7634 *Biochemistry* **2010,** *49,* 7634–7642
DOI: 10.1021/bi1008722

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Cyclophilin A Is Involved in Functional Expression of the Na^+-Ca^{2+} Exchanger NCX1[†]

Benayahu Elbaz, Michael Valitsky, Geula Davidov, and Hannah Rahamimoff*

Department of Biochemistry and Molecular Biology, Hebrew University-Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel

Received May 30, 2010; Revised Manuscript Received July 29, 2010

ABSTRACT: The Na^+-Ca^{2+} exchanger (NCX) is a major Ca^{2+} regulating protein. It is almost ubiquitously expressed. Cyclophilins (Cyps) make up a class of proteins that are involved in protein folding via their peptidyl prolyl cis−trans isomerase (PPIase) and chaperone domains. They are also the cellular receptors of cyclosporin A (CsA). Binding of CsA to cyclophilins inhibits both PPIase and chaperone activities. We have shown that treatment of transfected HEK 293 cells expressing the Na^+-Ca^{2+} exchanger NCX1 with CsA results in downregulation of surface expression and transport activity, without any reduction in the total level of cell NCX1 protein [Kimchi-Sarfaty, C., et al. (2002) *J. Biol. Chem. 277* (4), 2505−2510]. In this work, we show that knockdown of cell CypA using targeting siRNA (without any CsA treatment) results in a reduction in the level of NCX1 surface expression, a decrease in the level of Na^+-dependent Ca^{2+} uptake, and no change in the total amount of cell NCX1 protein in NCX1.5-transfected HEK 293 cells and nontransfected H9c2 cells that express NCX1.1 naturally. It also reduced Na^+-dependent Ca^{2+} fluxes measured by changes in Fluo-4 AM fluorescence in single NCX1.5-transfected HEK 293 and single H9c2 cells. Knockdown of CypB had no significant effect on either transport activity, surface expression, NCX1 cell protein expression, or Ca^{2+} fluxes. Overexpression of CypA or its R55A mutant, which exhibits a substantially reduced PPIase activity, alleviated the reduction of NCX1 surface expression caused by CsA treatment, suggesting that the PPIase domain was probably not mandatory for NCX1 functional expression. We suggest that CypA plays a role in the functional expression of NCX1 protein.

Cyclophilins make up a multimember class of highly conserved proteins present in all organisms and compartments (*1*). They are the cellular receptors of cyclosporin A (CsA).[1] Binding of CsA to its cyclophilin receptor results in inhibition of the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin and suppression of the immune reaction (*1*). In addition to their involvement in suppression of the immune reaction, cyclophilins play a role in protein folding via their PPIase (peptidyl prolyl cis−trans isomerase) domain that catalyzes the isomerization of X−proline peptide bonds (*2*) and via their chaperone activity (*1*, *3*, *4*). Binding of CsA to cyclophilins inhibits their PPIase and chaperone activity and can result in impaired protein maturation and folding, thereby reducing their level of functional expression. It was shown that CsA treatment resulted in impaired functional expression of membrane proteins such as the homo-oligomeric acetylcholine receptor containing the α7 subunit, the homo-oligomeric 5-hydroxytryptamine type 3 receptor (*5*, *6*), the Kir2.1 potassium channel (*7*), the creatinine transporter (*8*), and the insulin receptor (*9*). The involvement of the PPIase domain of CypA (the cytosolic subtype) in functional expression of homo-oligomeric acetylcholine receptor in oocytes (*5*, *6*) has been suggested, because overexpression of CypA but not its R55A mutant (*10*) (in which only 1% of the PPIase activity was retained) reversed the effect of CsA.

The Na^+-Ca^{2+} exchanger is a major Ca^{2+} regulating protein (*11*). It is encoded by three mammalian genes (*NCX1*, *NCX2*, and *NCX3*) exhibiting ~65% sequence homology. NCX1 protein is expressed almost ubiquitously, whereas NCX2 and NCX3 are expressed mostly in the brain (*12*). NCX1 splice isoforms are expressed in a tissue specific manner (*13*). We have shown previously that exposure of transfected HEK 293 cells expressing the Na^+-Ca^{2+} exchangers NCX1, NCX2, and NCX3 to CsA results in downregulation of surface expression and transport activity of NCX proteins without any reduction in the total amount of cell NCX protein (*14*, *15*). This suggests that the mode of action of CsA on the expression of NCX1, NCX2, and NCX3 is post-translational and presumably related to impaired maturation and folding, resulting in cell retention of NCX proteins (*16*).

There are potentially two pathways by which CsA could impair Na^+-Ca^{2+} exchange activity: by generation of reactive oxygen species (ROS) (*17*−*20*) and by binding to cyclophilins and inhibiting their PPIase and/or chaperone activity. Several studies examined directly the effect of free radicals on the Na^+-Ca^{2+} exchanger (*21*−*23*). These studies indicated that NCX1 activity was affected by exposure to ROS-generating molecules within 1−15 min. We have shown previously, however, that addition of CsA to the Na^+-Ca^{2+} exchange reaction mixture for 20 min (*14*, *15*) had no effect on NCX transport activity, suggesting that it did not impair NCX surface expression and transport activity by generation of free radicals.

[†]This work was supported in part by research grants from the Israel Science Foundation (ISF) and The Israel Ministry of Health (Ezvonot) to H.R.

*To whom correspondence should be addressed. E-mail: hannah.rahamimoff@huji.ac.il. Phone: 972-2-6758511 (office) and 972-54-5920020 (mobile). Fax: 972-2-6757379.

[1]Abbreviations: NCX, Na^+-Ca^{2+} exchanger; PPIase, peptidyl prolyl cis−trans isomerase; CsA, cyclosporin A; Cyp, cyclophilin; FN, Flag epitope introduced at the extracellular amino terminus of NCX protein; FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity.

 Published on Web 08/03/2010

However, to rule out this possibility, we have examined in this work the role of cyclophilins directly in the functional expression of NCX1 without any CsA treatment. The experiments were conducted in *NCX1.5*-transfected HEK 293 cells and in H9c2 cells that express NCX1.1 naturally. NCX1.1 is the major Na^+-Ca^{2+} exchanger expressed in the heart. It is the longest NCX1 splice isoform (*13*, *24*). NCX1.5 is a brain isoform that is shorter than NCX1.1 by 13 amino acids (*25*). Both isoforms exhibited similar dose-dependent downregulation of surface expression by CsA when transfected into HEK 293 cells (*14*) and no change in expression of total cell NCX1 protein. This suggests that the effect of CsA on NCX1 expression is not exclusive to one isoform or the other.

