Identification and Functional Analysis of Two Ca$^{2+}$-binding EF-hand Motifs in the B"/PR72 Subunit of Protein Phosphatase 2A*

Received for publication, November 18, 2002, and in revised form, January 8, 2003
Published, JBC Papers in Press, January 10, 2003, DOI 10.1074/jbc.M211717200

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Protein phosphatase 2A (PP2A) is a multifunctional serine/threonine phosphatase that is critical to many cellular processes including cell cycle regulation and signal transduction. PP2A is a heterotrimer containing a structural (A) and catalytic (C) subunit, associated with one variable regulatory or targeting B-type subunit, of which three families have been described to date (B/PR55, B/PR61, and B/PR72). We identified two functional and highly conserved Ca$^{2+}$-binding EF-hand motifs in human B"/PR72 (denoted EF1 and EF2), demonstrating for the first time the ability of Ca$^{2+}$ to interact directly with and regulate PP2A. EF1 and EF2 apparently bind Ca$^{2+}$ with different affinities. Ca$^{2+}$ induces a significant conformational change, which is dependent on the integrity of the motifs. We further evaluated the effects of Ca$^{2+}$ on subunit composition, subcellular targeting, catalytic activity, and function during the cell cycle of a PR72-containing PP2A trimer (PP2A_{D72}) by site-directed mutagenesis of either or both motifs. The results suggest that integrity of EF2 is required for A/PR65 subunit interaction and proper nuclear targeting of PR72, whereas EF1 might mediate the effects of Ca$^{2+}$ on PP2A_{D72} activity in vitro and is at least partially required for the ability of PR72 to alter cell cycle progression upon forced expression.

Protein phosphatase 2A (PP2A), an essential serine/threonine phosphatase present in all eukaryotic cells, is a multifunctional enzyme of fundamental importance in signal transduction that regulates a wide variety of cellular events, including cell cycle progression, development, transcription, translation, DNA replication, and viral transformation (reviewed in Refs. 1 and 2). This extraordinary functional diversity can be explained by the existence of several mechanisms by which PP2A activity, substrate specificity, and subcellular localization are regulated (reviewed in Ref. 1). One of the major ways to achieve this is by the interaction, through a scaffolding A/PR65 subunit, of the PP2A catalytic subunit (PP2A_{C}) with one of several regulatory B-type subunits. Although it has been suggested that a significant part of PP2A_{C} can occur as a dimer with the A/PR65 subunit (PP2A_{D}) within cells (3), recent evidence argues that PP2A is an obligate trimer (PP2A_{T}) in vivo (4, 5). Nevertheless, there are a number of well documented cases at variance with this central “dogma,” such as the association of PP2A_{C}, with α4 without any other subunits (6), the complex formation of PP2A_{C}, the B" subunit and cyclin G_{2} without the A subunit (7), and the association of PP2A_{D} with some viral tumor antigens (8).

PP2A regulatory subunits are encoded by four multigene families, referred to as B, B", B', and B"'. All B-type subunits, with exception of B", share two motifs for A/PR65 subunit binding (9). Because B" subunits lack these binding motifs, their status as real PP2A subunits needs further evaluation. Each B subunit is thought to confer a set of specific functional characteristics to the phosphatase. For example, the B subunits have been implicated in the regulation of cytoskeletal protein assembly (10, 11), and in Drosophila S2 cells they mediate actions of PP2A on the mitogen-activated protein kinase signaling pathway (4, 5). The B" subunits, on the other hand, mediate PP2A function in the Wnt signaling cascade (12–14) and are required for the protective function of PP2A against apoptotic cell death in S2 cells (4, 5).

The functions of the B" subunit family are probably the least well understood. Five different B" isoforms have been identified in mammals: human PR72 and PR130 (the founding members of the family) (15), mouse PR59 (16), human PR48 (17), and the recently identified human G5PR (18). Although B" homologues have been described in plants (19), Xenopus laevis oocytes (20), Drosophila melanogaster (4, 5) and Caenorhabditis elegans (protein CO6G1.5, GenBank accession number AA32946), they are manifestly absent in yeast. PR72 and PR130 are splice variants generated from a single gene and share an identical C terminus. PR72 has a muscle-specific expression, whereas PR130 is ubiquitous (15). In an in vitro assay, PP2A_{D72} is the only PP2A trimer that specifically stimulates simian virus 40 large T-antigen-dependent origin unwinding, an essential step in the initiation of viral DNA replication (21). PR59 was iden-
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EXPERIMENTAL PROCEDURES

Materials—\(45\)CaCl\(_2\) (2.5 mCi/ml, 110.8 µg of Ca\(^{2+}\)/ml), [\(^{35}\)S]methionine, and protein G-Sepharose beads were obtained from Amersham Biosciences. Restriction enzymes and DNA-modifying enzymes were purchased from Fermentas. Calcium ionophore A23187, propidium iodide, and Hoechst 33342 were from Sigma. BAPTA-AM was supplied by Alexis Biochemicals; nocardazole was from Applichem. The bacterial expression vector for the Ha-tagged domain I of calpastatin was a generous gift of Dr. J. Else (Cancer Research Laboratories, Department of Biochemistry, Queen’s University, Kingston, Ontario, Canada). The protein was expressed and purified on nickel-agarose beads (Affiland) following standard procedures. Anti-EGFP antibodies were a kind gift of Dr. M. Beuclens (Division of Biochemistry, Faculty of Medicine, Katholieke Universiteit Leuven, Belgium). Anti-PP2A\(\gamma\), and anti-PR65 monoclonal antibodies were generously supplied by Dr. S. Dilworth (Department of Metabolic Medicine, Imperial College Faculty of Medicine, Hammersmith Hospital, London, UK). Pico proofoptodymerase (used in all PCRs) was purchased from Roche Molecular Biochemicals.

