Ca\(^{2+}\) functions as a molecular switch that controls the mutually exclusive complex formation of pyridoxal phosphatase with CIB1 or calmodulin

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Pyridoxal 5'-phosphate (PLP) is an essential cofactor for neurotransmitter metabolism. Pyridoxal phosphatase (PDXP) deficiency in mice increases PLP and \(\gamma\)-aminobutyric acid levels in the brain, yet how PDXP is regulated is unclear. Here, we identify the Ca\(^{2+}\)- and integrin-binding protein 1 (CIB1) as a PDXP interactor by yeast two-hybrid screening and find a calmodulin (CaM)-binding motif that overlaps with the PDXP-CIB1 interaction site. Pulldown and crosslinking assays with purified proteins demonstrate that PDXP directly binds to CIB1 or CaM. CIB1 or CaM does not alter PDXP phosphatase activity. However, elevated Ca\(^{2+}\) concentrations promote CaM binding and, thereby, diminish CIB1 binding to PDXP, as both interactors bind in a mutually exclusive way. Hence, the PDXP-CIB1 complex may functionally differ from the PDXP-Ca\(^{2+}\)-CaM complex.

Keywords: calmodulin; chronophin; CIB1; haloacid dehalogenase; pyridoxal phosphatase; vitamin B6

Pyridoxal 5'-phosphate (PLP, active vitamin B6) is a versatile cofactor in the catalysis of numerous biochemical transformations [1]. Mammalian PLP-dependent enzymes drive over 140 different chemical reactions in essential processes such as glucose, lipid, amino acid, and neurotransmitter metabolism [2–4]. Sufficient cellular PLP levels are thus critical for a large and important class of enzymes.

In mammals, PLP precursors are taken up by the diet, and PLP is produced by pyridoxal 5'-kinase (PDXK) and pyridox(am)ine 5'-phosphate oxidase (PNPO) [5]. Conversely, intracellular PLP hydrolysis – and thus

Abbreviations
CaM, calmodulin; CIB1, Ca\(^{2+}\)- and integrin-binding protein 1; GABA, \(\gamma\)-aminobutyric acid; HBSS, Hank’s balanced salt solution; PDXP, pyridoxal 5'-phosphate phosphatase; PLP, pyridoxal 5'-phosphate; TEA, triethanolamine.
in vivo. In a mutually exclusive way. PDXP-CIB1 and PDXP-CIB1 binding to PDXP, as both interactors bind PDXP can directly associate with the Ca$^{2+}$- and integrin-binding protein 1 (CIB1) or with calmodulin (CaM) in a mutually exclusive manner. CIB1 or Ca$^{2+}$-CaM complexes may, therefore, be functionally altered ratio of excitatory/inhibitory neurotransmission. The findings suggest that PDXP is involved in the fine-tuning of GABA levels, the underlying molecular mechanisms are currently unknown.

Information on PDXP regulation is very limited and the heat-shock protein Hsp90 is the only direct PDXP interactor that has been reported so far. As a first step toward understanding PDXP-dependent cellular processes, we have screened a brain cDNA library for PDXP-binding partners. Here, we show that PDXP can directly associate with the Ca$^{2+}$- and integrin-binding protein 1 (CIB1) or with calmodulin (CaM) in a mutually exclusive manner. CIB1 or CaM did not alter PDXP phosphatase activity in vitro. However, we found that Ca$^{2+}$ can act as a molecular switch that promotes CaM binding and, thereby, diminishes CIB1 binding to PDXP, as both interactors bind PDXP in a mutually exclusive way. PDXP-CIB1 and PDXP-Ca$^{2+}$-CaM complexes may, therefore, be functionally different. We anticipate that these findings will lay the groundwork for future cellular studies on PDXP-dependent processes in inhibitory neurotransmission.

**Experimental procedures**

**Mammalian and bacterial expression constructs**

Murine PDXP was reverse-transcribed from adult mouse brain cDNA. Total RNA was isolated using TRIzol (Invitrogen/Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer’s instructions, and cDNA was obtained with the High Fidelity RNA PCR Kit (Takara Bio Europe SAS, Saint-Germain-en-Laye, France) and oligo dT primers. The PCR product was subcloned into the BamHI and EcoRI restriction sites of pcDNA3 (Invitrogen) to construct untagged PDXP; into the KpnI and XhoI sites of pENTR3C (Invitrogen), followed by insertion via homologous recombination into pDEST27 (Invitrogen) to produce GST-tagged PDXP for expression in mammalian cells; or into the NcoI and NotI sites of the bacterial expression vector pETM11 (EMBL) to create N-terminally His$^6$-tagged PDXP for in vitro studies. Full-length murine CIB1 was PCR-amplified with native Pfu polymerase (Stratagene/Agilent Technologies, Santa Clara, CA, USA) using pB42AD-CIB1 as a template. CIB1 was inserted into pcDNA3 via EcoRI-XhoI to construct untagged CIB1, and into pGEX-4T1 (GE Healthcare, Munich, Germany) via BamHI-XhoI to generate N-terminally tagged GST-CIB1. The C-terminal CIB1 truncation mutant CIB1-173 was subcloned from pB42AD (Clontech/Takara Bio Europe SAS, Saint-Germain-en-Laye, France) into pGEX-4T1 via EcoRI-XhoI. Murine phosphatase-inactivated PDXP$^{D25N}$ was obtained by standard site-directed mutagenesis using Platinum Pfx Polymerase (Invitrogen). CaM cDNA was a kind gift from Dr Atsushi Miyawaki (Brain Science Institute, Wako City, Saitama, Japan) and was cloned in the BamHI/XhoI sites of pET3a-1 (Novagen/Merck KGaA, Darmstadt, Germany) for expression in Escherichia coli, or subcloned in the HindIII/EcoRI sites of pcDNA3 for mammalian expression. All constructs were verified by sequencing.