We have addressed in this research three questions. (1) Can functional expression of NCX1 be modulated by manipulating cell cyclophilins? (2) Which of the cyclophilin subtypes, cytosolic (CypA) or endoplasmic reticulum (CypB), is involved in modulating NCX1 expression? (3) Which of the cyclophilin's functional domains, PPIase or chaperone, is involved in NCX1 functional expression? Our results suggest that cyclophilin A and not cyclophilin B is involved in functional expression of NCX1 and that the PPIase activity of CypA is probably not mandatory for functional expression of NCX1 protein. Our results also suggest that manipulating cell cyclophilin A has an impact on cell Na^+-dependent Ca^{2+} fluxes measured in single cells by changes in Fluo-4 AM fluorescence.

MATERIALS AND METHODS

Cell Lines and Cell Culture. HEK 293 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium with 1% glutamine, 1% penicillin-streptomycin, and 10% fetal bovine serum (Biological Industries Ltd., Kibbutz Beit Haemek, Israel) at 37 °C in humid air containing 5% CO_2. H9c2 (ATCC CRL-1446) cells derived from rat heart myocardium were grown in Dulbecco's modified Eagle's medium (Biological Industries Ltd.) with 2% glutamine, 1% penicillin-streptomycin, and 10% fetal bovine serum (Biological Industries Ltd.) at 37 °C in humid air containing 5% CO_2.

Expression Systems. HEK 293 cells were transfected with a plasmid encoding the Na^+-Ca^{2+} exchanger *FN2-NCX1.5* in pcDNA3.1 (Invitrogen). Introduction of the Flag epitope instead of the N9 glycosylation site has been described previously (*25*−*27*). In some experiments, the cells were also transfected with cloned cyclophilin A or CypA/R55A bearing a six-histidine (6xhis) tag in pcDNA3 kindly provided by Helekar and Patrick (*5*). These cyclophilins were subcloned into pcDNA3.1. The fidelity of the subcloning procedure was verified by sequencing of the full-length genes (Center for Genomic Technologies, The Hebrew University, Jerusalem, Israel).

H9c2 cells were examined for the expression of the Na^+-Ca^{2+} exchanger by RT-PCR (*28*) and found to express NCX1.1 naturally. The expression of cyclophilin A, R55A, and cyclophilin B was verified by Western analysis (see bellow).

Transfection of HEK 293 cells was conducted with PEI (polyethyleneimine) reagent prepared as described by von Harpe et al. (*29*). The average transfection efficiency was 71.6% (standard deviation of 8.7).

RNA Interference. SmartPool siRNA (Dharmacon) was used to knock down cyclophilin A and cylophilin B using DharmaFECT (Dharmacon) reagent according to the manufacturer's protocol. siGENOME SMARTpool against human CypA (M-004979) and ON-TARGETplus siCONTROL cyclophilin pool (D-001820-10) against human CypB were used to transfect HEK 293 cells. siGENOME SMARTpool against rat CypA (M-090084) and ON-TARGETplus siCONTROL cyclophilin pool (D-001820-30) against rat CypB were used to transfect H9c2 cells. SiGENOME nontargeting pool (D-001206) was used as a control for both cell lines. siGLO Red Transfection indicator (D-001630) was used to visualize siRNA-transfected cells. Calibration of knockdown efficiency was done with 10, 20, 30, 50, and 100 nM siRNA for 24, 48, and 72 h using Western blot analysis. On the basis of the calibration, 10 nM siRNA for CypA and 20 nM siRNA for CypB were used.

Determination of Na^+-Dependent Ca^{2+} Uptake. Determination of transport activity in whole cells was conducted essentially as described previously (*14*, *15*, *27*). In principle, expressing cells were preloaded with 0.16 M NaCl and 0.01 M Tris-HCl (pH 7.4) using 25 μM nystatin (Sigma-Aldrich). Cells were washed with the same buffered NaCl solution (without $MgCl_2$) to remove nystatin. Transport was initiated by overlaying the cells with the same buffered Na^+ or K^+ chloride-containing solution, to which 25 μM $^{45}Ca^{2+}$ (GE Healthcare, Chalfont St. Giles, Buckinghamshire, U.K.) had been added and conducted at 25 °C for 10 min. All solutions also contained 1 mM ouabain (Sigma-Aldrich). Na^+-dependent Ca^{2+} uptake was assessed by subtracting the amount of Ca^{2+} taken up in the absence of a Na^+ gradient from that taken up in its presence. The cell protein content of each well was determined (*30*), and the results are presented as picomoles of Ca^{2+} taken up per milligram of cell protein. Transport measurements were taken in triplicate, and each experiment was repeated six to nine times as specified.

Detection of Surface-Expressed and Total Cell Na^+-Ca^{2+} Exchanger Protein. (*i*) *Surface Biotinylation and Western Blot Analysis*. Biotinylation of surface membrane proteins in transfected HEK 293 and H9c2 cells was conducted in situ with NHS-SS-biotin (Pierce, Rockford, IL), essentially as described previously (*27*, *31*) and based on the protocol of Stephan et al. (*32*). Adherent cells from a single well of a 12-well plate were used for surface biotinylation. The biotinylated cells were lysed with a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% SDS, 0.1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich), 0.01 mg/mL pepstatin A (Sigma-Aldrich), and 0.02 mM leupeptin (Sigma-Aldrich). The SDS concentration was lowered by a 10-fold dilution of the lysate with a solution with an identical composition to that which was used to lyse the cells, except that it did not contain SDS. The lysate was loaded on streptavidin agarose beads (Pierce) and gently shaken overnight at 4 °C. The beads were washed as described in refs *27* and *31*. Biotinylated surface-expressed proteins were released from the beads by being heated for 10 min at 85 °C with Laemmli sample buffer, separated by SDS−polyacrylamide gel electrophoresis, and immunoblotted. To identify biotinylated N-Flag-tagged NCX1.5 protein, M2 (Sigma-Aldrich), the anti-Flag epitope monoclonal antibody, was used as the primary antibody. AbO-6, the polyclonal antibody against a 14-amino acid peptide derived from the large cytosolic loop of NCX1 (*26*), was used to identify biotinylated NCX1.1. Western blot analysis was conducted by standard procedures. For analysis of total cell extracts, 30 μg of approximately 600 μg of total cell protein derived from the entire contents of a single well of a 12-well plate was used. As for biotinylated NCX1 proteins, M2 (Sigma-Aldrich), the anti-Flag epitope monoclonal antibody, was used as the primary antibody for

FN-NCX1.5 and AbO-6, the anti-NCX1.1 polyclonal antibody, was used (*26*). A horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody (Jackson Immuno-Research Laboratories Inc., West Grove, PA) was used to detect antigen−antibody complexes using the ECL kit (Biological Industries Ltd.).

Western analysis of additional proteins was conducted using the following antibodies: anti-CypA (Upstate, Lake Placid, NY), anti-CypB (ABR, Golden, CO), anti-6xHIS antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (Sigma).