Site-directed Mutagenesis of EF-hands 1 and 2—Two point mutations were introduced in each EF-hand using a PCR-based method. For the introduction of a single-point mutation into EF1, two separate PCRs were performed with PR72 cDNA as template: the first with 5'-ATAT- CATATGATGATCAAGGAAACATCTC-3' (start primer) and 5'-GTATCGAGACAGACGGCTGC3' (reverse mutated primer) and the second with 5'-GCCACGGCGCTGTCTCTGATAC-3' (forward mutated primer) and 5'-TATAGGATCCATCTTCTCACCTGATTG-3' (stop primer). The combined reaction product was these two PCRs were then used as template for a second amplification round with start and stop primers only. The resulting PCR product was cloned into the EcoRV-digested pBluescript vector (Stratagene) and sequenced to confirm the introduction of the mutation. The second point mutation within EF1 was introduced very similarly with the start primer, 5'-GTCGCTGATGAGCGATCTGATGATCCTGAGC-3' (reverse mutated primer), 5'-CTGGGAACACTGAGACGACC-3' (forward mutated primer), and the stop primer using the single EF1 point mutant as template. Together, the PR72 EF1 sequence was changed into 5'GAGAGAGAGAGAGAGAGAG-3'. For mutation of EF2, the first point mutation round was performed with the start primer, 5'-CATAGAAGATCTC- CAATGCATACATGGAGAG-3' (reverse mutated primer), 5'-CTCTCTGATGAGCGATCTGATGATCCTGAGC-3' (forward mutated primer), and the stop primer using PR72 as template. The second point mutation was generated very similarly with the start primer, 5'-GTCTCATCATCACAGCCATGAGAGAGAGAGAGAGAG-3' (reverse mutated primer), 5'-CGCGCTGATGAGCGATCTGATGATCCTGAGC-3' (forward mutated primer) and the stop primer using the single EF2 point mutant as template. In this way the EF1 sequence was changed into 5'AVHDGDVLSVMDYAL-3'. The double mutation of EF1 and EF2 was generated via four consecutive mutation rounds using the same primers and the same PCR-based method.

Expression and Purification of Recombinant PR72 Polyepitides—Wild-type PR72, N- and/or C-terminal truncations of wild-type PR72, and the single or double EF hand mutants thereof were cloned into pET15b, pET3C (Novagen), pGEX-2T2 or pGEX-4T3 (Amersham Biosciences) following standard molecular biology procedures. The resulting plasmids were transformed into BL21-EcoRI (E. coli) bacteria for protein expression. Inductions were performed with 1 mM isopropyl-1-thio-p-n-galactopyranoside for 2 h to 30 min at 30 °C. Bacterial pellets were lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Inclusion bodies were purified (41) and solubilized in 7 M guanidinium hydrochloride. After dialysis against buffer B (200 mM Tris-HCl, pH 8.2, 500 mM NaCl), the majority of the proteins remained soluble.

\(^{45}\)Ca\(^{2+}\)—Overlay Assay—\(^{45}\)Ca\(^{2+}\)-binding to the recombinant (fusion) proteins was measured after transfer to Immobilon P membranes (42). Briefly, membranes were washed overnight at room temperature in nominally Ca\(^{2+}\)-free incubation medium containing 10 mM imidazole, pH 7.0, 60 mM KCl, and 1 mM MgCl\(_2\). Binding was carried out in the same medium supplemented with 1.5 µCi/ml \(^{45}\)CaCl\(_2\) for 10 min at room temperature. Membranes were washed during 2 min in 50% ice-cold ethanol/water mixture and air-dried. Bands were visualized by autoradiography.

Fluorescence Spectroscopy—Protein concentrations of purified recombinant proteins, comprising amino acids (aa) 262–449 of PR72, were evaluated by absorbance measurement at 280 nm and by the BCA
quantification method (Pierce). Equal amounts of PR72 aa262–449 or PR72 EF1 + 2 mutant aa262–449 were diluted in buffer B and excited at 295 nm. The fluorescence spectra between 300 and 410 nm were recorded in a Photon Technology International spectrofluorometer in the absence or presence of different amounts of CaCl2 buffered by 1 mM EGTA. Free Ca2+ concentration was calculated with the CaBuO program (available at ftp.cc.kuleuven.ac.be/pub/droogmans/cabu.zip).

**GST-Pull Downs**—His6- and GST-tagged proteins were obtained from pBluescript vectors containing the coding regions of PR72, PR72 EF1mut, PR72 EF2mut, or PR72 EF1 + 2 mutant, using the TNT-coupled rabbit reticulocyte lysate system (Promega) with the appropriate RNA polymerase (T3 or T7). The GST-PR65α subunit of PP2A (PR65-GST) and the free GST protein were produced in *E. coli* BL21-pLys cells following standard procedures, and purified glutathione-S-phosphate beads (Amersham Biosciences) according to the manufacturer’s instructions.

