Lanes 1–6 headed 'Extract': extracts before immunoprecipitation were loaded to confirm the presence of equal amounts of protein in the initial extracts. The LexA- α 1LCL and LexA- α 2LCL fusion proteins were expressed constitutively in both glucose and galactose media (lanes 3–6, molecular masses 66 and 68 kDa). (C) COS-7 cells were transfected with either vector plasmid (lanes 1) or HA-epitope-tagged cofilin cDNA (lanes 2). Immunoprecipitates were prepared from COS-7 extracts with an affinity-purified 12CA5 monoclonal antibody and blotted with rat Na,K-ATPase polyclonal antibody. The 100 kDa band of the α subunit of Na,K-ATPase was detected only from transfected cells (lane 2 under Immunoprecipitation) but not from mock-transfected cells (lane 1 under Immunoprecipitation).

extracts that contained the LexA- α 2LCL hybrid only in galactose medium (Figure 1B, dot to the left of the band in lane 6 in Immunoprecipitation) but not in glucose medium (Figure 1B, lane 5 in Immunoprecipitation). The 12CA5 antibodies also pre-

Figure 2 Amino acid sequence of clone 267 (A) and schematic representation of clone 267 and its deletion products (B)

(A) Clone 267 isolated by the yeast screen from the rat skeletal muscle expression library encodes residues 45–166 of rat cofilin. The amino acid sequence shown was deduced from the nucleotide sequences of clone 267. The starting points of each of the constructs are indicated by an arrow below the sequence. The actin-binding (amino acids 104–114) and tropomyosin (amino acids 122–127) homology regions are indicated by shaded boxes. (B) Schematic representation of clone 267 and its derivatives. The numbers refer to the amino acid sequence of the rat cofilin. The open reading frame is shown as a thick line with the actin-binding and tropomyosin homology regions depicted by shaded boxes. The thin line indicates a 3'-untranslated region.

precipitated the LexA- α 1LCL fusion proteins only in galactose medium (Figure 1B, dot to the left of the band in lane 4 in Immunoprecipitation) but not in glucose medium (Figure 1B, lane 3 in Immunoprecipitation), suggesting that the interaction between the Na⁺ pump and cofilin was not isoform-specific. The antibody did not precipitate LexA fusion proteins from extracts that contained LexA-bicoid hybrid in either glucose or galactose medium [26] (Figure 1B, lanes 1 and 2 in Immunoprecipitation). The mobility of the precipitated proteins was identical with that of immunoreactive bands in extracts of yeast containing LexA- α 1LCL and LexA- α 2LCL fusion proteins that were expressed constitutively, although the relative intensities of the bands differed (Figure 1B, lanes 3, 4, 5 and 6 in Extract) but not in extracts of yeast containing LexA-bicoid hybrid protein (Figure 1B, lanes 1 and 2 in Extract). The

Figure 3 Expression of clone 267 and its deleted constructs (A), and interaction of these polypeptides with the pEG α 2LCL bait (B)

(A) Immunoblots of protein expressed from clone 267 and the various truncated derivatives. Each sample was analysed by SDS/PAGE [15% (w/v) gel], transferred to nitrocellulose filters and hybridized with anti-(HA tag) antibody. The yeast cells grown in glucose (lanes 1, 3, 5, 7, 9 and 11) or galactose (lanes 2, 4, 6, 8, 10 and 12) medium were transformed with one of the following plasmids: pJG4-5 (an empty library vector) (lanes 1 and 2), clone 267 (lanes 3 and 4), clone 267A (lanes 5 and 6), clone 267B (lanes 7 and 8), clone 267C (lanes 9 and 10) and clone 267D (lanes 11 and 12). The positions of molecular mass markers are indicated at the left. (B) Individual colonies of EGY48 yeast cells that contained pEG α 2LCL and one of the plasmids: pJG4-5 (an empty library vector) (lanes 1 and 2), clone 267 (lanes 3 and 4), clone 267A (lanes 5 and 6), clone 267B (lanes 7 and 8), clone 267C (lanes 9 and 10) and clone 267D (lanes 11 and 12). The positions of molecular mass markers are indicated at the left. (C) Interaction of these polypeptides with the pEG α 2LCL bait. The left panel shows a circular diagram of the yeast cells. The right panel shows the growth of the yeast cells on a glucose Ura⁻His⁻Trp⁻Leu⁻ and a galactose Ura⁻His⁻Trp⁻Leu⁻ plate. Interaction resulted in activation of the *LexAop-Leu2* reporter. Strong growth with clones 267 and 267A, weak growth with clone 267B and no growth with clones 267C, 267D and pJG4-5 were observed.

results indicate that cofilin was directly associated with 66 and 68 kDa proteins. Although the association took place in RIPA buffer, the addition of 2% (w/v) SDS to the RIPA buffer eliminated the 66 and 68 kDa bands (results not shown).