**Yeast two-hybrid assays**

Library screening was carried out as reported previously [19], using an adult mouse brain cDNA Matchmaker LexA library (Clontech). PDXP$^{wt}$ or PDXP$^{D25N}$ was subcloned into the EcoRI-SalI sites of the modified bait vector pHisLexA/Zeo. This vector was created by introducing the histidine reporter gene HIS3 into pHybLex/Zeo (Invitrogen). Briefly, pHisLexA/Zeo-PDXP was transformed together with the p8op-LacZ reporter gene plasmid into the yeast strain EGY48, followed by transformation with the adult mouse brain cDNA Matchmaker LexA library (Clontech). Co-transformed yeast cells were screened for their ability to grow on selection plates containing 5-bromo-4-chloro-3-indoyl-$\beta$-D-galactopyranoside (X-gal), but lacking histidine, tryptophan, uracil, and leucine ($-$H/W/L, +X-gal). Positive clones showing preferential growth and color formation on selection plates with galactose plus raffinose compared

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to glucose were identified by sequencing. The CIB1-PDXP interaction was analyzed by co-transforming the indicated constructs into EGY48. Co-transformants were selected and spotted in parallel onto growth plates (+L, −X-gal) and onto selection plates (−L, +X-gal).

Recombinant protein expression and purification

His6-PDXP, GST-CIB1 and CaM were transformed into E. coli BL21(DE3) cells (Stratagene) and expressed for 18 h at 20 °C (PDXP) or 25 °C (CIB1 and CaM) after induction with 0.5 mM isopropyl β-D-thiogalactopyranoside. All purification steps were carried out at 4 °C. Transformed cells were harvested by centrifugation for 10 min at 8000 g and resuspended in the respective lysis buffers supplemented with protease inhibitors (EDTA-free protease inhibitor tablets; Roche, Mannheim, Germany) and 150 U·mL−1 DNase I (Applichem, Renningen, Germany). Cells were lysed using a cell disruptor (Constant Systems Ltd., Daventry, UK), and cell debris was removed by centrifugation for 30 min at 30 000 g.

Untagged PDXP was purified as described [20]. For the purification of recombinant mouse CIB1, bacteria were lysed in 100 mM triethanolamine (TEA), 500 mM NaCl at pH 7.4, and GST-CIB1 was batch-purified on a glutathione sepharose 4B resin (GE Healthcare). After extensive washing with > 25 column volumes of wash buffer (50 mM TEA, 250 mM NaCl; pH 7.4), the GST-tag of column-bound CIB1 was cleaved with thrombin (GE Healthcare, 10 U·mL−1) of glutathione sepharose) for 20 h at room temperature. CIB1 was eluted in wash buffer, concentrated, and further purified on a HiLoad 16/60 Superdex 200 pg gel filtration column operated on an AKTA liquid chromatography system (GE Healthcare) in buffer A (50 mM TEA, 250 mM NaCl, 5 mM MgCl2; pH 7.4). For pulldown experiments with immobilized GST-CIB1 (see below), the GST-tag was not cleaved off.

Recombinant, untagged CaM expressing bacteria were lysed in 100 mM TEA, 1 M NaCl, 10 mM CaCl2 at pH 7.4. Cleared lysates were heat-denatured for 10 min at 80 °C, and insoluble material was pelleted by centrifugation for 30 min at 30 000 g. Cleared supernatants were applied to a phenylsepharose column (Resource PHE; GE Healthcare) in buffer B (50 mM TEA, 1 M NaCl, 10 mM CaCl2; pH 7.4), and CaM was eluted in buffer C (50 mM TEA, 250 mM NaCl, 10 mM EGTA; pH 7.4). CaM was further purified on a HiLoad 16/60 Superdex 200 pg size exclusion chromatography column in buffer A. Pure fractions were pooled, concentrated to a protein concentration of 10–20 mg·mL−1, flash-frozen in liquid nitrogen, and stored at −80 °C.

Generation of α-CIB1 antibodies

CIB1-specific rabbit polyclonal antibodies were generated by Charles River (Sulzfeld, Germany) using purified full-length murine GST-CIB1 as an immunogen in New Zealand white rabbits, and antibodies were purified by affinity chromatography.

Solid-phase binding experiments

Solid-phase interaction assays were conducted essentially as described [21]. Ninety-six-well microtiter plates were coated overnight at 4 °C with 2 µg·well−1 of purified, untagged CIB1 in TMN buffer (40 mM TEA, 30 mM NaCl, 1 mM MgCl2; pH 7.4), and nonspecific binding sites were subsequently blocked with 3% protease-free bovine serum albumin (Serva, Heidelberg, Germany) in TMN buffer for 2 h at room temperature. Wells were incubated for 2 h with purified, untagged PDXP (10 pmol/well), and bound PDXP was detected with rabbit monoclonal α-PDXP antibodies (clone C85E3; Cell Signaling Technology, Frankfurt/Main, Germany). Subsequently, the wells were incubated for 1 h with horseradish peroxidase-conjugated goat α-rabbit IgG (Pierce/ThermoFisher Scientific, Schwerte, Germany). After addition of 3,3′,5′,5′-tetramethylbenzidine (TMB; Calbiochem/Merck KGaA, Darmstadt, Germany) as a horseradish peroxidase substrate, the binding interactions were analyzed continuously by measuring the absorbance at 380 or 650 nm.

For peptide competition experiments, the PDXP peptide (C-GFAGLRAEQFLFSSAL) or the control peptide (C-LALQGFSRSALNAFAG, both custom-synthesized by Anaspec Inc. (Fremont, CA, USA) with > 95% purity and employed at 50–1000 pmol/well) was incubated in TMN buffer with the precoated CIB1 for 1 h. Afterward, purified, untagged PDXP, or purified human PAK1 protein (Biaffin GmbH, Kassel, Germany) was added for 2 h. Wells were incubated with α-PDXP or α-PAK1/2/3 antibodies (both from Cell Signaling Technology), and bound proteins were measured as described above.