The GST pull-down binding reactions contained 20 μl of [35S]methionine-labeled proteins, 1 μg of GST or PR65-GST, 20 μl of prewashed glutathione-Sepharose beads, and NENT100 buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% Nonidet P-40, 25% glycerol, 100 mM NaCl) to a final volume of 500 μl. If appropriate, either 2 mM EGTA or 4 mM CaCl2 was added to the reaction mix. Incubation was done for 4 h at 4 °C on a rotating wheel. The beads were washed five times with 1 ml of NENT300 (NENT with 300 mM NaCl) containing 2 mg/ml bovine serum albumin and either 2 mM EGTA or 4 mM CaCl2. Bound proteins were eluted by addition of 20 μl of SDS sample buffer and boiling. The eluted proteins were analyzed by SDS-PAGE and imaged using an Amersham Biosciences PhosphorImager.

**Cell Culture**—Monkey COS7, human U2OS, and rat L6 cells were supplied by the American Type Culture Collection (Manassas, VA). COS7 and U2OS cells were cultured in Dulbecco’s modified Eagle’s medium containing 1 g/liter glucose (BioWhittaker) supplemented with ‘s modified Eagle’s medium (MEM) containing 10% fetal calf serum. Nuclear and cytoplasmic extracts of subconfluent KG). Rat L6 myoblasts were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. EGTA. Free Ca2+ concentrations were calculated with the CaBuf program (comprising only EF2) seemed to bind significantly more Ca2+ than GST-PR72 aa238 – 449 or GST-PR72 aa353 – 410 – 529, and aa219 – 529. All transformations were performed in the PJD9–4A *Saccharomyces cerevisiae* strain (46) harboring adenine, $\beta$-galactosidase, and histidine reporter plasmids under the control of GAL4 upstream-activating sequence (UAS) to ensure positive interactions, only the expression of the adenine and $\beta$-galactosidase reporters were assayed. EGFP Experiments—PCR fragments of PR72, PR72 EF1mut, PR72 EF2mut, and PR72 EF1 + 2 mut were cloned into the Smal restriction site of pEGFP-C1 (Clontech). 1 μg of each plasmid was transfected into COS7 cells grown on a glass coverslip in a 6-cm dish (Nunc) using FuGENE 6 transfection reagent. 24–36 h after transfection cells were washed in phosphate-buffered saline and fixed in ice-cold methanol for 20 min. Following the addition of 3 μl Hoechst 33342 for 10 min to stain the nuclei, cells were briefly rinsed in water, mounted in mounting medium (Sigma), and examined by a fluorescence microscope (Diaplant, Leitz, Germany) equipped with a digital camera (DC200, Leica Microsystems). Anti-EGFP immunoprecipitations, transfected cells were lysed in Tris-buffered saline supplemented with 0.1% Nonidet P-40. Before addition of the anti-EGFP antibody, lysates were preclotted with 10 μl of protein G-Sepharose. Immunoprecipitates were washed four times in lysis buffer and once with 0.1 mM LiCl before addition of SDS sample buffer, boiling, SDS-PAGE, and Western blotting.

**Measurement of PP2A Activity**—Protein phosphatase assays were performed with [32P]-labeled phosphorylase a as the substrate in the absence of protein stimulation, essentially as described (15). PP2A, purified from rabbit skeletal muscle (15), was preincubated with calpastatin (an inhibitor of the m-calpain protease) and different amounts of CaCl2 for 10 min at 30 °C. Subsequently, different amounts of EGTA were added together with the substrate. This mixture was further incubated for 10 min at 30 °C. The reactions were stopped with trichloroacetic acid precipitation, and the amount of free [32P]-labeled phosphorylase a was counted. Similar experiments were performed by including the Ca2+ - and/or EGTA concentrations directly in the phosphate assay. Because this approach basically resulted in the same data, this indicated that Ca2+ - and/or EGTA effects are “instantaneous,” and prior incubation of Ca2+ - and/or EGTA with the enzyme is not required.

**FACS Analysis**—Asynchronously growing U2OS cells were transfected with pEGFP-C1, PR72-EF2mut, PR72 EF1mut-EF2, PR72 EF2mut-EF2 and PR72 EF1 + 2mut-EF2 in four 6-cm dishes per plasmid. 24 h after transfection, cells were trypsinized, seeded into 10-cm dishes, and allowed to grow for another 24 h before FACS analysis. If nocodazole (1 μg/ml) was used, it was added at this point for another 16 h before FACS analysis. If BAPTA-AM (10 μM) was used, it was added 6 h before the addition of nocodazole. Cells were fixed for 5 min in 4% paraformaldehyde at room temperature to preserve the EGFP signal. After washing, the cell pellet was incubated in 0.5 ml of phosphate-buffered saline containing 100 μg/ml propidium iodide and 0.1% RNase for at least 1 h at room temperature. The samples were analyzed with a Beckman Instruments Coulter Epics XL flow cytometer (Analysis) on FL1 (for EGFP) and FL3 (for propidium iodide) using standard procedures and the System 11TM software (Analysis) for quantification.