Quantitative analysis of immunoblots was conducted with ImageJ (W. S. Rasband, National Institutes of Health, Bethesda, MD).

(*ii*) *Immunostaining of the Na^+-Ca^{2+} Exchanger Protein by FACS*. HEK 293 cells expressing the extracellular N-Flag-tagged NCX1.5 were used to assess surface expression and total cell immunoreactive Na^+-Ca^{2+} exchanger by FACS analysis essentially as described in ref *15*. Cells were harvested and washed with PBS. For the determination of the total cell immunoreactive NCX protein, cells were permeabilized and fixed using an IntraPrep permeabilization and fixation kit following the manufacturer's procedure (Beckman Coulter, Immunotech). Following permeabilization or directly after being harvested for surface expression studies, cells were incubated with 1 μg of mouse M2 (anti-Flag) monoclonal antibody (Sigma) or 1 μg of control mouse IgG1κ antibody (BD PharMingen), in a total volume of 100 μL of PBS with 0.1% BSA (Sigma), for 30 min at 37 °C. After being washed, cells were incubated with 1 μg of FITC-conjugated anti-mouse antibody IgG1κ (BD PharMingen), or Alexa-green 488 anti-mouse secondary antibody (Invitrogen) for 30 min at 37 °C. Preliminary experiments showed that incubation of cells with a secondary antibody only revealed similar intensity background results, as incubation of cells with the control mouse IgG1κ antibody. Therefore, experiments were run using control of secondary antibodies only for each treatment. Following the second incubation, cells were washed with PBS and 0.1% BSA and 10^5 cells were analyzed by Becton Dickinson LSRII (BD Sciences, San Jose, CA). Statistical analysis was performed using FCS Express to determine the mean fluorescence intensity (MFI) values (arbitrary units).

In all of the experiments presented here, transport activity, surface expression, and total immunoreactive protein were derived from cells transfected in parallel.

Calcium Imaging. Calcium imaging was conducted essentially as described by Tsoi et al. (*33*). Pilot experiments indicated that pluronic acid was not needed to facilitate the entry of Fluo-4 AM into the cells, and therefore, in all the experiments that are included in this work, it was not added. In brief, the following protocol was used. Transfected HEK 293 cells and H9c2 cells were loaded with 5 μM Fluo-4 AM in Na^+ loading solutions composed of 10 mM Tris-HEPES (pH 7.4), 0.1 mM $CaCl_2$, and 145 mM NaCl; 45 min later, the cells were washed and incubated for an additional 15 min in the same solution without Fluo-4 AM. Fluo-4 AM fluorescence was recorded in Na^+ loading solution for 20 s, after which the cells were perfused with K^+ loading solutions composed of 10 mM Tris-HEPES (pH 7.4), 0.1 mM $CaCl_2$, and 145 mM KCl for an additional 180 s. All solutions also contained 1 mM ouabain. Confocal microscopy was performed using a Molecular Dynamics Phoibos 2001 confocal microscope, built on a Nikon Diaphot-TMD inverted microscope with a 40×, 1.25 NA, Plan oil immersion objective (Zeiss, Oberkohen, Germany). All filters were obtained from

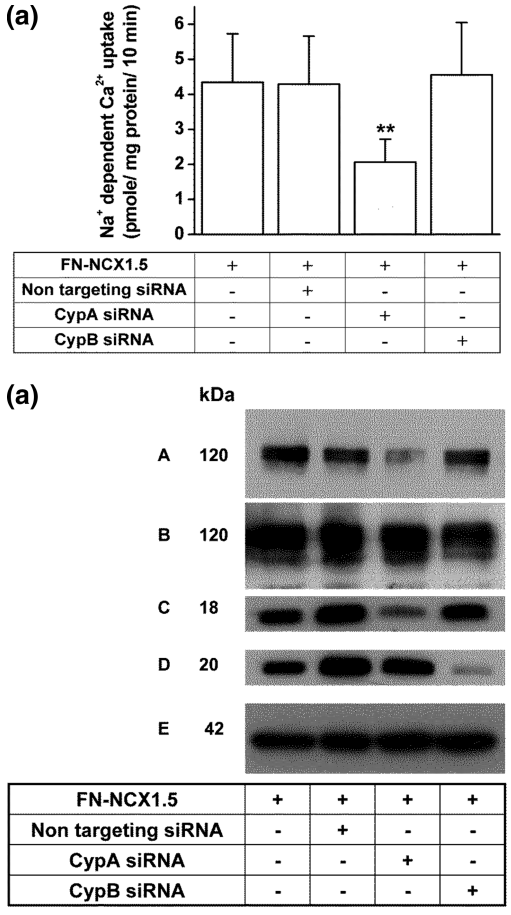

FIGURE 1: Effect of cyclophilin knockdown on FN-NCX1.5 transport activity, surface expression, and total FN-NCX1.5 immunoreactive protein in transfected HEK 293 cells. Cells were transfected with nontargeting siRNA (20 nM) or siRNA targeting CypA (10 nM) or CypB (20 nM); 24 h later, the cells were transfected with a plasmid encoding the Na^+-Ca^{2+} exchanger *FN-NCX1.5* (1 μg per well in one of 12 wells). (A) Twenty-four hours after transfection with *FN-NCX1.5*, Na^+-dependent Ca^{2+} uptake (in picomoles of Ca^{2+} calculated per milligram of cell protein) was assessed (see Materials and Methods). Statistical analysis of nine experiments was conducted as described in Materials and Methods (**$p < 0.01$). Error bars represent the SEM. (B) In parallel transfection, the level of surface expression (A) was determined by biotinylation using NHS-SS-biotin. Biotinylated proteins were captured on streptavidin beads (for details, see Materials and Methods) and subjected to Western blot analysis. Detection of FN-NCX1.5 protein was conducted with M2, the anti-Flag monoclonal antibody. Western blot analysis was used to determine the level of total immunoreactive FN-NCX1.5 protein (B), CypA (C), and CypB (D). Thirty micrograms of transfected cell extract was loaded in each lane. Immunodetection of actin (E) was used as an equal loading control.

Omega Optical Inc. The 488 nm line of a 25 mW argon ion laser (Melles Griot) was used as the excitation light source. Excitation light was first reflected by a primary 510 nm dichroic beam splitter (510 DRLP) and used to excite the fluorescence indicator in the specimen. The emitted fluorescence light passed through the primary beam splitter, 510 nm long-pass emission filter (510 ALP), and a 200 μm diameter pinhole.