Cell culture, pulldown assays, and co-immunoprecipitation

HeLa cells (American Type Culture Collection/LGC Standards GmbH, Wesel, Germany) and HEK293-AD cells (referred to as HEK293 cells; Stratagene) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, 2 mM l-glutamine, 100 U·mL−1 penicillin, and 100 µg·mL−1 streptomycin. For pulldown experiments with isolated proteins, purified GST or GST-CIB1 was incubated with
untagged PDXP (~ 5 µg of each protein) for 2 h at 4 °C under rotation in pulldown buffer (40 mM TEA, 30 mM NaCl, 1 mM MgCl₂, 0.1% Triton X-100, 1 mM β-mercaptoethanol, 1 µg·mL⁻¹ aprotinin, 1 µg·mL⁻¹ leupeptin, 1 µg·mL⁻¹ pepstatin; pH 7.4).

For pulldown experiments with exogenously expressed PDXP, HeLa cells were transfected with 0.2 µg pcDNA3-PDXP or vector DNA and 3 µL of Lipofectamine 2000 in OptiMEM (both Invitrogen) per well of a 6-well plate. After 16 h, cells were scraped in pulldown buffer and lysed by passing through a 24-gauge needle on ice. Unbroken material and cellular debris were removed by centrifugation (12 000 g, 15 min, 4 °C), and the protein concentration of the clarified lysates was determined using the Micro BCA assay (Pierce). For each sample, an aliquot corresponding to 300 µg of total protein was incubated with 5 µg of immobilized GST or GST-CIB1 for 4 h under rotation at 4 °C. For pulldown experiments with endogenously expressed PDXP, one adult mouse brain was minced in pulldown buffer and lysed as described above. Three hundred micrograms of the whole-brain lysate was incubated with 5 µg of immobilized GST or GST-CIB1 for 4 h at 4 °C under rotation. In all cases, the beads were collected by centrifugation and washed three times in pulldown buffer. The associated proteins were resolved by SDS/PAGE and blotted onto nitrocellulose membranes (Hybond C; Amersham/GE Healthcare, München, Germany). PDXP binding to CIB1 was assessed by western blot analysis with PDXP-specific antibodies.

To assess the effect of calcium on the CIB1-PDXP interaction in cells, HEK293 cells were seeded in 10 cm dishes (2–3 × 10⁵ cells/dish). On the next day, cells were transfected with CIB1 (in pcDNA3) or either GST or GST-PDXP (in pDEST27) using TransIT-LT1 (Mirus Bio/MobiTec GmbH, Goettingen, Germany). Twenty hours after transfection, cells were serum-starved for 16 h in DMEM (containing 2 mM Ca²⁺), treated with 5 µM of the calcium ionophore A23187 (Calbiochem) or with the vehicle DMSO for 3 min, briefly rinsed in TMN (30 mM TEA, 150 mM NaCl, 1 mM MgCl₂; pH 7.5) prewarmed to 37 °C, and then lysed by shear stress (10 passages through a 25G × 5/8” syringe) at 4 °C in interaction buffer (TMN containing 1 mM DTT, 5 µg·mL⁻¹ aprotinin, 1 µg·mL⁻¹ pepstatin, 1 µg·mL⁻¹ leupeptin, 1 mM pefabloc, and supplemented with 1 mM CaCl₂ for cells treated with A23187). After centrifugation at 15 000 g for 15 min at 4 °C, the lysates were incubated for 1 h under constant rotation with glutathione beads [pre-equilibrated in interaction buffer supplemented with 3% (w/v) bovine serum albumin] at room temperature. The beads were washed four times with TMN (supplemented with 1 mM DTT ± CaCl₂) using a vacuum manifold; bead-associated proteins were eluted with Laemmli’s sample buffer, and the eluates were analyzed on a 12% SDS/PAGE and immunoblotted against PDXP or CIB1. To analyze the effect of reduced cellular Ca²⁺ levels on the CIB1-PDXP interaction, cells were seeded and transfected as described above. Twenty hours after transfection, cells were washed once in Hank’s balanced salt solution (HBSS). Cells were then incubated for 30 min in HBSS containing 20 µM of the cell-permeable Ca²⁺-chelator BAPTA-AM (Molecular Probes/ThermoFisher Scientific, Schwerte, Germany) or the DMSO solvent control. Cells were rinsed and lysed in TMN as described above, except that the TMN lysis buffer was supplemented with 5 mM EGTA for BAPTA-treated cells, and with 1 mM CaCl₂ for DMSO control-treated cells. Lysates were then processed as described above, and beads were washed as described except that the TMN washing buffer was supplemented with 1 mM DTT and additionally with 5 mM EGTA for BAPTA-treated cells, or with 1 mM CaCl₂ for control-treated cells. Bead-associated proteins were eluted with Laemmli’s sample buffer, and eluates were analyzed on a 12% SDS/PAGE and immunoblotted against PDXP or CIB1.

For co-immunoprecipitation experiments, HEK293 cells were seeded in 3.5-cm wells (4.5 × 10⁵ cells/well) and transfected with untagged murine pcDNA3-PDXP, alone or in combination with untagged murine pcDNA3-CIB1. The amount of transfected plasmid DNA was kept constant with pcDNA3 empty vector. Cells were lysed as described above in interaction buffer (without DTT); lysates were incubated with α-CIB1 antibodies for 1 h at 4 °C, and protein A beads (GE Healthcare) were used for immunoprecipitation.