**RESULTS**

**Two Conserved EF-hands within PR72 Bind Ca2+ with Different Affinities**—Analysis of the primary amino acid structure of the B family members revealed the presence of two well-conserved EF-hand domains (termed EF1 and EF2) (Fig. 1A). These motifs are well known Ca2+-binding domains and are present, very often in tandem, in many Ca2+-binding proteins (with calmodulin being the most renown example) (reviewed in Ref. 26). Their high degree of conservation, within the family as well as throughout evolution, and given their (almost) perfect match with the known consensus sequence (Fig. 1A), it was not unlikely to predict that the B members are genuine Ca2+-binding proteins. To check this, we used the human B/PR72 protein as a “model” for the other family members, and we performed all further experiments with this particular B isoform.

To test whether PR72 could bind Ca2+ in vitro, a 45Ca2+ overlay assay was performed on several recombinant PR72-derived proteins, expressed and purified from *E. coli* bacteria (see “Experimental Procedures”) (Fig. 1B). The results show that full-length PR72, as well as GST fusion proteins of PR72 aa238–358 (comprising EF1), of PR72 aa353–410 (comprising EF2), and of PR72 aa238–420 (comprising both EF1 and EF2), but not GST alone bind Ca2+. Moreover, GST-PR72 aa353–410 (comprising only EF2) seemed to bind significantly more Ca2+ than GST-PR72 aa238–358 (comprising only EF1), although
approximately equal amounts of both proteins were loaded (Fig. 1, B and C, compare lane 6 and lane 8). This could suggest that the affinity of Ca\(^{2+}\) for EF2 is much higher than for EF1.

To ensure that Ca\(^{2+}\) binding to full-length PR72 or the fragments thereof indeed occurred via the proposed EF-hands, several mutations known to destroy Ca\(^{2+}\) binding to these motifs were introduced either in the full-length protein or in the fragments thereof (see "Experimental Procedures"). Briefly, in each motif the aspartate at position 1 plus the aspartate or glutamate at position 12 were changed into alanines. Similarly, these mutated proteins were expressed and purified from bacteria and subjected to \(^{45}\)Ca\(^{2+}\) overlay (Fig. 1B). The data show that mutation of EF1 abolishes Ca\(^{2+}\) binding to GST-PR72 aa238–420 (comprising only EF1) (lane 7), and similarly, mutation of EF2 abolishes Ca\(^{2+}\) binding to GST-PR72 aa353–410 (comprising only EF2) (lane 9). However, single mutation of EF1 within GST-PR72 aa238–420 (comprising both EF1 and EF2) has only minor effects on Ca\(^{2+}\) binding to this protein, whereas mutation of EF2 almost completely destroys Ca\(^{2+}\) binding (Fig. 1B, lane 3 and lane 4). This clearly demonstrates that both EF motifs bind calcium ions with different affinities.

Ca\(^{2+}\)-mediated Regulation of the PP2A B′/PR72 Subunit

It is well known that Ca\(^{2+}\) binding can result into drastic conformational changes within EF-hand proteins, changing the relative position of helix E and helix F from a closed to a more open configuration (reviewed in Refs. 26 and 47). This property of Ca\(^{2+}\) forms the basis for its regulatory capacity, because conformational changes within proteins often affect their biological activities.

To assess whether Ca\(^{2+}\) binding to PR72 could result into conformational changes, measurements of the intrinsic tryptophan fluorescence of wild-type and mutated PR72 recombinant proteins were conducted in the presence of different amounts of calcium ions. Such fluorescence spectra are dependent on the micro- and/or macro-environment of the emitting Trp residues within the protein. Optimal results were obtained with a rather short PR72 fragment comprising both EF-hand motifs (aa262–449). This polypeptide contains only three Trp residues, two of...
which are in the very near vicinity of each EF-hand (one is found third aa N-terminally from EF1 and one 5 aa N-terminally from EF2). Using this fragment obviously diminishes the risk that (small) changes in fluorescence of only a few Trp residues in the near vicinity of the EF motifs may become undetectable because of the intrinsic fluorescence of a lot of other Trp residues of which the physicochemical environment is not changed by Ca\(^{2+}\) binding. The data show that addition of Ca\(^{2+}\) to the PR72 aa262–449 apoprotein, in any of the concentrations used, leads to a significant increase in fluorescence intensity (Fig. 2A), whereas this is not the case for the PR72 aa262–449 protein in which both EF-hands are mutated (Fig. 2B). This is indicative for a conformational change induced by binding of calcium ions to the EF hands. Moreover, the spectra of PR72 aa262–449 EF(1 + 2)mut and PR72 aa262–449 in the absence of Ca\(^{2+}\) are very similar (overlay Fig. 2C), suggesting that the introduction of the four point-mutations per se does not affect the overall protein conformation of the wild-type apoprotein.

Effects of Ca\(^{2+}\) on the Interaction of PR72 with the A/PR65 Subunit—The A/PR65 subunit binding domain of PR72 was determined by analysis of the interaction of several deletion mutants of PR72 with A/PR65a in yeast two-hybrid assays. This yielded a rough estimation of the interaction domain. The smallest PR72 fragment tested was still able to interact with the A subunit, composed of amino acids 219–473 (results not shown). This domain contains about 80% of the proposed A Subunit Binding Domain 1 (ASBD1, aa197–302) and the complete ASBD2 (aa342–399) (9). Remarkably, also both Ca\(^{2+}\)-binding motifs are located within these ASBDs, suggesting that calcium ions might affect the interaction of PR72 with the A/PR65 subunit (and consequently, with the core enzyme).