To detect cells that were dyed both with Fluo-4 AM and with siGLO red transfection indicator, excitation light was reflected by a primary 535 nm dichroic beam splitter (535 DRLP). The emitted fluorescent light passed through a dichroic mirror (565B/S). siGLO red fluorescence was captured in PMT (photomultiplier) 1 using filter 600 EFLP, and Fluo-4 AM fluorescence was

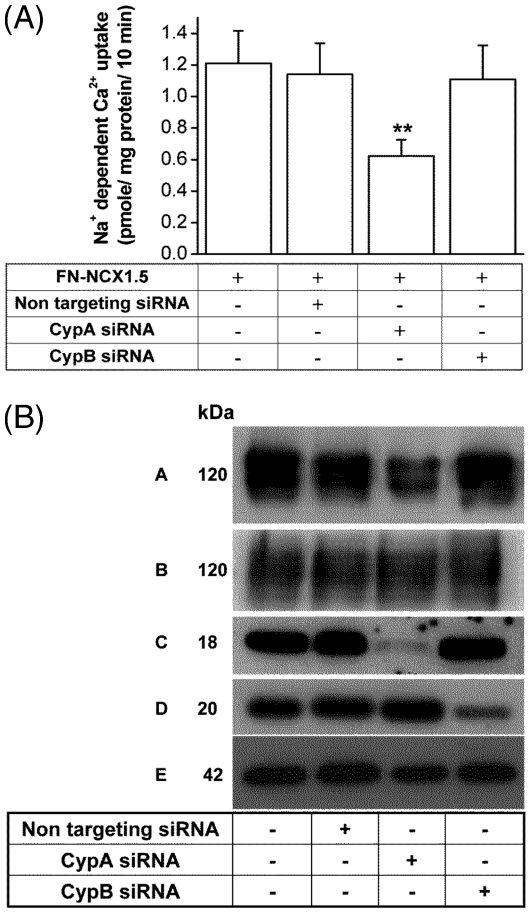

FIGURE 2: Effect of cyclophilin knockdown on NCX1.1 transport activity, surface expression, and total NCX1.1 immunoreactive protein in transfected H9c2 cells. Cells were transfected with nontargeting siRNA (20 nM) or siRNA targeting CypA (10 nM) or CypB (20 nM). (A) Eight days after transfection, Na^+-dependent Ca^{2+} uptake (in picomoles per milligram of cell protein) was determined (see Materials and Methods), for five experiments. The error bars represent the SEM (**$p < 0.01$). (B) In parallel transfections, the level of surface expression (A) was determined by biotinylation using NHS-SS-biotin. Biotinylated proteins were captured on streptavidin beads and subjected to Western blot analysis (for details, see Materials and Methods). Western blot analysis was used to determine the level of total immunoreactive NCX1.1 protein (B), CypA (C), and CypB (D). Thirty micrograms of cell extract was loaded in each lane. Immunodetection of actin (E) was used as an equal loading control.

captured in PMT2 using filter 540 DF30; 31−59% of transfected HEK 293 cells and 60−69% of H9c2 cells in any given field were doubly stained.

Data were analyzed using MATLAB (The MathWorks Inc., Natick, MA). A line scan passing through one or several cells was performed. The fluorescence across the cells was uniform. The region of interest, the midpoint (the middle pixel of each scan) of each cell, was extracted and divided into groups of 500 experimental points. The average fluorescence of the first 20 s was defined as F_0, and the fluorescence of each time point (F_t) was calculated in relative values. Bars represent the standard error of the mean (SEM) calculated with Origin version 6.1 (OriginLab Corp., Northampton, MA).

Images shown in Figure 3 were taken on a TE2000-E confocal microscope (Nikon) using a 40× oil immersion objective.

Statistical Analysis. To asses the statistical significance of transport data, calculated in picomoles of Ca^{2+} taken up per milligram of cell protein (Figures 1A and 2A), exact Wilcoxon Matched-Pairs Signed-Ranks Test with Bonferroni correction was used. Two asterisks denote $p < 0.01$; error bars represent the SEM.

For panels A and B of Figure 6, the Friedman exact test (as implemented by PASW, formerly SPSS-18) was used to test the null hypothesis. In those cases where the Friedman test was significant, post hoc analysis between pairs was conducted, based on ref *42* as implemented by StatsDirect (StatsDirect Ltd., Chesire, U.K.). The theoretical basis of the methods used by StatsDirect is described in ref *43*.

RESULTS

Knockdown of Cyclophilin A mRNA Downregulates the Surface Expression and Transport Activity of FN-NCX1.5 in Transfected HEK 293 Cells and of NCX1.1 in H9c2 Cells. To examine the possible involvement of cyclophilins in the expression of the Na^+-Ca^{2+} exchanger, HEK 293 cells and H9c2 cells were transfected with siRNAs targeting cyclophilin A or cyclophilin B (for details, see Materials and Methods and the legends of Figures 1 and 2) and with nontargeting siRNA used as a control. HEK 293 cells were also transfected with a plasmid (pcDNA3.1) encoding *FN-NCX1.5* (*26*, *27*). The following protocol was used in HEK 293 cells in all experiments. Cells were split from the same batch. All transfections were conducted in parallel starting with the siRNAs. Twenty-four hours after transfection with the siRNAs, the cells were transfected also in parallel with *FN-NCX1.5* and kept for 24 h at 37 °C; 48 h after the onset of the experiment (24 h after transfection with *FN-NCX1.5*), the levels of Na^+-dependent Ca^{2+} transport activity (Figure 1A), FN-NCX1.5 surface expression, total cell FN-NCX1.5 protein expression, cyclophilin A, cyclophilin B, and actin (Figure 1B) were determined using the appropriate antibodies (see the legend of Figure 1 for details).

Figure 1A shows the average Na^+-dependent Ca^{2+} uptake, calculated in picomoles of Ca^{2+} taken up per milligram of cell protein (see Materials and Methods) in transfected HEK 293 cells ($N = 9$). The average transport activity measured in cells transfected with *FN-NCX1.5* was 4.3 (SEM = 1.4), with nontargeting siRNA and *FN-NCX1.5* 4.3 (SEM = 1.4), with CypA siRNA and *FN-NCX1.5* 2.1 (SEM = 0.6), and with CypB siRNA and *FN-NCX1.5* 4.5 (SEM = 1.5). On the basis of these data, only transfection of HEK 293 cells with CypA siRNA reduced the relative transport activity of FN-NCX1.5 significantly (**$p < 0.01$) when compared to cells that were not transfected with siRNA, transfected with nontargeting siRNA, or transfected with CypB siRNA. Transfections parallel to those used for determination of Na^+-Ca^{2+} exchange activity were conducted (Figure 1B) and used for determination of the level of FN-NCX1.5 protein surface expression (panel A), total cell immunoreactive FN-NCX1.5 protein (panel B), cell cyclophilin A (panel C), cell cyclophilin B (panel D), and actin (panel E), the control used for equal loading of the wells. It can be seen that transfection with cyclophilin A siRNA and with cyclophilin B siRNA (panels C and D, respectively) reduced effectively the cell content of each, yet only in cells transfected with cyclophilin A siRNA was the surface expression of FN-NCX1.5 (Figure 1B, panel A) downregulated. This correlates with the reduction of Na^+-dependent Ca^{2+} uptake activity shown in Figure 1A. No difference in total cell FN-NCX1.5 protein (panel B) was detected in siRNA-treated (targeting or nontargeting) or nontreated cells. Quantitative analysis using ImageJ (see Materials and Methods)

