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Mapping the Ca^{2+}-dependent binding of an invertebrate homolog of protein phosphatase 4 regulatory subunit 2 to the small EF-hand protein, calsensin

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Abstract

The EF-hand family of calcium-binding proteins regulates cellular signal transduction events via calcium-dependent interactions with target proteins. Here, we show that the COOH-terminal tail of the leech homolog of protein phosphatase 4 regulatory subunit 2 (PP4-R2) interacts with the small neuronal EF-hand calcium-binding protein, Calsensin, in a calcium-dependent manner. Using two-dimensional NMR spectroscopy and chemical shift perturbations we have identified and mapped the residues of Calsensin that form a binding surface for PP4-R2. We show that the binding groove is formed primarily of discontinuous hydrophobic residues located in helix 1, the hinge region, and helix 4 of the unicornate-type four helix structure of Calsensin. The findings suggest the possibility that calcium-dependent modulation of phosphatase complexes through interactions with small calcium-binding proteins may be a general mechanism for regulation of signal transduction pathways.

Keywords: NMR; Calcium-binding protein; Calsensin; Binding surface; Leech; Protein phosphatase regulation

1. Introduction

Intracellular calcium concentration regulates a variety of cellular processes and many of these signal transduction events are mediated by members of the EF-hand family of calcium-binding proteins via interaction with target proteins in a calcium-dependent manner [1]. We have previously cloned and characterized such a neuronal EF-hand Ca^{2+}-binding protein, Calsensin [2,3]. Calsensin is expressed in a subset of peripheral sensory neurons fasciculating into a single axon tract in the leech central nervous system [2,3]. Furthermore, NMR spectroscopic analysis has demonstrated that the structure of Calsensin consists of four helices forming a unicornate-type four-helix bundle and that the third helix is important for calcium-induced conformational changes [4]. Thus, the molecular features of Calsensin and its restricted expression in the nervous system are consistent with the hypothesis that it may participate in protein-complex mediated calcium-dependent signal transduction events in neurons [3]. However, the molecular nature of such potential complexes had not been determined. Therefore, to identify proteins that interact with Calsensin we carried out overlay screens with a GST-Calsensin fusion protein. Here we report that Calsensin interacts with the leech homolog of the protein phosphatase 4 regulatory subunit, PP4-R2.

Protein phosphatase 4 (PP4; also referred to as PPP4 or PPX) is a member of the PP2A subfamily of serine/threonine phosphatases that is ubiquitously expressed in eukaryotes [5]. PP4 is very highly conserved from mammals to *Drosophila* with 91% identity on the amino acid level [6] and is an essential enzyme required for organization of microtubules and centrosomes [7,8]. Recently, PP4 also has been found to interact with the survival motor neurons complex affecting the temporal

Abbreviations: PP4-R2, Protein phosphatase 4 regulatory subunit 2; PP4, Protein phosphatase 4; NMR, Nuclear magnetic resonance; GST, Glutathione-S-transferase; EDTA, Ethylenediaminetetraacetic acid; EGTA, Ethyleneglycol-bis (b-aminoethyl)-*N,N,N′,N′*-tetraacetic acid; HRP, Horseradish peroxidase; HSQC, Heteronuclear single quantum coherence; ORF, Open reading frame; TBS, Tris buffered saline; DAB, Diamino benzidine; RACE, Rapid amplification of cDNA ends; SMN, Survival motor neuron; SnRNPs, Small nuclear ribonucleoproteins; PMSF, Phenylmethylsulfonyl fluoride; Immunoprecipitation; Ip, mAb; Monoclonal antibody; PAb, Polyclonal antiserum

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localization of snRNPs [9]. Furthermore, PP4 has been shown to play an important role in apoptosis, TNF-α signaling, as well as in activation of c-Jun N-terminal kinase and NF-κB [10–13]. So far three regulatory subunits have been identified for PP4: α4, PP4-R1, and PP4-R2 [14–18]. Thus, it is likely that PP4 participates in many essential cellular process and that the activity of its catalytic domains is controlled by regulatory subunits forming complex holoenzymes [19,20].