To test this hypothesis, we performed mammalian two-hybrid assays in COS7 cells. This system has the advantage that interactions can be easily quantified and/or evaluated in the presence of extracellular stimuli, such as calcium ionophore or BAPTA-AM treatment. In the absence of any stimulus, the A/PR65 subunit interaction is observed with wild-type PR72 and PR72 EF1mut but not with PR72 EF2mut or PR72 EF(1 + 2)mut (Fig. 3A). Identical results were obtained in the yeast two-hybrid system (results not shown), suggesting that the integrity of EF2 and therefore its Ca\(^{2+}\)-binding capacity are vital for binding to A/PR65. Furthermore, the comparison of A subunit binding to B'/PR72 with that to B/PR55a and B'/PR61y1 in the mammalian two-hybrid system reveals that B'/PR72 relatively shows the strongest interaction with A/PR65, whereas the interaction with B/PR55a is the weakest (Fig. 3A). Treatment of the cells with 5–10 \(\mu\)M BAPTA-AM, a cell-permeable Ca\(^{2+}\) chelator, resulted in a slight (albeit non-specific) decrease of the observed PR72-P2R65 interaction, because a parallel decrease was also observed for the PR55-P2R65 and the PR61-P2R65 interactions upon BAPTA-AM addition (data not shown). Similarly, treatment of the cells with 2–10 \(\mu\)M A23187, a calcium ionophore, led to inconclusive results, because in this case an overall inhibition of the transcriptional response of the reporter genes (luciferase as well as \(\beta\)-galactosidase) was observed (data not shown).

To confirm the former data in vitro, GST pull-down assays were performed with GST-PR65a and in vitro translated and radioactively labeled PR72 protein or the EF-hand mutants thereof, either as such (without any special treatment), in the presence of 1 mM EGTA (pH 8.2, see “Experimental Procedures”). The calculated free Ca\(^{2+}\) concentrations are indicated. An overlay of the fluorescence spectra of recombinant PR72 aa262–449 and PR72 aa262–449 EF(1 + 2)mut in the absence of Ca\(^{2+}\) (presence of 1 mM EGTA) is shown in C.

Effects of Ca\(^{2+}\) on Phosphatase Activity of PP2A\(_{T72}\) in Vitro—In order to test the effect of CaCl\(_2\) on phosphatase activity, different (buffered) concentrations of Ca\(^{2+}\) were tested on purified PP2A\(_{T72}\) in an in vitro assay with phosphorylase a as the substrate. Calpastatin was added in order to block any residual m-calpain activity, known to be present in some PP2A\(_{T72}\) preparations (15). It should be noted that the standard purification of PP2A\(_{T72}\) from rabbit skeletal muscle is performed in buffers containing 1 mM EGTA all through the procedure (15). We therefore presume that EF1 (the low affinity
binding site) may be (partially) in a Ca\(^{2+}\)-free state, whereas EF2 (the high affinity binding site) is likely still loaded with Ca\(^{2+}\), because, according to our data, this is necessary for the interaction with PP2A\(_p\). Only high concentrations of Ca\(^{2+}\) seem to have an inhibitory effect on the phosphatase activity of PP2A\(_{T72}\) in vitro (Fig. 4A). These inhibitory effects are therefore probably mediated by the low affinity binding site EF1. In the absence of any added Ca\(^{2+}\), EGTA is without effect or only slightly stimulatory (Fig. 4B). This slight stimulation could correlate with some dissociation of PR72 from the trimer because it is known that PP2A\(_{T72}\) has a lower specific activity than PP2A\(_{B}\) with phosphorylase \(\alpha\) as the substrate (48). However, because the effect is so small, these data seem to confirm that EGTA, even at very high concentrations, is hardly able to affect the interaction of PR72 with the core enzyme. In the presence of both Ca\(^{2+}\) and EGTA, complex titration curves are observed (Fig. 4C); lower Ca\(^{2+}\) concentrations stimulate the activity maximally 2-fold, depending on the EGTA concentration, whereas inhibition by the higher Ca\(^{2+}\) concentrations seems to be more pronounced at higher EGTA concentrations. To investigate the individual contribution of EF1 and EF2 on the inhibitory as well as on the stimulatory effects of Ca\(^{2+}\) in the in vitro assay, we tried to reconstitute PP2A\(_{T72}\) purified from rabbit skeletal muscle was appropriately diluted and assayed in the presence of calpastatin and different amounts of Ca\(^{2+}\) (A) or EGTA (B) for 10 min at 30 °C with \(^{32}\)P-labeled phosphorylase \(\alpha\) as the substrate. C, the enzyme was preincubated in the presence of calpastatin and different amounts of calcium for 10 min at 30 °C. Different amounts of EGTA were added together with \(^{32}\)P-labeled phosphorylase \(\alpha\), and the incubation was continued for another 10 min at 30 °C before trichloroacetic acid precipitation and measurement of the free \(^{32}\)P-labeled phosphate. The final EGTA and Ca\(^{2+}\) concentrations in the assay mixture are indicated.