To determine whether cofilin and the Na⁺ pump interact *in vivo*, we transiently transfected COS-7 cells with either vector plasmid or HA-epitope-tagged cofilin cDNA. Immunoprecipitates were prepared from COS-7 extracts with an affinity-purified 12CA5 monoclonal antibody and blotted with rat Na,K-ATPase polyclonal antibody. HA-tagged cofilin co-precipitated the 100 kDa of the α subunit of Na,K-ATPase only from transfected cells (Figure 1C, lane 2 in Immunoprecipitation) and not from mock-transfected cells (Figure 1C, lane 1 in Immunoprecipitation), although Na,K-ATPase was present in the extracts of mock-transfected cells (Figure 1C, lane 1 in Extract). The results support the view that the interaction between the α chain of Na,K-ATPase and cofilin occurs specifically *in vivo*.

Table 1 Activation of *lacZ* reporter gene by clone 267 and its derivatives

Yeast transformants harbouring the indicated expression plasmids were grown under galactose induction conditions and assayed for β -galactosidase activity. The results are expressed as units of β -galactosidase. Values given are the averages of two independent determinations.

Activator	β -Gal activity (units)	
	Bait: pEG α 1LCL	Bait: pEG α 2LCL
pJG4-5	2	2
Clone 267	113	156
Clone 267A	30	20
Clone 267B	6	2
Clone 267C	2	1

Deletion analysis of cofilin clone 267

To identify the regions of cofilin that interact with the LCL of the α chains of Na,K-ATPase, we used deletion analysis of clone 267. A panel of deletions from the 5' end of the coding sequence was generated by PCR and cloned into the pJG4-5 library vector encoding the HA epitope. Clone 267 and its N-terminal deletion constructs 267A, 267B and 267C encode 122, 98, 67 and 37 residues of the open reading frame respectively; clone 267D encodes 51 residues of the 3' untranslated region (Figure 2). The expression of these constructs was confirmed by immunoblots of extracts of yeast transformed with each construct (Figure 3A). The 12CA5 monoclonal antibody detected 29 kDa (Figure 3A, lane 4), 26.5 kDa (lane 6), 23.7 kDa (lane 8), 19.4 kDa (lane 10) and 23.3 kDa (lane 12) polypeptides in cells expressing clone 267, 267A, 267B, 267C and 267D respectively in galactose medium but not in cells expressing the library vector (lane 2). We noticed that the expression level of the 267B and 267C cells was much lower than that of the rest (Figure 3A, lanes 8 and 10) and that there was some expression of the proteins in glucose medium (lanes 3, 5 and 11) owing to the leakiness of the system. We also noticed that an 12CA5 antibody recognized an endogenous protein in the yeast extract as a background (Figure 3A). This might have been due to the fact that some of the antigenic epitopes of endogenous yeast proteins are similar to an influenza HA epitope readily detected by Western blotting.

We introduced clone 267, its deleted derivatives and the library vector as a control into EGY48/pSH18-34/pEG α 1LCL and EGY48/pSH18-34/pEG α 2LCL and examined the behaviour of the strains on glucose Ura⁻His⁻Trp⁻Leu⁻ and galactose Ura⁻His⁻Trp⁻Leu⁻ media. Figure 3(B) shows that colonies on the galactose Ura⁻His⁻Trp⁻Leu⁻ plate were greatly diminished in number after removal of the N-terminal 99 residues, suggesting that the putative LCL-binding domain is in this region. We also measured the activity of the *LacZ* reporter gene in the cells grown in glucose or galactose medium by liquid-culture β -galactosidase assay. As shown in Table 1, removal of the first 24 residues from clone 267 decreased the interaction of cofilin with α 1 4-fold and with α 2 8-fold. Deletion of the 55 N-terminal residue from clone 267 resulted in a nearly complete loss of activity in the β -galactosidase assay. It seems that deletion of the N-terminal 24 residues, with the sequence KNIILEEGKEILV-GDVGQTVDDPY, had a profound effect on the ability of cofilin to interact with the Na⁺ pump, but deletion of an additional 31 residues caused a nearly complete loss of the interaction. Although the loss of activity after deletion of the N-terminal sequence is highly suggestive of the loss of a region that forms an important contact with the Na⁺ pump, we cannot exclude the