For pulldown assays with CaM, sepharose-immobilized CaM (GE Healthcare) was equilibrated in HNT buffer (20 mM HEPES, 100 mM NaCl, 0.1% Triton X-100; pH 7.4) supplemented with 5 mM EGTA or 100 µM CaCl₂. PDXP (14.5 ng) and/or CIB1 (100 ng; corresponding to a molar ratio of 1 : 10) were incubated with 50 µL of CaM-sepharose beads for 90 min at 4 °C under rotation. As a bead control, sepharose (GE Healthcare) was inactivated using Tris base. Beads were washed three times in wash buffer (20 mM HEPES, 500 mM NaCl, 1% Triton X-100; pH 7.4), and associated proteins were solubilized in Laemmli’s buffer, separated by SDS/PAGE, and analyzed by probing with CIB1- or PDXP-specific antibodies. For pulldown assays of endogenously expressed CIB1 or PDXP, one rat testis was homogenized in 5 mL of ice-cold HNT buffer supplemented with protease inhibitors (5 µg·mL⁻¹ aprotinin, 1 µg·mL⁻¹ pepstatin, 1 µg·mL⁻¹ leupeptin, 1 mM PMSF) using a Dounce homogenizer.
Unbroken material and cell debris were removed by centrifugation, and 1 mL of the cleared lysate was added to 50 µL CaM-sepharose (or to an equal volume of inactivated sepharose control beads) per reaction. Samples were incubated overnight at 4 °C under constant rotation. Subsequently, beads were washed three times in wash buffer and processed for western blot analysis as described above.

For peptide competition assays, CaM-sepharose was equilibrated in HNT buffer supplemented with 100 µM CaCl2. Fifty microliters of CaM-sepharose or Tris-inactivated sepharose control beads (see above) was preincubated for 60 min at 4 °C under rotation with the indicated concentrations of the PDXP 76-90 peptide or the scrambled control peptide (see above under solid-phase binding experiments). Afterward, PDXP (14.5 ng, or 0.44 pmol) was added for 90 min at 4 °C under rotation. Beads were washed three times in wash buffer supplemented with 100 µM CaCl2, and associated proteins were solubilized in Laemmli’s buffer, separated by SDS/PAGE, and analyzed with PDXP-specific antibodies.

Crosslinking experiments

Five micrometers of PDXP was incubated for 2 h at 37 °C with 10 µM of CIB1 and/or 10 µM of CaM (all proteins were untagged) in a total volume of 15 µL in buffer A supplemented with 50 µM EGTA ± different concentrations of CaCl2 and 0.67% formaldehyde. Crosslinking was stopped by the addition of Laemmli’s sample buffer, and proteins were resolved by SDS/PAGE and stained with Coomassie brilliant blue. Band intensities were analyzed densitometrically using the software IMAGEJ, version 1.44 (http://rsb.info.nih.gov/ij/).

In vitro phosphatase assays

One hundred nanomolar of PDXP was preincubated in the absence or presence of 1 µM CIB1 or 1 µM CaM (all proteins were untagged) for 10 min at 22 °C in buffer A supplemented with 0.001% Triton X-100 and 1 mM EGTA or 100 µM CaCl2 at pH 7.4. The reactions were started by the addition of PLP (final concentrations ranging from 0 to 500 µmol L⁻¹ in a total volume of 50 µL) and stopped after 2 min by the addition of 100 µL Biomol Green (Enzo Life Sciences GmbH, Lörrach, Germany). Color was allowed to develop for 10 min and the absorbance of the resulting phosphomolybdate complex was read at 620 nm on an Envision 2104 multilabel microplate reader (Perkin Elmer, Rodgau, Germany). Free phosphatase release was quantitated using phosphate standard curves, and $v_{max}$ and $K_m$ were calculated using GRAPHPAD PRISM, version 4.01-GraphPad Software, San Diego, CA, USA.

Statistical analysis

Statistically significant differences were analyzed using Student’s unpaired, two-tailed $t$-tests, using GRAPHPAD PRISM version 7.05. Statistically significant differences in the effect of Ca²⁺ on CIB1-PDXP or CaM-PDXP-complex formation were analyzed with the Mann–Whitney two-tailed test, using GRAPHPAD PRISM version 4.01.

Results

Identification of CIB1 as a PDXP interactor

PDXP is a small phosphatase of the haloacid dehalogase (HAD) superfamily [8,22] that possesses no apparent protein–protein interaction domains. To identify candidate PDXP interacting proteins, we screened a mouse brain cDNA library using the yeast two-hybrid system. Sixteen different positive clones were obtained, including the previously reported PDXP-binding partner Hsp90 [18] (Table S1). Two full-length open reading frames coded for the CIB1, a potentially novel PDXP-binding partner. CIB1 interacted equally well with PDXPwt and with the phosphatase-inactive point mutant PDXP D25N [10], indicating that the enzymatic activity of PDXP did not affect the interaction (Fig. 1A). CIB1 is an EF hand-containing, broadly expressed protein that can modulate the function and the subcellular localization of diverse effectors, including a number of kinases and the phosphatase calcineurin [23–26]. In the brain, CIB1 has been implicated in the regulation of synaptic plasticity [27] and the protection against stress-induced neurotoxicity [25,28]. Because of these links to physiological and pathological neuronal processes, we decided to characterize the potential CIB1-PDXP interaction in further detail.