Effects of Ca\(^{2+}\) on the Subcellular Localization of PR72—The localization of PR72 and the putative Ca\(^{2+}\) effects on this phenomenon were initially evaluated by expression of EGFP fusion proteins of wild-type PR72 and of the PR72 EF-hand mutants in COS7 cells. The data show that wild-type PR72 and PR72 EF1mut are predominantly nuclear, whereas PR72 EF2mut and PR72 EF(1 + 2)mut are clearly excluded from the nucleus (Fig. 5A). Moreover, immunoprecipitations with anti-EGFP antibodies revealed co-immunoprecipitation of PP2AC and A/PR65 with PR72 and PR72 EF1mut but not with PR72 EF2mut or PR72 EF(1 + 2)mut (Fig. 5B), nicely confirming our former observations. Treatment of the transfected cells with 5–10 \(\mu\)M BAPTA-AM or 2–10 \(\mu\)M A23187, however, failed to affect the distribution of EGFP-PR72 or any of the EGFP-PR72 mutants (data not shown). The presence of PR72 in the nucleus was further confirmed by cell fractionation of L6 cells (rat myoblasts), where the majority of endogenous PR72 is present in the nuclear fraction (Fig. 5C).

![Fig. 3. A. mammalian two-hybrid results in COS7 cells. PR72, the PR72 EF mutants, PR55a, and PR61y1 were fused to the DNA binding domain of GAL4 (pAB-GAL4-derived plasmids). PR65a was fused to the transactivating region of VP16 (pSNATCH-II-derived plasmids). The indicated combinations of both expression plasmids were transfected into COS7 cells together with the luciferase reporter plasmid pUAS-TATA-luc and the \(\beta\)-galactosidase vector pEF1-GAL as internal control. B. GST pull-down assay with A/PR65a-GST and \(^{35}\)S-labeled PR72 or the PR72 EF-mutants. \(^{35}\)S-Labeled proteins were produced by in vitro coupled transcription-translation in reticulocyte lysates. Bacterial recombinant GST or GST-PR65a immobilized on glutathione-Sepharose beads were incubated with the labeled proteins, in the presence of 2 mM EGTA or 4 mM CaCl\(_2\). After stringent washings, the resin-bound proteins were eluted with SDS-loading buffer, analyzed by SDS-PAGE, and visualized by autoradiography.

![Fig. 4. Effects of calcium ions on phosphorylase \(\alpha\) phosphatase activity of PP2A\(_{T72}\) in vitro. PP2A\(_{T72}\) purified from rabbit skeletal muscle was appropriately diluted and assayed in the presence of calpastatin and different amounts of Ca\(^{2+}\) (A) or EGTA (B) for 10 min at 30 °C with \(^{32}\)P-labeled phosphorylase \(\alpha\) as the substrate. C, the enzyme was preincubated in the presence of calpastatin and different amounts of calcium for 10 min at 30 °C. Different amounts of EGTA were added together with \(^{32}\)P-labeled phosphorylase \(\alpha\), and the incubation was continued for another 10 min at 30 °C before trichloroacetic acid precipitation and measurement of the free \(^{32}\)P-labeled phosphate. The final EGTA and Ca\(^{2+}\) concentrations in the assay mixture are indicated.

Effects of Ca\(^{2+}\) on the Ability of PR72 to Induce Cell Cycle Arrest—Similar to B\'/PR48 (17) and B\'/PR59 (16), we noticed that forced expression of B\'/PR72 in U2OS cells leads to a G\(_1\)/S phase arrest. This arrest became apparent by comparing the propidium iodide-stained DNA profiles of PR72-EGFP-transfected and pEGFP-C1-transfected cells by flow cytometry (results not shown). This effect was more pronounced after prior blockage of the dividing cells in G\(_2\)/M by the addition of nocodazole, a spindle de-polymerizing agent (Fig. 6A). Interestingly, if the same experiment was repeated in PR72 EF1mut-EGFP, PR72 EF2mut-EGFP, and PR72 EF(1 \+ 2)mut-EGFP expressing cells, the EF1 and EF(1 \+ 2) mutants partially lost the ability to induce the G\(_1\)/S arrest, whereas the EF2 mutant even generated a more pronounced G\(_1\)/S arrest (Fig. 6A). These results suggest that the ability of PR72 to induce a G\(_1\)/S cell cycle arrest is at least partially dependent on the integrity of EF1. The administration of 10 \(\mu\)M BAPTA-AM induced a slight increase in the amount of cells in the G\(_1\) phase in EGFP-, PR72 EF1mut-EGFP-, and PR72 EF(1 \+ 2)mut-EGFP-transfected cells, whereas the opposite is true for the PR72-EGFP- and PR72 EF2mut-EGFP-transfected cells. These results confirm that Ca\(^{2+}\) binding to the low affinity EF1-binding site is (partially) necessary to generate the growth arrest, probably via a Ca\(^{2+}\)-dependent interaction with a substrate or another binding partner. The more pronounced G\(_1\)/S arrest in cells where PR72 EF2mut is overexpressed suggests that the mechanism by which PR72 induces the G\(_1\)/S arrest likely occurs via competition of monomeric PR72 with a B\'-containing PP2A holoenzyme for binding to this substrate or binding partner. The EF2 mutant indeed lacks the interaction with the core enzyme and therefore would have a more pronounced dominant negative effect than the wild-type protein.