Figure 4 Competition assay for the analysis of interaction between Na,K-ATPase and cofilin

(A) Cofilin was expressed in *E. coli* as described in the Experimental section. Protein preparations (8 μ g) were fractionated by SDS/PAGE [12% (w/v) gel] and stained with Coomassie Blue. Lane 1, total proteins before induction with isopropyl β -D-thiogalactoside (IPTG); lane 2, total proteins after induction with IPTG; lane 3, fraction of bacterial lysate not retained on the Ni²⁺-charged His-Bind resin; lane 4, purified protein retained on the column and eluted with elution buffer. The position of the expressed cofilin (17 kDa) is indicated with the arrow at the right; the positions of low-molecular-mass markers are indicated at the left. (B, C) Total proteins from *E. coli* BL21(DE3)pLysS cells harbouring the cofilin construct before (lanes 2) and after (lanes 1) induction were fractionated by SDS/PAGE [12% (w/v) gel] and transferred to nitrocellulose. The blot was incubated with 4 mg of Na,K-ATPase in the absence (B) and the presence (C) of excess cofilin. The position of the fusion cofilin is indicated with the arrow at the right; the positions of low-molecular-mass markers are indicated at the left.

possibility that a conformational change in the protein results in a loss of activity.

Interaction between Na,K-ATPase and cofilin

A question of interest is whether cofilin binds to native Na,K-ATPase or whether the interaction takes place only with the LCL of the α chains. To answer this question, we used two binding assays. In one, the fragment encoding residues 45–166 of cofilin obtained in the interaction assay described above (clone 267) was expressed in *E. coli* (Figure 4A), fractionated by SDS/PAGE and

Figure 5 Binding assay between Na,K-ATPase and cofilin by the BIAcore system

Rat kidney Na,K-ATPase at five concentrations (3.5, 8, 16, 31 and 62.5 nM) was allowed to contact the CM5 sensor chip with bound cofilin (10 mg/ml) and the response was determined (RU, relative units). The interaction between cofilin and Na,K-ATPase was dependent on concentration and the dissociation constant for the interaction was evaluated by the curve-fitting algorithm of the BIAevaluation 3.0 software.

transferred to nitrocellulose. The blot was incubated with 0.4 μ M purified rat kidney Na,K-ATPase in the presence and the absence of 1 μ M recombinant cofilin; the amount of Na,K-ATPase associated with cofilin was determined by immunoblotting with antibody 620 raised against the α chain of rat kidney Na,K-ATPase. The results shown in Figures 4(B) and 4(C) indicate that the purified rat kidney Na,K-ATPase bound to the expressed cofilin (Figure 4B, lane 1) and that there was competition for Na,K-ATPase by free cofilin (Figure 4C, lane 1). We also performed surface plasmon resonance measurements with the BIAcore system to assess the binding strength of cofilin and Na,K-ATPase, with the use of immobilized cofilin and five different concentrations (3.5, 8, 16, 31 and 62.5 nM) of the purified rat kidney Na,K-ATPase (Figure 5). The apparent dissociation constant (K_d) determined from these data was

28.6 ± 19.6 nM ($n = 3$). Unfortunately, as is obvious from the larger concentrations of Na,K-ATPase, the association phase was biphasic and the dissociation curves did not approach the same plateau values with time. This might have been due to a problem such as ligand heterogeneity. Therefore the kinetics of the cofilin–Na,K-ATPase complex seems far more complicated and does not warrant an exact K_d determination at this point. However, we can note that the shapes of the association and dissociation phases were observed consistently in all three BIAcore studies of cofilin and Na,K-ATPase, suggesting that they do interact.