To test for a direct interaction between CIB1 and PDXP, we first attached CIB1 to an ELISA plate and incubated with increasing amounts of purified, recombinant PDXP. This type of solid-phase binding assay has previously been used to characterize other CIB1 interacting proteins [21,29]. We found that PDXP directly bound to CIB1 in a concentration-dependent and saturable manner (Fig. 1B). Subsequently, we performed pulldown experiments with recombinant, purified GST-CIB1 and untagged PDXP. Purified GST was used as a negative control. As shown in the upper panel of Fig. 1C, PDXP bound to GST-CIB1, but not to GST alone. By densitometrically quantifying the western blots, we estimated the fraction of PDXP bound to

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PDXP complex formation with CIB1 or Ca\(^{2+}\)-CaM

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CIBI. With purified proteins, we found a molar ratio of ~ 2.5 × 10⁻³ bound PDXP/total CIB1. We next conducted similar GST-CIB1 versus GST pulldown experiments with lysates obtained from HeLa cells expressing exogenous PDXP (Fig. 1C, middle panel), and with murine whole-brain extracts containing endogenously expressed PDXP (Fig. 1C, bottom panel). In both cases, we observed that cellular PDXP specifically bound to immobilized CIB1. In HeLa cell lysates (containing ~ 415 times less PDXP than the reactions in Fig. 1C with purified proteins), we found a molar ratio of ~ 7.1 × 10⁻⁵ bound PDXP/total CIB1. In brain whole-cell lysates (containing ~ 35 times less PDXP than the reactions in Fig. 1C with purified proteins), we found a molar ratio of ~ 4.6 × 10⁻⁴ bound PDXP/total CIB1. Finally, we tested whether PDXP and CIB1 were also able to interact when both proteins were untagged. HEK293 cells expressing PDXP and/or CIB1 were subjected to immunoprecipitation using a rabbit polyclonal α-CIB1 antibody. We found that untagged PDXP indeed associated with untagged CIB1 (Fig. 1D). Taken together, these results clearly demonstrate a direct interaction between CIB1 and PDXP.

CIB1 contains four EF hands, and its two C-terminal EF hands bind either Mg²⁺ or Ca²⁺ (EF-III, K_D for Mg²⁺: 120 µM; K_D for Ca²⁺: 1.9 µM), or only Ca²⁺ (EF-IV, K_D 0.5 µM) [30]. Metal binding contributes to CIB1 folding, and can thereby affect ligand binding [31,32]. While some CIB1 effectors such as the p21-activated kinase PAK1 show an increased association with CIB1 in the presence of elevated [Ca²⁺] [21], a rise in [Ca²⁺] reduces binding of the apoptosis signal-regulating kinase ASK1 to CIB1 [25]. Interactors such as α1B integrin bind CIB1 in a Ca²⁺-independent manner [33–35]. To test the potential effect of reduced or elevated intracellular Ca²⁺ levels on the CIB1-PDXP association, we treated HEK293 cells expressing GST-PDXP and CIB1 with the Ca²⁺-chelator BAPTA-AM (Fig. 1E) or with the Ca²⁺-ionophore A23187 (Fig. 1F) and compared the amounts of PDXP-associated CIB1 in GST-sepharose pulldowns. We found that BAPTA treatment tended to increase the amount of CIB1 in GST-PDXP pulldowns, although this difference was not statistically significant (fold increase of CIB1-PDXP interaction in BAPTA-AM-treated cells compared to control cells: 2.01 ± 0.59, mean values ± SEM; n = 3, P = 0.163, unpaired t-test). Likewise, we did not detect pronounced changes in the amounts of PDXP-bound CIB1 when cells were treated with A23187 (fold increase of CIB1-PDXP interaction in A23187-treated cells compared to control cells: 0.83 ± 0.07, mean values ± SEM; n = 3, P = 0.068, unpaired t-test). These data suggest that the CIB1-PDXP association is largely independent of Ca²⁺ concentrations.

**Mapping of the CIB1-PDXP interaction domains**

We next set out to identify the domains on PDXP and CIB1 that are involved in their reciprocal binding and first tested the interaction of N- and C-terminal PDXP truncation mutants with full-length CIB1 in yeast (Fig. 2A). Figure 2B shows that deletion of the first 80 N-terminal residues of PDXP, which encompass the catalytically essential active site motifs I and II of the phosphatase [10,22], had no detectable effect. However, deletion of five additional amino acids in PDXP85–292 abolished the CIB1-PDXP interaction. Consistent with this result, the deletion of more than two-thirds of the PDXP C terminus up to amino acid 85 was compatible with a CIB1-PDXP interaction. The deletion of five additional amino acids (PDXP1–80) reduced the CIB1-PDXP interaction, and the removal of the PDXP C terminus up to amino acid 75 (PDXP1–75) resulted in the loss of a detectable interaction. Together, these data suggest that the region encompassing amino acids 75–
85 in PDXP plays an important role in CIB1-PDXP binding. This region consists of a loop and helix H4, is surface-exposed, and located on the back of the active site entrance (Fig. 2C). To validate the mapping results, we tested the effect of a 15-amino acid peptide corresponding to residues 76–90 in PDXP on the interaction of full-length, purified CIB1 and PDXP proteins in solid-phase binding assays. This peptide (PDXP76–90) comprises the mapped CIB1 binding site together with an adjacent strand of β-sheet A and the first three residues of helix H5. Figure 2D shows that PDXP76–90 efficiently competed with CIB1 binding to PDXP. This competition was specific, because it was neither observed in the presence of a scrambled control peptide, nor did PDXP76–90 impair the CIB1-PAK1 interaction [21].

Crystal [32,36] and solution structures of CIB1 [31,34] show a compact α-helical protein with extended N- and C-terminal regions, and a hydrophobic channel that is thought to be involved in the binding of CIB1 interactors (Fig. 3A). In the absence of a binding partner, this hydrophobic cleft is occluded by helix H10 at the extreme CIB1 C terminus (amino acids 180–191). Studies with a peptide derived from the high-affinity CIB1 interactor αIIb integrin have demonstrated that the peptide displaces H10, leading to an increased accessibility of the hydrophobic cleft for target binding [37]. In contrast, CD38 (an enzyme that metabolizes cyclic ADP-ribose) can interact with the CIB1 N terminus [29].