In this study, we show that the COOH-terminal tail of leech PP4-R2 interacts with Calsensin in a calcium-dependent manner. Furthermore, using two-dimensional NMR spectroscopy we have identified and mapped the residues of Calsensin that form a binding surface for PP4-R2. These results indicate the possibility that the function of the PP4 molecular complex may be regulated by changes in intracellular calcium through interactions with EF-hand calcium-binding proteins.

2. Materials and methods

2.1. Identification and molecular characterization of leech PP4-R2

To identify potential direct interacting partners of Calsensin we used a purified full-length Calsensin-GST fusion protein previously described by Venkitaramani et al. [4] to screen a Haemopis marmorata expression library. The oligo-dT primed λgt10 *Eco*RI–*Not*I directional cDNA library [21] was plated at a density of 30,000 pfu per 150 mm plate and a total of 106 plaques were screened essentially according to the procedures of Sambrook and Russell [22]. The resulting nitrocellulose filters were blocked with 5% dry non-fat milk in TBS (blotto) for 1 h and incubated overnight with 10 μg/ml of GST-Calsensin fusion protein in blotto (blotto contains approximately 1 mg/ml of calcium) at 4 °C and washed 3 times with TBS containing 0.2% Tween-20. Positive clones binding Calsensin-GST were identified by immunoreactivity with the Calsensin specific mAb Lan3–6 [3], plaque purified, and the phage DNA isolated and subcloned into pBluescript KS+/− vectors according to the method provided by the manufacturer (Stratagene). DNA sequencing of the inserts was performed by the Iowa State University DNA Sequencing and Synthesis Facility. The identified sequences were compared with known and predicted sequences using the National Center for Biotechnology Information BLAST e-mail server. Additional NH_2-terminal sequence for one of the clones with homology to vertebrate PP4-R2 [17] was obtained using the Ambion 5′ RLM-RACE kit and protocols. Alignments used to produce maximum parsimony trees were generated with the Clustalw version 1.7 program and encompassed the entire PP4-R2 sequences. Trees were constructed by maximum parsimony using the PAUP computer program version 4.0b [23] on a Power Macintosh G4. All trees were generated by heuristic searches and bootstrap values in percent of 1000 replications are indicated on the bootstrap majority rule consensus tree.

2.2. Biochemical analysis and antibody generation

SDS-PAGE was performed according to standard procedures [24]. Electroblot transfer was performed as in Towbin et al. [25] with transfer buffer containing 20% methanol and in most cases including 0.04% SDS. Immunoblots were labeled with PP4-R2 mAb 15F4 (see below), with the Calsensin mAb Lan3–6 or with the Calsensin specific antiserum Frigg [3], with anti-GST antibody [26], or with commercially obtained anti-biotin antibody (Cell Signaling Technologies). For these experiments, we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 μm nitrocellulose, and using anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody diluted 1:1000 in Blotto. The signal was visualized using chemiluminescent detection methods (ECL kit, Amersham). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680).

A full-length biotinylated Calsensin fusion protein (biot-Calsensin) was generated by inserting the entire open reading frame of Calsensin in frame into the Xa3-Pinpoint vector (Promega). Biotinylated and GST fusion proteins of leech PP4-R2 sequence corresponding to the COOH-terminal amino acids 153–423 identified as interacting with Calsensin in the library screen were subcloned into the pGEX4T3 and the Xa3-Pinpoint vectors, respectively, to generate the constructs GST-PP4-R2 and biot-PP4-R2. The three fusion-proteins were expressed in XL1Blue or BL21 host strains and purified according to the manufacturer's protocols. Recombinant full-length Calsensin was purified as described below and in Venkitaramani et al. [4].