of four additional immunoblots similar to that shown in Figure 1B was conducted. Transfection with siRNA targeting CypA reduced the level of FN-NCX1.5 surface expression from an assigned value of 100% (no siRNA added) to 52% [standard deviation (SD) of 7]. Nontargeting siRNA and CypB targeting siRNA had no significant effect on FN-NCX1.5 surface expression, yielding values of 94% (SD = 6) and 93% (SD = 3), respectively. The level of expression of total FN-NCX1.5 protein was similar: 102% (SD = 2) in cells transfected with nontargeting siRNA, 100% (SD = 17) in cells with CypA targeting siRNA, and 88% (SD = 30) in CypB targeting siRNA. Expression of CypA decreased by CypA siRNA transfection to 35% (SD = 9) relative to that for CypA in nontransfected cells. The CypA content of cells transfected with nontargeting siRNA and CypB targeting siRNA was not reduced: 86% (SD = 22) and 109% (SD = 19), respectively. The level of expression of CypB was decreased by CypB siRNA transfection to 52% (SD = 11) relative to that for CypB in nontransfected cells. The CypB content of cells transfected with nontargeting siRNA and CypA targeting siRNA was not reduced: 88% (SD = 20) and 107% (SD = 26), respectively.

Taken together, these experiments suggest that cyclophilin A plays a role in the functional expression of FN-NCX1.5 in transfected HEK 293 cells.

H9c2 cells express NCX1.1 naturally (*28*). To examine the role of cyclophilins A and B in NCX1.1 expression, the following protocol for their knockdown was used. Twenty-four hours after being plated, H9c2 cells were transfected with targeting or nontargeting cyclophilin A or cyclophilin B siRNA. Eight days later, the levels of Na^+-Ca^{2+} exchange activity, surface and total immuoreactive NCX1.1 protein, cyclophilin A, and cyclophilin B were determined. Figure 2A shows the average Na^+-dependent Ca^{2+} uptake (in picomoles of Ca^{2+} taken up per milligram of cell protein) compiled from five experiments. The transport activity in H9c2 cells was 1.2 (SEM = 0.2). In cells that were transfected with nontargeting siRNA, it was 1.1 (SEM = 0.2). When the transfection was conducted with CypA siRNA, it decreased to 0.6 (SEM = 0.1), and when the transfection was conducted with CypB siRNA, the average Na^+-dependent Ca^{2+} uptake was 1.1 (SEM = 0.2). On the basis of these data, only transfection with CypA siRNA reduced the relative transport activity of NCX1.1 significantly (**$p < 0.01$) when compared to cells that were not transfected with siRNA, transfected with nontargeting siRNA, or transfected with CypB siRNA. Transfections parallel to those used for determination of Na^+-Ca^{2+} exchange activity were conducted (Figure 2B) and used for the determination of the levels of NCX1.1 protein surface expression (panel A), total cell immunoreactive NCX1.1 protein (panel B), cell cyclophilin A (panel C), cell cyclophilin B (panel D), and actin (panel E), the control used for equal loading of the wells. It can be seen that transfection with cyclophilin A siRNA and cyclophilin B siRNA (panels C and D, respectively) reduced effectively the cell content of each, yet only in cells transfected with cyclophilin A siRNA was the level of surface expression of NCX1.1 (Figure 2B, panel A) downregulated. This correlates with the reduction of Na^+-dependent Ca^{2+} uptake activity shown in Figure 2A. No difference in total cell NCX1.1 protein (panel B) was detected in siRNA-treated (targeting or nontargeting) or nontreated cells. Quantitative analysis of four immunoblots similar to that shown in Figure 2B was conducted. Transfection with siRNA targeting CypA reduced the level of FN-NCX1.5 surface expression from an assigned value of 100% (no siRNA added) to 38% (SD = 18).

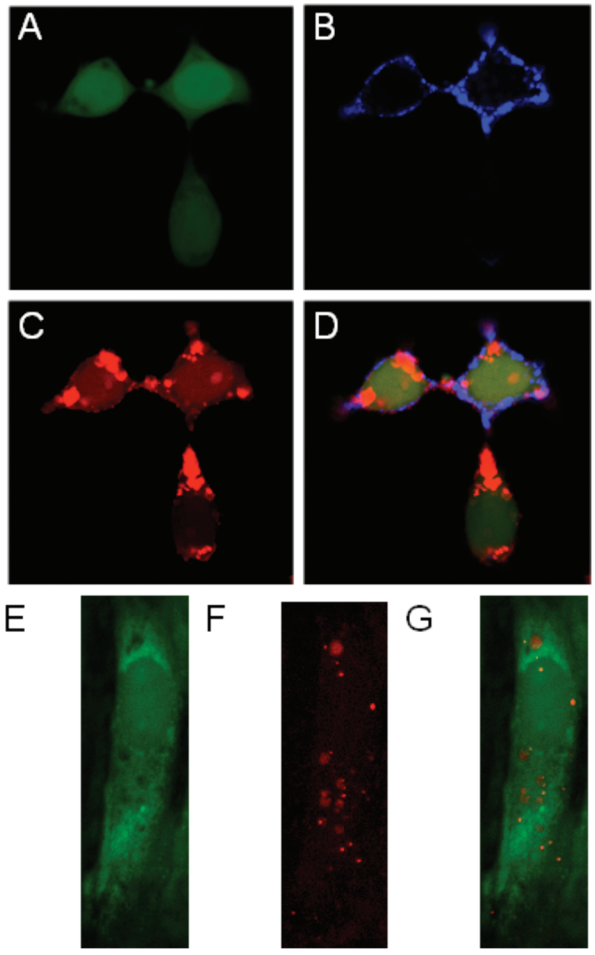

FIGURE 3: Effect of cyclophilin A or cyclophilin B knockdown on Na^+-dependent Ca^{2+} fluxes in single cells. For the choice of cells, HEK 293 cells were transfected with siRNAs and *FN-NCX1.5* as described in Materials and Methods. They were loaded with 5 μM Fluo-4 AM (A) in a Na^+ loading solution. To detect FN-NCX1.5 expression, the Fluo-4 AM-loaded cells were labeled with M2 anti-Flag monoclonal Ab and Cy5 conjugated anti-mouse secondary antibody (B). siRNA transfection reagent contained siGLO red transfection indicator (C). Panel D shows an overlay of Fluo-4 AM-loaded cells expressing FN-NCX1.5 that contain siRNA. These were used for Ca^{2+} imaging (Figure 4). H9c2 cells were transfected with siRNAs as described in Materials and Methods. They were loaded with 5 μM Fluo-4 AM (E) in a Na^+ loading solution. siRNA transfection reagent contained siGLO red transfection indicator (F). Panel G shows an overlay of Fluo-4 AM-loaded cells that contain siRNA. These were used for Ca^{2+} imaging (Figure 5).