To define the CIB1 domain involved in PDXP binding, we first analyzed the interaction of CIB1 C-terminal truncations with PDXP in yeast (Fig. 3B). Figure 3C shows that deletion of H10 (designated H10D in the secondary structure plot) in CIB11–178 had little effect, whereas the deletion of five additional residues (CIB11–173) strongly diminished the CIB1-PDXP interaction. A complete loss of the CIB1-PDXP interaction was observed upon truncation of the high-affinity Ca2+-binding EF hand IV [30], located in between amino acids 161 and 172 (CIB11–168). These data suggest that the C terminus of CIB1 comprises the PDXP-binding region. To test this further, we measured the CIB1-PDXP association in solid-phase binding assays. Consistent with the results obtained in yeast, the binding of PDXP to immobilized full-length CIB1 increased proportionally with the quantity of PDXP until reaching saturation, whereas PDXP showed only residual binding to immobilized CIB11–173 (Fig. 3D). We conclude that the surface-exposed, C-terminal helices H9 and H10 of CIB1 (corresponding to H12 and H13 in the secondary structure plot in Fig. 3B) are important for PDXP binding (Fig. 3E).

Identification of calmodulin as a binding partner of PDXP and CIB1

CIB1 is the closest structural homolog of calcineurin B and calmodulin (CaM), two Ca2+-binding proteins that function as regulatory components of calcineurin A [32]. Calcineurin is a heterodimer, composed of the catalytic A subunit (calcineurin A) that is constitutively bound to the regulatory B subunit (calcineurin B). Ca2+ binding to calcineurin B results in a small activation of the enzyme, and this basal activity is strongly stimulated by Ca2+-CaM binding to calcineurin A [38].

Because PDXP can interact with the calcineurin B homolog CIB1 (albeit in a transient and substoichiometric manner; see Fig. 1C), we asked whether PDXP might also bind CaM. Primary sequence analysis of murine PDXP using the Calmodulin Target Database (calmodulin.uhnres.utoronto.ca/ctdb/ctdb/home.html) identified a putative CaM binding motif of the 1–10 subclass with bulky hydrophobic residues spaced eight amino acids apart (FAGLRAEQFL [Fig. 3A]). Interestingly, the predicted CaM binding site overlaps with the mapped CIB1 binding site on PDXP (amino acids 75–85, see Fig. 2A). We also noted a potential

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**Fig. 2.** Mapping of the CIB1 binding site on PDXP. (A) Secondary structure plot for murine PDXP (PDB entry 4BX3), generated by DSSP [47]. The PDXP N terminus comprising amino acids (a.a.) 1–126 is shown enlarged below the full-length structure plot. Helices are labeled H1–H14, and β-strands are labeled A–D according to the β-sheet they belong to. Motifs: β, β turn; γ, γ turn; β, β hairpin. Disulfide bond. Residue contacts: red to ligand; black to metal. The position of the active site motifs I and II is indicated below the amino acid sequence of the enlarged structure plot. The thick blue lines represent the generated PDXP constructs that were tested in (B); numbers indicate amino acids. (B) Interaction site mapping in yeast. Full-length murine PDXP or truncation mutants were co-transformed with murine CIB1. For each condition, replicates of three different yeast colonies plated on growth or selection medium are shown. (C) CIB1 binding site on PDXP (PDB entry 4BX3). Light gray, PDXP catalytic core domain; dark gray, capping domain; red, CIB1 binding site (a.a. 75–100 peptide, but not of a scrambled peptide, compete with PDXP binding to immobilized CIB1. The PDXP76–90 peptide does not inhibit the CIB1-PAK1 interaction, which was tested in the presence of 100 µM added Ca2+. Please note that the fourth data point for the CIB1-PAK1 experiment corresponds to a molar ratio of 1 : 1600. Data are mean values ± SD, n = 3.
CaM binding motif of the 1-8-14 subclass with bulky hydrophobic residues spaced six and five amino acids apart in CIB1 (\textsuperscript{21}FLTKQEILLAHRRF; Fig. S1B,C).

We next performed pulldown assays with purified CaM, PDXP and CIB1 in order to test the potential association of PDXP and CIB1 with CaM.
PDXP complex formation with CIB1 or Ca\(^{2+}\)-CaM

Ca\(^{2+}\) triggers PDXP complex formation with CaM

CaM is a prototypical Ca\(^{2+}\) sensor, and proteins with 1-10 and 1-8-14 motifs such as those present in PDXP and CIB1 primarily bind CaM in a Ca\(^{2+}\)-dependent manner [39]. To investigate the role of Ca\(^{2+}\) for the binding of CaM to PDXP and CIB1, we assayed the association of purified PDXP and CIB1 with CaM-sepharose under Ca\(^{2+}\)-free conditions and in the presence of 100 \(\mu\)M Ca\(^{2+}\). We observed that the PDXP-CaM association increased, whereas the CIB1-CaM association diminished in the presence of elevated [Ca\(^{2+}\)]. Likewise, when all three proteins were present, CIB1 competed with PDXP binding to CaM in the absence, but not in the presence of elevated [Ca\(^{2+}\)] (Fig. 4C).

To experimentally test whether the predicted CaM binding site (Fig. S1) indeed overlaps with the mapped CIB1 interaction site on PDXP, we employed the PDXP peptide PDXP\(^{76-90}\) (Fig. 2D). Figure 4D shows that the PDXP\(^{76-90}\) peptide reduced PDXP binding to CaM-sepharose beads. Densitometric analysis showed that PDXP\(^{76-90}\) reduced the Ca\(^{2+}\)-CaM-PDXP binding by almost 30% (relative change in Ca\(^{2+}\)-CaM-PDXP binding compared to scrambled peptide: 0.72 \pm 0.01, data are mean values \pm SEM in \(n = 3\) independent experiments; \(P < 0.0001\), Student’s unpaired \(t\)-test).