Co-localization of cofilin and Na,K-ATPase

To determine whether cofilin and Na,K-ATPase are associated *in vivo*, we determined the labelling pattern of endogenous cofilin and Na,K-ATPase in COS-7 cells by confocal microscopy, after labelling the proteins with fluorescent antibodies (Figure 6). When COS-7 cells were stained with the anti-cofilin monoclonal antibody, we observed that endogenous cofilin was distributed throughout the cell, including plasma membrane (Figure 6A). Endogenous Na,K-ATPase in the cells was also distributed from the plasma membranes to the cytoplasm (Figure 6B). As can be seen in Figure 6(C), both cofilin and Na,K-ATPase were present at the plasma membrane of the COS-7 cells and were close enough to cause merging of the fluorescent stains. The co-localization of cofilin and Na,K-ATPase in COS-7 cells was consistent with the association of these proteins in a co-immunoprecipitable complex.

Effect of cofilin on Na,K-ATPase activity

Ion transport in both transiently transfected COS-7 cells and mock-transfected cells was measured by the uptake of $^{86}\text{Rb}^+$ as a surrogate for K^+ . Active uptake is 52% inhibitable by the Na,K-ATPase-specific inhibitor ouabain in mock-transfected COS-7 cells, which express an $\alpha 1$ isoform with a high affinity for the glycoside. After transfection with FNcof/cDNAneo, the existence of cofilin was confirmed by immunoblotting. As shown in Figure 7(A), one discrete band with a molecular mass of

Figure 6 Co-localization of endogenous cofilin and endogenous Na,K-ATPase at the plasma membrane of COS-7 cells

Anti-cofilin monoclonal antibody (green) and anti-(Na,K-ATPase) polyclonal antibody (red) were used to detect cofilin (A) and Na,K-ATPase (B) respectively. The images of (A) and (B) were superimposed and are shown in yellow (C), indicating that Na,K-ATPase and cofilin were co-localized at the surface of the cell.

Figure 7 Effect of cofilin on ouabain-sensitive $^{86}\text{Rb}^+$ uptake

(A) COS-7 cells were transfected with 12 μg of vector DNA (mock) or HA-tagged cofilin DNA in a 100 mm dish. After 46–50 h, cells were resuspended in lysis buffer and 10 μg of solubilized cell extracts was examined by SDS/PAGE followed by immunoblotting with anti-HA antibody. HA-tagged cofilin was not present in untransfected cells (lane 1), whereas it was found in transfected cells (lane 2). The arrow indicates the position of HA-tagged cofilin of 23.3 kDa. The positions of molecular mass markers are indicated at the left. (B) Cells were preincubated with (grey columns) or without (black columns; control) 10 μM ouabain for 15 min at 37 $^{\circ}\text{C}$. Rubidium uptake was performed in triplicate and is expressed as a percentage of control value of mock (results are means $\pm$ S.E.M., $n = 4$).

23.3 kDa for cofilin was observed with an 12CA5 antibody. Rubidium flux assays showed that the pumping activity of the Na^+ pump in the transfected cells was 26–38 % greater than that in mock-transfected cells (Figure 7B). Therefore the cofilin expressed in these cells seems to be functional in terms of Na,K-ATPase activity.

DISCUSSION

To identify possible protein regulators of Na,K-ATPase , the LCL between residues 349 and 754 of the $\alpha 2$ subunit was chosen as a bait in the yeast two-hybrid system because it is thought to be one of the potential regulatory domains as well as the main functional domain of the enzyme [3,27]. By screening 2×10^7 cells viable on galactose Ura⁺-His⁻-Trp⁻ plates, 119 galactose-dependent Leu⁺ and β -galactosidase expressing colonies were obtained. From these, 37 plasmids were isolated that retained the ability to transform the yeast strain to the proper phenotype and the exogenous DNA was examined by sequencing. The DNA of four of the 37 clones was identified as part of cofilin, an actin-binding protein, suggesting that cofilin binds to the LCL.