**DISCUSSION**

This is the first report of a direct regulatory effect of calcium ions on protein phosphatase 2A. In the present study, we demonstrate that the B\'/PR72 regulatory subunit of PP2A is a “classical” Ca\(^{2+}\)-binding protein of the EF-hand type. PR72 contains two well conserved EF-hand motifs, which apparently exhibit different affinities for Ca\(^{2+}\) in an overlay assay. Because it is known that this type of assay merely detects high affinity binding sites (42), EF1 (aa290–302) could be catalogued as a low affinity binding site because of its poor Ca\(^{2+}\) binding, whereas EF2 (aa364–376) is clearly a high affinity binding site. Although both amino acid sequences of EF1 and EF2 conform to the overall EF-hand consensus, the difference in affinity could be explained by the presence of a glycine residue at position 6 within EF2, whereas EF1 has a leucine at this position. According to some reports (47), it is important that a relatively small amino acid is present at this particular position in between both helices E and F in order for calcium ions to bind effectively. Moreover, we have shown that Ca\(^{2+}\)-binding to PR72 results in a significant conformational change, which is dependent on the integrity of the EF-hands. This structural change could be visualized by an increase in the intrinsic tryptophan fluorescence of the PR72 aa262–449 apoprotein upon the addition of Ca\(^{2+}\).

We further investigated the functionality of both EF-hands by a site-directed mutagenesis approach. We mutated in each EF-hand the glutamate or aspartate residues at positions 1 and 12, which are both involved in the Ca\(^{2+}\) coordination (47), into

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**Fig. 5.** A. Localization of wild-type and mutant EGFP-PR72 fusion proteins in COS7 cells. COS7 cells were transfected with pEGFP-C1-derived plasmids encoding EGFP fusions of PR72 or the PR72 EF mutants. After fixation of the cells and nuclear staining with Hoechst 33342, EGFP expression was examined with the fluorescence microscope. B. Co-immunoprecipitation of PP2A\(_{\text{C}}\) and APR65 with PR72-derived EGFP fusion proteins. Lysates of COS7 cells transfected with the upper EGFP fusion constructs were immunoprecipitated (IP) with a polyclonal anti-EGFP antibody. Whole cell lysates and the immunoprecipitations were then subjected to Western blotting and blots were developed with a mixture of anti-PP2A\(_{\text{C}}\) and anti-PR65 monoclonal antibodies. C. Western blot of PR72 in nuclear and cytoplasmic extracts of L6 cells. Nuclear and cytoplasmic extracts of rat myoblast L6 cells, expressing PR72 were subjected to Western blotting. Blots were developed with a polyclonal anti-PR72 antibody raised against a C-terminal peptide of PR72 (15).

These remarkable results suggest that the integrity of EF2 is not only required for binding to the core enzyme but also for proper subcellular (nuclear) targeting of PR72. Apparently, PR72 cannot enter the nucleus unless incorporated within a trimeric PP2A complex. Moreover, these data suggest that withdrawal of B\'/PR72 from the trimer does not necessarily lead to its degradation within living cells, which is in contrast with some observations done for the B\'/PR55 subunit (49). To ensure that the EGFP tag did not influence our observations, we also transiently overexpressed wild-type PR72, PR72 EF1mut, PR72 EF2mut, and PR72 EF(1 \+ 2)mut in human U2OS cells from the pCEP4 vector, and in all cases the wild-type or mutant PR72 proteins were expressed (results not shown). This confirms that PR72 mutants unable to bind to the APR65 subunit are not rapidly degraded within cells.
alanines. These mutations did not only abolish Ca\(^{2+}\) binding to these motifs, but also changed the overall protein structure relatively little. This is an important observation, making it highly unlikely that specific defects of the EF mutants (as observed in further experiments) can be explained by conformational changes, which are sometimes inherent to the introduction of the mutations themselves.

By having established by a yeast two-hybrid approach that the minimal A subunit interacting domain of PR72 comprises both EF-hands, we were prompted to investigate whether these motifs are directly involved in A subunit binding. By a combination of both yeast and mammalian double-hybrid experiments, in vitro GST-PR65 pull-down assays, and anti-EGFP co-immunoprecipitations, we have shown that mutation of Asp-290 and Asp-301 within EF2 destroys binding of PR72 to the A subunit. Given that these mutations also destroy Ca\(^{2+}\) binding to EF2, this could suggest that Ca\(^{2+}\) binding to EF2 and the resulting conformational change are required for A subunit binding. Alternatively, Asp-290 and Asp-301 may be structurally important amino acids that are directly involved in protein-protein contacts with the A subunit. However, the identical intrinsic fluorescence spectra of PR72 aa262–449 and PR72 aa262–449 EF(1+2)mut argue against this explanation. Because EF2 is a high affinity Ca\(^{2+}\)-binding site in vitro, it would not be unlikely that it constitutively binds calcium ions in vivo, where the normal intracellular Ca\(^{2+}\) concentration is a few hundred nanomolars. An accurate determination of the affinity constant of EF2 for Ca\(^{2+}\) binding would make this more clear. The fact, however, that calcium chelators, even at high concentrations, are hardly capable of affecting the PR72-PR65 interaction in vitro as well as in vivo suggests that either the affinity of EF2 for Ca\(^{2+}\) is very high or that Ca\(^{2+}\) is inaccessible for these chelators because it is embedded within the PP2A\(_{72}\) or PR72 protein structure.

Another remarkable result was the abolishment of the proper nuclear localization of PR72 by mutation of EF2, sug-
gesting that its incorporation within the trimer is required for its subcellular targeting. This could indicate that the trimer context is required for the interaction with a specific import protein or, alternatively, with a modifying enzyme that takes care of a specific modification necessary for nuclear import. It should be noticed that PR72 contains two putative nuclear localization signals within its primary structure (15), which may be functional nuclear targeting motifs as well. This issue awaits further clarification.