Higher peptide concentrations such as 10 \(\mu\)M PDXP\(^{76-90}\) did not lead to a further reduction of Ca\(^{2+}\)-CaM-PDXP sepharose binding (up to 40 \(\mu\)M PDXP\(^{76-90}\) were tested with similar results). These data support the predicted 1–10 CaM binding motif, although additional CaM binding sites on PDXP may exist. Taken together, our observations suggest that CIB1, PDXP and CaM can interact in a reciprocal manner in the absence of Ca\(^{2+}\), and that elevated [Ca\(^{2+}\)] favors Ca\(^{2+}\)-CaM-PDXP complex formation. We have, however, no evidence for the existence of a ternary CaM-CIB1-PDXP complex analogous to the calcineurin holoenzyme.

To further characterize the associations of CIB1 or CaM with PDXP, we performed chemical crosslinking experiments with formaldehyde. Formaldehyde has a short (~2–3 Å) crosslinking span. This enables the detection of protein–protein interactions with high specificity, provided that suitable amino acid residues are present and that formaldehyde can access the binding interface [40]. As shown in Fig. S2, the incubation of the individual proteins with formaldehyde did not cause nonspecific protein aggregation. Although PDXP exists as a homodimer [6–7,20,41], this dimerization may not be seen under the conditions of our experiments, and proteins with formaldehyde did not cause nonspecific protein aggregation. Although PDXP exists as a homodimer [6–7,20,41], this dimerization was not destabilized by formaldehyde. Figure 5A shows that CIB1-PDXP complexes were readily detectable after crosslinking. CIB1-PDXP binding appeared to be moderately enhanced by the addition of Ca\(^{2+}\), but this trend was not statistically significant. These results are consistent with the pulldown experiments shown in Fig. 1E,F, which indicate that CIB1 and PDXP can interact in the absence of Ca\(^{2+}\), and that this interaction is not markedly increased in the presence of elevated Ca\(^{2+}\) levels. In contrast, CaM-PDXP crosslinking was barely detectable in the absence of added Ca\(^{2+}\), but increased several-fold in the presence of elevated [Ca\(^{2+}\)]. The same results were obtained when a mixture of CIB1, CaM and PDXP was incubated with formaldehyde in the absence or presence of Ca\(^{2+}\), in line with the pulldown experiments shown in Fig. 4C.
Solid phase binding (OD\textsubscript{380})

PDXP (\mu g)

1 15 25 35 70

1–128 1–160 1–173 1–178

PDXP CIB1 WT

CIB1, a.a.:

Selection medium

Growth medium

CIB1, a.a. 1–191

CIB1 1–173

kDa

CIB1 WT

CIB1 1–173

Control

Selection medium

Growth medium

EF III

EF IV

1–191 1–178 1–173 1–128 1–160

PDXP CIB1 WT CIB1 1–173

Solid phase binding (OD\textsubscript{380})

PDXP (\mu g)

0 0.5 1.0 1.5 2.0 2.5

0 0.2 0.4 0.6 0.8 1.0 1.2

E. Jeanclos et al. PDXP complex formation with CIB1 or Ca\textsuperscript{2+}-CaM
Finally, we examined the CIB1-PDXP and CaM-PDXP crosslinking efficiencies in the presence of all three proteins at different [Ca\(^{2+}\)]. Figure 5B shows that [Ca\(^{2+}\)] above 20 \(\mu\)M were required to favor CaM-PDXP over CIB1-PDXP complex formation. We note that in the presence of CIB1, the Ca\(^{2+}\)-induced increase in CaM-PDXP complex formation was attenuated from \(~7\) fold to \(~2\) fold compared to CaM-PDXP crosslinking in the absence of CIB1 (see quantifications in Fig. 5A,B). These data are in agreement with the observation that CIB1 and Ca\(^{2+}\)-CaM can compete for PDXP binding (Fig. 4C). Hence, PDXP can engage in complex formation with CIB1 or with CaM, and the latter association is preferred in the presence of [Ca\(^{2+}\)] above 20 \(\mu\)M. [Ca\(^{2+}\)] exceeding 20 \(\mu\)M indeed occurs physiologically in the brain, for example, in the nanodomains of presynaptic Ca\(^{2+}\) channels upon channel opening [42].

**Fig. 4.** Identification of CaM as a PDXP and CIB1 binding partner. Pulldown binding assays with immobilized CaM. (A) Purified PDXP and/or CIB1 (molar ratio PDXP : CIB1 = 1 : 10) were incubated with CaM-sepharose or sepharose control beads. Bead-associated PDXP and CIB1 were detected by immunoblotting. The input is a fraction of the PDXP + CIB1 mixture. Note that excess CIB1 competes with PDXP binding to CaM (compare lanes 3 and 7). (B) Rat testis whole-cell lysates (WCL) were incubated with either CaM-sepharose or control beads. (C) Effect of added Ca\(^{2+}\) on the association of purified PDXP and CIB1 with CaM. Note that the Ca\(^{2+}\)-dependent increase of PDXP binding to CaM occurs even in the presence of a 10-fold molar excess of CIB1 (compare lanes 1 and 2 with lanes 5 and 6). (D) The PDXP\(^{76-90}\) peptide competes with PDXP binding to CaM-sepharose. All blots are representative of \(n = 3\) experiments.
Fig. 5. Ca^{2+} regulates PDXP complex formation with CIB1 or CaM. Crosslinking experiments with purified, untagged PDXP (5 μM), CIB1 and CaM (10 μM each) were performed using 0.67% formaldehyde. (A) Left panel, Crosslinking experiments in the absence or presence of ~25 μM Ca^{2+}. Proteins were separated on a 12% SDS/PAGE and stained with Coomassie brilliant blue. MWM, molecular weight marker. The positions of the individual proteins and protein complexes are indicated. Right panel, Densitometric analysis of PDXP-CIB1 and PDXP-CaM complexes when crosslinking reactions were performed with PDXP and CIB1, or with PDXP and CaM. Data represent mean values ± SEM; n = 6. Differences were analyzed with the Mann–Whitney two-tailed test. (B) Effect of different [Ca^{2+}] on crosslinking efficiencies. Left panel, Proteins were resolved on a 15% SDS/PAGE and stained with Coomassie brilliant blue. Note that CaM migrates as multiple bands at low [Ca^{2+}]. Right panel, Densitometric analysis of PDXP-CIB1 and PDXP-CaM complex formation when crosslinking reactions were performed with PDXP, CIB1, and CaM. The total complex formation represents the aggregate of the two binary complexes. To test for statistically significant differences, the effects of different [Ca^{2+}] on PDXP-CIB1 or PDXP-CaM complex formation were compared to the conditions without added [Ca^{2+}]. **P < 0.005; *P < 0.05. Data are mean values ± SEM; n = 3.
CIB1 and CaM do not affect PDXP phosphatase activity in vitro