Nontargeting siRNA and CypB targeting siRNA had no significant effect on FN-NCX1.5 surface expression, yielding values of 87% (SD = 14) and 92% (SD = 20), respectively. The levels of expression of total FN-NCX1.5 protein were similar: 95% (SD = 12) in cells transfected with nontargeting siRNA, 82% (SD = 6) in cells with CypA targeting siRNA, and 91% (SD = 23) in cells with CypB targeting siRNA. The level of expression of CypA was decreased by CypA siRNA transfection to 40% (SD = 22) relative to that for CypA in nontransfected cells. The CypA content of cells transfected with nontargeting siRNA and CypB targeting siRNA was not reduced: 125% (SD = 36) and 123% (SD = 15), respectively. The level of expression of CypB was decreased by CypB siRNA transfection to 30% (SD = 13) relative to that for CypB in nontransfected cells. The CypB content of cells transfected with nontargeting siRNA and CypA targeting siRNA was not reduced: 119% (SD = 19) and 121% (SD = 18), respectively.

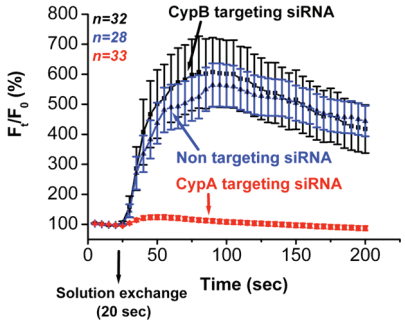

FIGURE 4: Effect of cyclophilin A or cyclophilin B knockdown on Na^+-dependent Ca^{2+} fluxes in single HEK 293 cells. The figure shows the relative Fluo-4 AM fluorescence changes detected in HEK 293 cells transfected with *FN-NCX1.5* and nontargeting siRNA (blue triangle) that reach 564%. When the cells were transfected with *FN-NCX1.5* and CypA targeting siRNA, the relative fluorescene detected was 129% (red circles). Transfection with *FN-NCX1.5* and CypB targeting siRNA (black squares) reached (603%), not significantly different from the relative fluorescence of *FN-NCX1.5* and nontargeting siRNA (blue triangles) measured. Bars represent the SEM.

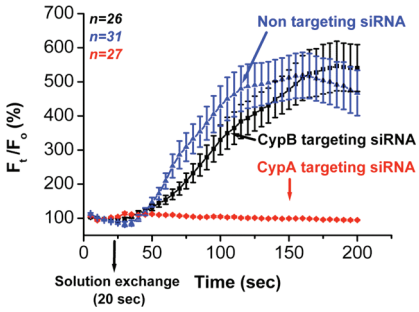

FIGURE 5: Effect of cyclophilin A or cyclophilin B knockdown on Na^+-dependent Ca^{2+} fluxes in single H9c2 cells expressing NCX1.1. The figure shows the relative Fluo-4 AM fluorescence changes detected in H9c2 cells transfected with nontargeting siRNA (blue triangles) that reach 519%. When the cells were transfected with CypA targeting siRNA, the relative fluorescene detected was 114% (red circles). Transfection with CypB targeting siRNA (black squares) reached (546%), not significantly different from the relative fluorescence of nontargeting siRNA (blue triangles) measured. Bars represent the SEM.

Taken together, these experiments suggest that cyclophilin A plays a role in the functional expression of NCX1.1 in H9c2 cells. Cyclophilin B knockdown has no impact on NCX1.1 surface expression and transport activity.

Cyclophilin A Knockdown Has an Impact on Cell Ca^{2+} Fluxes. To study the impact of cyclophilin knockdown on Ca^{2+} fluxes, we conducted experiments in single transfected HEK 293 cells and in single H9c2 cells. This was done because the experimental protocol involved double transfections 24 h apart (see Materials and Methods) for HEK 293 cells and a single transfection with siRNA for H9c2 cells. Although the transfection efficiency with PEI reagent was quite high [71.6% (SD = 8.7)], it was possible that not all cells contain both siRNA and NCX1. Therefore, we conducted these studies in single cells that were identified by appropriate staining (Figure 3). All cells were loaded with Fluo-4 AM. FN-NCX1.5 expression in HEK 293 cells was detected by immunostaining with the M2, the anti-Flag antibody. All siRNAs also contained siGLO red transfection indicator. Only HEK 293 cells containing all (Fluo-4 AM, FN-NCX1.5, and siRNA) were used to measure Na^+-dependent Ca^{2+} fluxes (Figure 3A−D). Preliminary experiments using mock-transfected HEK 293 cells (with the plasmid pcDNA3.1 only) showed no increase in Fluo-4 AM fluorescence upon formation of a Na^+ gradient (not shown).

Similar studies were also conducted with H9c2 cells that express NCX1.1 naturally. Detection of the protein was done in preliminary experiments, using the polyclonal antibody AbO-6 (*26*) following permeabilization. This was done because endogenous NCX1.1 protein does not have an external epitope suitable for immunostaining without permeabilization. To measure Ca^{2+} fluxes, we chose cells by Fluo-4 AM and siRNA transfection indicator content (Figure 3E−G).

Cyclophilin A but Not Cell Cyclophilin B Knockdown Reduces Na^+-Dependent Ca^{2+} Fluxes Measured in Single HEK 293 Cells and in Single H9c2 Cells. Figure 4 compares Na^+ gradient-dependent Ca^{2+} fluxes in single HEK 293 cells expressing FN-NCX1.5 and containing nontargeting siRNA, CypA-targeting siRNA, and CypB-targeting siRNA. The relative Fluo-4 AM fluorescence (F_t/F_0, in percent), in cells containing nontargeting siRNA, increases from the assigned value of 100% (before formation of a Na^+ gradient) to 564% (blue triangles). When the cells contained CypA targeting siRNA, the relative value of fluorescence increased to 129% only (red circles). When the cells contained siRNA targeting CypB, the relative fluorescence increased to 603% (black squares), a value similar to that obtained in cells that were treated with nontargeting siRNA. These experiments strongly suggest that CypA (but not CypB) plays a role in regulating cell Ca^{2+} via the Na^+−Ca^{2+} exchanger in HEK 293 cells.