For antibody generation the GST-PP4-R2 fusion protein was expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column (Sigma-Aldrich), according to the pGEX manufacturer's instructions (Amersham Pharmacia Biotech). The mAb 15F4 was generated by injection of 50 μg of GST-PP4-R2 into BALB/c mice at 21 d intervals. After the third boost, mouse spleen cells were fused with Sp2 myeloma cells and monospecific hybridoma lines were established as previously described [26]. All procedures for mAb production were performed by the Iowa State University Hybridoma Facility.

Pulldown experiments: biot-Calsensin (2 μg) and biot-PP4-R2 (2 μg) fusion proteins were coupled to streptavidin beads (Pierce) and used for pulldown assays of 2 μg GST-PP4-R2 and 2 μg recombinant Calsensin, respectively, in the presence (150 mM NaCl, 1 mM $CaCl_2$, 0.2% Triton-X-100, 0.2% NP-40, 25 mM Tris–HCl, pH 7.4, and containing the protease inhibitors PMSF and aprotinin) or absence of calcium (150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton-X-100, 0.2% NP-40, 25 mM Tris—HCl, pH 7.4, and containing the protease inhibitors PMSF and aprotinin). The GST-PP4-R2 and Calsensin proteins were pre-cleared by incubation with streptavidin beads before incubation with the streptavidin beads coupled to the respective biotinylated fusion proteins or with the beads alone overnight at 4 °C on a rotating wheel. The beads were washed 3 times for 10 min each in 1 ml of the appropriate calcium-free or calcium containing buffer, and proteins retained on the streptavidin beads were analyzed by SDS-PAGE and immunoblotting.

For immunoprecipitation experiments from leech nerve cord homogenate, 10 μl of mAb Lan3–6 was coupled to 30 μl protein-G Sepharose beads (Sigma) for 2.5 h at 4 °C on a rotating wheel in 50 μl immunoprecipitation (ip) buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.1% Triton X-100, 0.1% Nonidet P-40, 1 mM Phenylmethylsulfonyl fluoride, and 1.5 μg Aprotinin). Subsequently the antibody-coupled beads or beads-only were incubated overnight at 4 °C with 200 μl of dissected Haemopis nerve cord homogenate as previously described [3] on a rotating wheel. Beads were washed 3 times for 10 min each with 1 ml of ip buffer with low speed pelleting of beads between washes. The resulting bead-bound immunocomplexes were analyzed by SDS-PAGE and immunoblotting. In some experiments, to test for the specificity of the 15F4 mAb, 15 μg of the biot-PP4-R2 fusion protein was added to the diluted antibody solution before staining.

2.3. Preparation of samples for NMR spectroscopy

Purified ^{15}N-labeled Calsensin was obtained as described previously by Venkitaramani et al. [4]. *Escherichia coli* strain BL21 (DE3) was transformed with a full-length Calsensin-GST construct and the cultures grown in modified M9 medium supplemented with 1 mM $CaCl_2$ and 100 μg/ml ampicillin. For ^{15}N- labeled protein preparations, the modified M9 minimal media contained ^{15}N-enriched ammonium chloride [1 g/l, Cambridge Isotope Laboratories] as the sole nitrogen source. Induced cells were harvested by centrifugation 12 h post-induction. The cell pellets were resuspended in 50 ml of 50 mM sodium phosphate buffer (75 mM NaCl, 2 mM DTT and 0.02% NaN_3, pH 6.0) per liter of culture with lysozyme added to a final concentration of 1 mg/ml. After freezing at −80 °C overnight [27] the cells were disrupted upon thawing and protease inhibitor (1 mM PMSF) and DNase I (500 μl of 1 mg/ml stock) were added. The cell extracts were clarified by centrifugation and the supernatant loaded on a gluthathione-agarose (Sigma) column. The GST-fusion proteins were eluted with 5 mM reduced glutathione in 50 mM sodium phosphate buffer, concentrated with a Millipore stirred ultrafiltration cell, and separated on a size-exclusion column (Sephacryl S-100 HR, Amersham Pharmacia Biotech) equilibrated with 50 mM sodium phosphate buffer. Fractions containing the fusion proteins were pooled, the NaCl concentration increased to 150 mM, and the GST-tag cleaved off with thrombin by incubation at room temperature for 12–16 h. The GST-tag was subsequently removed from the recombinant Calsensin protein using a glutathione–agarose column. The Calsensin protein