CIB1 can directly stimulate or inhibit the activities of kinases such as PAK1 [21] or ASK1 [25], and CaM is a well-known activator of Ca^{2+}-CaM-dependent kinases and of the phosphatase calcineurin [43,44]. We therefore examined the potential impact of CIB1 or CaM on the phosphatase activity of PDXP. Using recombinant, purified proteins and pyridoxal...
5’-phosphate (PLP) as the physiological PDXP substrate [20], we found that CIB1 and/or CaM had little or no effect on PDXP activity (Table 1). Although the presence of 100 μM Ca²⁺ led to a slight overall increase in the $k_\text{on}$ values and to a decrease in the $k_\text{cat}$ values compared to Ca²⁺-free conditions, this effect can be explained by a competition between the essential cofactor Mg²⁺ and catalytically inert Ca²⁺ ions [45]. Thus, the interaction of CIB1 or CaM with PDXP did not alter its phosphatase activity in vitro.

**Discussion**

In this work, we report the identification and biochemical characterization of CIB1 and Ca²⁺-CaM as novel PDXP interactors. CaM is a well-characterized activator of the Ca²⁺-dependent phosphatase calcineurin. Interestingly, although the catalytic subunits of calcineurin (calcineurin A) and PDXP are unrelated, they both associate with structurally similar proteins, that is, with calcineurin B or CIB1. Despite this resemblance, there are important differences in the composition and regulation of the two (holo)enzymes. First, calcineurin A and B subunits form a constitutive heterodimer with basal phosphatase activity. In contrast, the association of PDXP and CIB1 is of low affinity, and PDXP is active in the absence of CIB1 in vitro (see below). Second, the binding of Ca²⁺-CaM to calcineurin A results in a full activation of the heterotrimeric enzyme, due to the displacement of an autoinhibitory domain from the catalytic cleft of calcineurin A [38-39,43]. In contrast, Ca²⁺-CaM binding to PDXP was competitive with CIB1 and did not lead to a further activation of the enzyme. Consistent with this finding, no autoinhibitory domains are evident in any of the eight available human or murine PDXP crystal structures (PDB entries 2P27, 2OYC, 2P69, 5AES, 5GYN, 4BX0, 4BX2, 4BX3). Hence, we have no indications for the existence of a PDXP-CIB1-Ca²⁺-CaM holoenzyme analogous to the calcineurin A-calcineurin B-Ca²⁺-CaM heterotrimer. Our results rather suggest that PDXP transiently associates with either CIB1 or Ca²⁺-CaM, as both interactors bind in a mutually exclusive way. As elevated Ca²⁺ levels promote CaM binding to PDXP and concomitantly diminish CIB1 binding to PDXP, Ca²⁺ appears to function as a molecular switch that can determine the composition of the PDXP complex (Fig. 6). This finding also suggests the possibility that PDXP-CIB1 and PDXP-Ca²⁺-CaM complexes are functionally different.

We did not observe a stimulatory or inhibitory effect of CIB1, CaM, or Ca²⁺-CaM on the phosphatase activity of PDXP in vitro. Still, it is conceivable that PDXP association with CIB1 or Ca²⁺-CaM affects the subcellular distribution of the phosphatase and thereby directs its activity to a particular compartment or nanodomain. CIB1 is a myristoylated protein that can be targeted to intracellular membranes or to the plasma membrane via its lipid anchor [23]. CIB1 membrane association has been reported to alter the activity of some of its interaction partners. For example, the analysis of CIB1-deficient mouse hearts showed that CIB1 is required to anchor calcineurin B at the sarcolema [24]. Whereas recombinant, purified CIB1 did not directly induce calcineurin phosphatase activity in vitro, CIB1 enhanced cellular calcineurin-NFAT activity through a membrane localization-dependent mechanism [24]. It remains to be elucidated if CIB1 (or Ca²⁺-CaM) binding can similarly influence PDXP phosphatase activity in cells.

Further studies on cellular functions of PDXP will likely benefit from the development of experimental tools that allow the visualization of PDXP, CIB1, and CaM at high resolution, ideally at endogenous expression levels, and in living cells. In addition, the development of PLP biosensors to measure PLP levels at subcellular resolution could provide insights into the local regulation of this important cofactor. Exciting progress in this direction has recently been reported [46].

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**Author contributions**

EJ, GK, HS, and AG conceived and designed experiments. EJ, GK, AH, OF, AO, and A-KL performed experiments. EJ, GK, HS, and AG analyzed the data. AG wrote the paper.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Results of a binding site search in the Calmodulin Target Database.

Fig. S2. Crosslinking experiments with PDXP, CIB1 and CaM.

Table S1. Yeast two-hybrid hits obtained by screening a mouse brain library with murine PDXP<sup>D255N</sup> as a bait.