Figure 5 shows similar measurements compiled from single H9c2 cells. The relative Fluo-4 AM fluorescence (F_t/F_0, in percent), in cells containing nontargeting siRNA, increases from the assigned value of 100% (before formation of a Na^+ gradient) to 519% (blue triangles). When the cells contained CypA targeting siRNA, the relative value of fluorescence increased to 114% only (red circles). When the cells contained siRNA targeting CypB, the relative fluorescence increased to 546% (black squares), a value similar to that obtained in cells that were treated with nontargeting siRNA. The very small increase in Ca^{2+} following creation of a Na^+ gradient in CypA siRNA-treated cells resulted from a substantial decrease (but not absence of) in the level of NCX surface expression. We have exposed CypA siRNA-treated H9c2 cells ($N = 11$) to 5 mM caffeine, and the relative fluorescence F_t/F_0 increased to 377%. A similar relative increase in fluorescence F_t/F_0 [356% ($N = 15$)] was obtained when the cells were transfected with nontargeting siRNA and treated with 5 mM caffeine. This suggests that the cells respond to an increase in intracellular Ca^{2+} concentration by other mechanisms. These experiments strongly suggest that in H9c2 cells CypA (but not CypB) also plays a role in regulating the cell Ca^{2+} concentration.

Overexpression of Cyclophilin A or CypA/R55A in HEK 293 Cells Relieves the CsA-Dependent Downregulation of FN-NCX1.5 Surface Expression and Transport Activity. Unlike H9c2 cells that naturally express NCX1.1, *FN-NCX1.5*-transfected HEK 293 cells produce large amounts of FN-NCX1.5 protein, of which much is retained in the cell and does not reach the surface membrane. It has been suggested that quality control mechanisms are responsible for cell retention of maturation and folding deficient protein (*16*). On the basis of the experiments described in the legends of Figures 1A,B and 4, cyclophilin A plays a role in the acquisition of functional competence of FN-NCX1.5. To examine whether increasing cell cyclophilin A levels over the

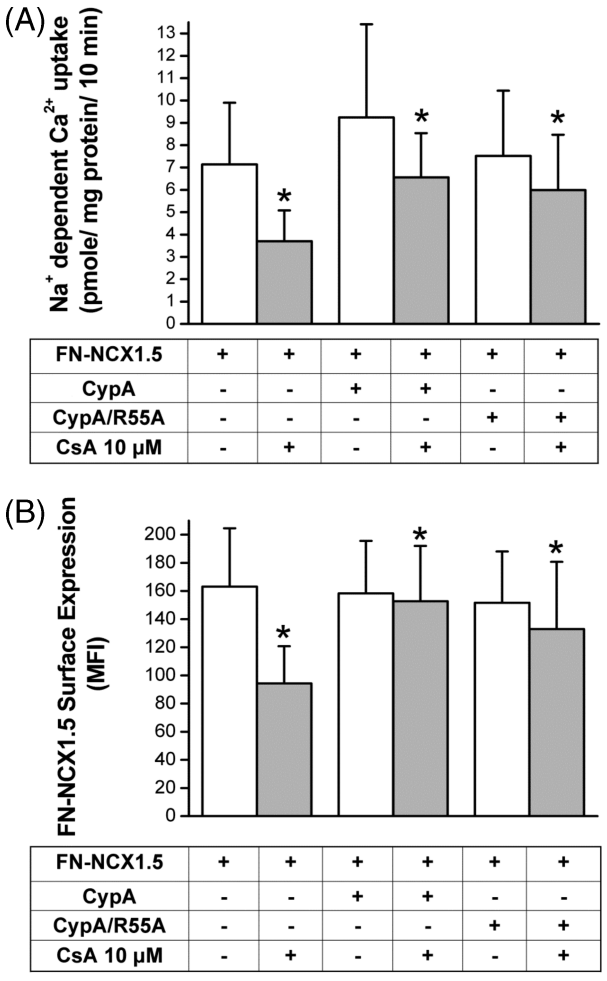

FIGURE 6: Effect of overexpression of cyclophilin A and cyclophylin A/R55A on Na^+-dependent Ca^{2+} transport activity and surface expression without and with CsA treatment. (A) HEK 293 cells were transfected with a plasmid encoding the Na^+-Ca^{2+} exchanger *FN-NCX1.5* or cotransfected also with 6xhis-tagged *cyclophilin A* or 6xhis-tagged *cyclophylin A*/*R55A*. Na^+-dependent Ca^{2+} transport activities (in picomoles of Ca^{2+} per milligram of cell protein) were measured without (white bar) and with 10 μM CsA (gray bar) treatment of the transfected cells. The drug was dissolved in DMSO, and an equal amount of DMSO was added to nontreated cells. The volume of DMSO was kept to 0.1% of that of the medium. The drug was added to the cell medium 3 h after transfection and kept until 24 h. Statistical comparisons were done by the Friedman test (see Materials and Methods). No statistical difference was found in FN-NCX1.5 transport activity between cells transfected with *FN-NCX1.5* only and the cells that were also cotransfected with *CypA* or *R55A*. Treatment of the cells with CsA showed that the transport activity of FN-NCX1.5-transfected cells was statistically different from that of either CypA or R55A-cotransfected cells with CsA treatment. The Conover test for multiple comparisons after the Friedman test was used to compare these pairs ($*p < 0.05$) ($N = 6$). Error bars represent the SEM. (B) FN-NCX1.5 surface expression assessed by FACS analysis (see Materials and Methods). Gray bars represent data for cells treated with 10 μM CsA. Significant differences in surface expression from that of FN-NCX1.5-transfected cells only were assessed by the Conover test for multiple comparisons after the Friedman test ($*p < 0.05$) as described in detail for panel A ($N = 6$). Error bars represent the SEM.

naturally available one could mobilize cell-retained FN-NCX1.5 and thereby increase its surface expression and transport activity, we have cotransfected cloned cyclophilin A with FN-NCX1.5 (Figure 6A). We have also examined in these experiments whether overexpression of CypA could relieve the downregulation of FN-NCX1.5 surface expression and transport activity caused by CsA treatment. Preliminary experiments were conducted to ensure that both naturally expressed and transfected Cyps (6xhis-tagged) were expressed. Figure 6A summarizes the average ($N = 6$) Na^+-dependent Ca^{2+} transport activities in picomoles of Ca^{2+} taken up per milligram of cell protein. The table below the bar graph lists the plasmid DNAs that were cotransfected with *FN-NCX1.5*. All gray bars represent cells that were treated also with 10 μM CsA.