A role for EF1, the low affinity calcium-binding site, is suggested by the in vitro measurement of PP2A_T72 phosphatase activity toward phosphorylase a, where only the addition of high Ca\(^{2+}\) concentrations resulted in inhibitory effects. Whether calcium ions also affect PP2A activity toward the as yet unknown physiological substrate(s) of PP2A_T72 awaits further investigation. Unfortunately, the effects of Ca\(^{2+}\) on the phosphatase activity of a PP2A_T72 enzyme with a mutated EF1 or EF2 motif could not be assessed, because we were unable to reconstitute such an enzyme in vitro with PP2A_\(\alpha\) and bacterially expressed and purified PR72 or its mutants. Nevertheless, EF1 is due to its relatively low affinity for Ca\(^{2+}\) likely the site with the highest regulatory potential in vivo, because it might act as a “calcium sensor” that transiently binds Ca\(^{2+}\) upon local or temporal rises in the intracellular Ca\(^{2+}\) concentration. But whether Ca\(^{2+}\) binding to EF1 would result in activation or inhibition of PP2A_T72 toward a particular in vivo substrate cannot be predicted from our in vitro experiments.

However, that EF1 may operate as a calcium sensor is supported by the cell cycle experiments, where it became clear that the ability of PR72 to induce a G_1/S arrest in U2OS cells is at least partially dependent on the integrity and the Ca\(^{2+}\) recruiting ability of EF1. Note that in this case the addition of BPAT-A/AM did have an effect on the properties of the wild-type protein. We propose that forced expression of monomeric PR72 may act as a dominant negative, influencing phosphatase activity indirectly. Because PR72 has a higher affinity for the A/PR65 subunit than PR55 or PR61 (Fig. 3A), it could be integrated in a trimer by expelling B/PR55 and B/PR61 from their holoenzymes or by interacting with a pre-existing dimer. Dephosphorylation of specific substrates for PP2A_T55, PP2A_T61, or PP2A_\(\alpha\) would therefore be inhibited, whereas specific PP2A_T72 substrates might be stimulated. On the other hand, “free” PR72 may compete with PP2A_T72 for an interacting protein that would normally mediate a specific PP2A_T72 effect. This would lead to inhibition of dephosphorylation of specific PP2A_T72 substrates. Therefore, here again, it cannot be predicted whether overexpression of PR72 would lead to inhibition or stimulation of dephosphorylation of some PP2A substrates. However, PP2A activity is required for the firing of replication origins (50, 51), and this may be mediated by PR72 (21) or another B family member. Binding of PR72 with a relevant substrate in this case likely occurs via the Ca\(^{2+}\)-bound EF1 motif, and Ca\(^{2+}\) binding to EF1 would likely stimulate PP2A activity. Cdc6 is one of the candidates to be this binding partner, because it interacts with PR48 and with aa354–1150 of PR130 (a region that encompasses the common part of PR130 and PR72) in yeast two-hybrid assays (17). The more pronounced dominant negative effect of the PR72 EF2 mutant, which lacks the interaction with the core enzyme and the proper subcellular localization, additionally suggests that this binding partner is (initially) not necessarily present in the nucleus.

Compared with B/PR61\(\alpha\) and especially with B/PR55, B/PR72 strongly interacts with the A/PR65 subunit in the mammalian two-hybrid system. This opens up the possibility that PR72 effectively competes with both PR61\(\alpha\) and PR55\(\alpha\) for binding to PP2A_\(\alpha\). Moreover, and in contrast with the reported data on the PR55 subunit (49), our data suggest that PR72 mutants failing to interact with the core enzyme are not highly unstable within cells. This suggests that in contrast with PP2A_\(\alpha\), A/PR65 (4, 5), and B/PR55 (49), the cell lacks a control mechanism to avoid the presence of free B/PR72, provided of course that such a population would exist. These findings therefore contribute to a better understanding of the dynamics of the various PP2A complexes in vivo.

Together, we have demonstrated a role for Ca\(^{2+}\) in PP2A_T72 subunit assembly, nuclear targeting, catalytic activity, and PR72-mediated cell cycle regulation. Whether the other B/PR72 family members also bind Ca\(^{2+}\) will have to be determined but is highly likely, given the conservation of the EF motifs within these proteins. This is obviously true for the PR130 subunit, a splice variant generated from the same gene as PR72 that shares both EF-hands. In the light of a recent report (52) demonstrating an interaction of PR130 with the ryanodine type 2 receptor, this is certainly an interesting observation. Moreover, given the muscle-specific expression of PR72 and the important role of calcium in muscle-specific processes, such as muscle contraction, our data open up a new avenue for further research at the interface between calcium signaling and protein phosphorylation/dephosphorylation.

Acknowledgments—We appreciate the help and critical comments of Dr. M. Beullens, Dr. S. Dilworth, and Dr. J. Elce for their assistance of Fabienne Withof and Roos Verbiest. We thank Dr. F. Cegielska, Dr. M. Beullens, Dr. S. Dilworth, and Dr. J. Elce for their willingness to provide plasmids or antibodies.

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Identification and Functional Analysis of Two Ca\textsuperscript{2+}-binding EF-hand Motifs in the B''/PR72 Subunit of Protein Phosphatase 2A
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J. Biol. Chem. 2003, 278:10697-10706.
doi: 10.1074/jbc.M211717200 originally published online January 10, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211717200

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