The results of the immunoprecipitation experiments with yeast extracts (Figure 1B) support the view that there is a binding interaction between cofilin and the LCL of the α subunit of Na,K-ATPase . This binding was not isoform-specific because both the $\alpha 1$ and $\alpha 2$ subunits of Na,K-ATPase were immunoprecipitated with 12CA5 antibodies against the HA tag on cofilin. The interaction was also observed with native Na,K-ATPase and recombinant cofilin, both on nitrocellulose and in solution (Figures 4 and 5). Several lines of evidence suggest that the interaction occurs in intact cells. Endogenous Na,K-ATPase was immunoprecipitated from COS-7 cells transfected with HA-tagged cofilin cDNA (Figure 1C). The activity of Na,K-ATPase was increased by the overexpression of cofilin (Figure 7B), suggesting a functional significance for the interaction. Furthermore, cofilin and Na,K-ATPase co-localize at the plasma

membrane (Figure 6). These results suggest that the interaction is likely to occur *in vivo* and thus might have physiological significance.

The deletion studies of the cofilin clone revealed that residues 45–99 of cofilin are essential for high-affinity binding between cofilin and Na,K-ATPase (Figures 2 and 3, and Table 1), and that removal of the sequence KNIILEEGKEILVGDVGQTVDDPY alone has a major inhibitory effect on the interaction. A search in the database for sequences similar to that of residues 45–68 of cofilin revealed no significant similarity to proteins other than those containing cofilin sequences. Accordingly, we cannot attribute the observed interaction to a coincidental similarity between cofilin and another protein that binds the Na^+ pump.

We tried to determine which part of the LCL of Na,K-ATPase interacts with the 45–68 sequences of cofilin. We divided the LCL region of the $\alpha 2$ isoform into three pieces: $\alpha 2\text{A}$ (residues 349–482), $\alpha 2\text{B}$ (residues 483–657) and $\alpha 2\text{C}$ (residues 658–756). We found that $\alpha 2\text{A}$ and $\alpha 2\text{B}$ were unable to activate expression of the *Leu2* gene and *lacZ* gene in either glucose or galactose medium. However, $\alpha 2\text{C}$ was able to activate both genes fully in the presence of glucose and galactose, suggesting that this polypeptide is able to recruit endogenous transcriptional activators to the task (K. Lee and G. Guidotti, unpublished work). We therefore conclude that the interaction between cofilin and Na,K-ATPase requires the presence of the entire LCL of 407 residues. Further studies with site-directed mutagenesis might be useful to identify which residues within the $\alpha 2$ loop are essential for the interaction with cofilin.

Cofilin is a widely distributed 21 kDa pH-sensitive actin-depolymerizing protein that can bind to both G-actin and F-actin [28–30]. It is involved in modulating the assembly and organization of actin filaments, potentially through the phosphorylation [31] of serine and threonine residues. Indeed, forms of cofilin phosphorylated by LIM kinase have been identified [32,33] and their function has been suggested [34]. It has been reported that there are functional differences between tropomyosin-containing actin filaments and cofilin-containing filaments [28].

Human erythrocyte and kidney ankyrin has been shown to form a complex with domain II (residues 140–290) and domain III (residues 345–784) of Na,K-ATPase , suggesting that it links an integral membrane protein to a cytoplasmic meshwork of spectrin and actin [10,11]. It was reported recently that the adducin was associated with the purified Na,K-ATPase [11]. It is not evident why our screen did not identify ankyrin and/or adducin as interacting proteins with the LCL of Na,K-ATPase .

Previous studies showed that the actin cytoskeleton modulates Na^+ pump activity in kidney epithelial cells, although the molecular mechanism of the modulation remains to be established [35,36]. In the context of the membrane and the targeting of membrane proteins to various membrane domains, the interaction of cofilin with actin and with Na,K-ATPase may have interesting implications. It is possible that cofilin acts to provide an interface between the cytoskeleton and Na,K-ATPase that might be necessary for movement of the pump to the plasma membrane, thus increasing the activity of Na,K-ATPase . It could also serve to localize the Na^+ pump to specific regions of the plasma membrane, or to anchor other regulatory proteins to Na,K-ATPase to enhance the pump activity. The molecular mechanisms leading to the regulation of Na,K-ATPase activity by associating with cofilin is currently under investigation in our laboratory.

In conclusion, the studies in this report indicate that cofilin interacts directly with Na,K-ATPase *in vitro* and *in vivo*, and

affects the activity of Na,K-ATPase. These results suggest that cofilin, part of the cytoplasmic actin network, might be part of a novel intracellular regulatory pathway by which cytoskeletal proteins contribute, either directly or indirectly, to the localization and regulation of Na,K-ATPase activity.

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