The average Na^+-dependent Ca^{2+} uptake in cells transfected with FN-NCX1.5 was 7.1 (SEM = 2.8) pmol of Ca^{2+}/mg of cell protein. Overexpression of cyclophlin A in addition to FN-NCX1.5 resulted in an uptake of 9.2 (SEM = 4.2) pmol of Ca^{2+}/mg of cell protein. Treatment of the cells with 10 μM CsA leads to a reduction of Na^+-dependent Ca^{2+} uptake to 3.8 (SEM = 1.4). However, when CsA treatment is conducted with cells that are cotransfected with FN-NCX1.5 and cloned with CypA, the Na^+-dependent Ca^{2+} uptake increases from 3.8 (SEM = 1.4) to 6.6 (SEM = 2.0).

To examine the importance of the PPIase domain of cyclophilin A in FN-NCX1.5 expression, cotransfection was conducted with cyclophilin A/R55A instead of CypA. This construct is a mutant in which the PPIase activity is substantially reduced relative to that of wild-type CypA (*6*, *10*). Overexpression of CypA/R55A in FN-NCX1.5-transfected cells resulted in Na^+-dependent Ca^{2+} uptake of 7.5 (SEM = 2.9) pmol of Ca^{2+}/mg of cell protein, and following CsA treatment, it increased from 3.8 (SEM = 1.4) to 6.0 (SEM = 2.5).

Figure 6B summarizes parallel experiments in which FN-NCX1.5 surface expression was assessed by FACS analysis. The bars represent the mean fluorescence intensity (MFI) calculated by compiling MFI data from six separate experiments. The average MFI of FN-NCX1.5-transfected cells was 163 (SEM = 41). CsA treatment of *FN-NCX1.5*-transfected cells reduces the level of surface expression to 94.2 (SEM = 26.5). Cotransfection of *CypA* did not increase significantly the level of surface expression [158.4 (SEM = 37.3)], but it elevated the reduced level of surface expression caused by CsA from 94.2 (SEM = 26.5) to 152.7 (SEM = 39.3). As also in Figure 6A, cotransfection with CypA/R55A has no significant impact on NCX1.5 surface expression, but it elevated the level of CsA-dependent reduced surface expression from 94.2 (SEM = 26.5) to 133 (SEM = 47.8). No changes in total immunoreactive FN-NCX1.5 protein expression without or with CsA treatment were detected by cotransfection with CypA or CypA/R55A (not shown). On the basis of the results shown in panels A and B of Figure 6, coexpression of CypA or CypA R55A with FN-NCX1.5 did not mobilize cell-retained FN-NCX1.5 to the surface membrane. Both the transport activity and surface expression values of FN-NCX1.5 without or with coexpression of CypA or with R55A were not statistically different as shown by the Friedman test. Coexpression of CypA or R55A with FN-NCX1.5 alleviated the reduction of transport activity and surface expression caused by CsA (for statistical analysis, see the legend of Figure 6A,B).

These experiments further support the role of cyclophilin A in functional expression of NCX1. Moreover, they suggest that the PPIase domain of cyclophilin A was probably not mandatory for NCX1 expression.

DISCUSSION

In this work, we examined the involvement of cyclophilins directly in functional expression of NCX1. Our hypothesis was

that if PPIase activity and/or chaperone function of cyclophilins was mandatory for acquisition of functional competence by NCX1, one should be able to demonstrate this by manipulating cell cyclophilin expression, and indeed, knockdown of cell cyclophilin A led to reduction of NCX1 surface expression, transport activity, and Na^+-dependent Ca^{2+} fluxes but did not change the amount of total cell NCX1 expression. This suggests that cyclophilin A modulated NCX1 expression in a post-translational manner. Knockdown of cyclophilin B, which shares a high degree of sequence homology with cyclophilin A, especially in its functional domains (*1*, *34*, *35*), and which is the endoplasmic reticulum subtype of the cyclophilin family, had no impact on the functional expression of NCX1. This is not what one would expect, because quality control of membrane and secretory proteins takes place in the endoplasmic reticulum (*36*). Different studies suggest though, that cyclophilin B is secreted from the cell (*37*) and is involved in extracellular processes such as adhesion of T cells to the extracellular matrix (*34*, *38*).

To examine which of the two cyclophilin A domains, the PPIase and/or chaperone, plays a role in functional expression of NCX1, we have coexpressed in HEK 293 cells cloned 6xhis-tagged cyclophilin A or cloned 6xhis-tagged cyclophilin A/R55A together with FN-NCX1.5. Cyclophilin overexpression (in addition to the naturally expressed cell cyclophilins) had no significant impact on functional expression of NCX1 protein, but both cyclophilin A species with and without PPIase activity alleviated the CsA-dependent downregulation of NCX1 surface expression and transport activity. This suggests that PPIase activity itself is not mandatory for functional expression of NCX1. It should be noted, however, that an alternative explanation is also possible, namely, that overexpression of R55A in addition to the endogenous CypA could sequester some of the cellular CsA which would leave enough endogenous CypA with intact PPIase activity.

A similar approach, however, taken by Helekar and Patrick (*5*, *6*) suggested that CypA/R55A could not replace CypA in alleviating the CsA reduction of functional expression of the α7 homo-oligomeric nicotinic acetylcholine receptor in oocytes, suggesting that in that case the PPIase activity was mandatory for functional expression of the protein.

We have attempted to co-immunoprecipitate FN-NCX1.5 with 6xhis-taged CypA from transfected HEK 293 cell lysates (results not shown). We used different methods to prepare cell extracts; we used anti-Flag beads or anti-Flag-coated plates, or Ni-NTA columns. Each matrix immunoprecipitated the corresponding tagged antigen from the cell extract but not both. It is possible, of course, that the CypA−NCX1 complex is based on very week interactions and that these did not survive the experimental conditions used for co-immunoprecipitation. Similarly, attempts to co-immunoprecipitate the homo-oligomeric α7 acetylcholine receptor with CypA from oocyte lysates (*6*) were unsuccessful. Here also it was suggested that weak interactions could be involved.

Several cytosolic proteins were shown to bind to either NCX1 or CypA. Among the cytoskeletal proteins, NCX1 was shown to colocalize with actin filaments (*39*) in Chinese hamster ovary cells. Cyclophilin A was shown to colocalize with microtubules in NIH 3T3 cells and to exist in the form of a heterocomplex containing cytoplasmic dynein in L929 cells (*40*). The binding of cyclophilin A to cytoplasmic dynein was indirect (*41*) via the dynamitin component of the dynein-associated dynactin complex. If binding of an additional protein beyond cyclophilin A was important for NCX1 functional expression, it could provide an explanation for why overexpression of cyclophilin A alone did not mobilize cell-retained NCX1 protein to the surface membrane. To improve our understanding of the process of NCX1 functional expression, the role of these and other cytoskeletal proteins together with cyclophilin A should be investigated.

ACKNOWLEDGMENT

We are grateful to Prof. Norman Grover from the Hebrew University-Hadassah Medical School for his help and guidance in the statistical analysis of our results. We thank Mr. Yaniv Gilbert for his help with Matlab programming.

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