was further purified by gel-filtration (Sephacryl S-100 HR). The collected fractions were analyzed by SDS-PAGE for purity, pooled, and concentrated to 1–2 mM for NMR experiments. The final NMR samples contained 10% ^2H_2O.

2.4. NMR spectroscopy

All NMR data were acquired at 298K on a Bruker DRX500 spectrometer operating at ^1H frequency of 499.867 MHz. A 5 mm triple-resonance ($^1H/^{15}N/^{13}C$) probe with XYZ field gradients was used for all experiments. A gradient-enhanced HSQC experiment with minimal water saturation [28] was used for all $^1H–^{15}N$ correlation experiments. $^1H–^{15}N$ HSQC spectra were obtained for 0.8 mM ^{15}N-labeled purified recombinant Calsensin either in calcium-free buffer (50 mM Na_2PO_4, 75 mM NaCl, 2 mM DTT, 8 mM EDTA, 0.02% NaN_3, pH 6.0) corresponding to the apo form of Calsensin or in the same buffer with 32 equivalents of $CaCl_2$ added to obtain the calcium-bound holo form. At this level of Ca^{2+}-concentration no additional spectral shifts were observed due to increased calcium-levels indicating that Calsensin was fully calcium-bound. The spectra from fully calcium-bound Calsensin were compared to spectra after adding increasing amounts of unlabeled carboxyl-terminal fragment (aa 153–423) of PP4-R2 purified from the GST-PP4-R2 fusion protein as described above for Calsensin to a final concentration of 0.8 mM. Due to precipitation of the protein 0.8 mM was the maximum concentration obtainable. The normalized chemical shift deviations were calculated from $\Delta av=[(\Delta_{HN})^2+\Delta_N/5)^2]^{1/2}$ [29]. To estimate the dissociation constant of the Calsensin/PP4-R2 interaction, uniformly labeled 0.8 mM ^{15}N Calsensin was similarly titrated with increasing amounts of unlabeled carboxyl-terminal PP4-R2 and the changes monitored by recording $^1H–^{15}N$ HSQC spectra. The resulting normalized chemical shift deviations are a measure of the ligand occupancy of Calsensin. The digital resolution of the spectra was 0.001 ppm. Seven residues (L9, D37, K68, E69, L72, N73 and C80) with minimal spectral overlap were selected and used for calculating the dissociation constant as described in Breheny et al. [30].

The data were processed on a Linux workstation using the NMRPIPE software package [31] and assignments were carried out using NMRView [32]. All structures were visualized and rendered using MOLMOL [33].

3. Results and discussion

To identify proteins that have direct interactions with Calsensin and that may play a role in calcium-dependent signal transduction processes we performed an overlay screen of a leech expression library with GST-Calsensin fusion protein. Potential positive clones detected by labeling with the Calsensin specific mAb Lan3–6 were retested, plaque-purified, sub-cloned, and sequenced. Seven different candidate clones identified in this way were subsequently inserted into the Xa3-Pinpoint vector to generate biotinylated fusion proteins for pull-down interaction assays with Calsensin. These assays confirmed three of the identified clones as potential interaction partners of Calsensin and one of these which contained an extensive carboxyl-terminal fragment (Fig. 1A, underlined sequence) interacted with Calsensin in a calcium-dependent manner (see below). For this reason, the latter clone was chosen for further study and the complete open reading frame nucleotide sequence obtained by 5′ RACE extension. The predicted amino acid sequence (GenBank: DQ363933) encodes a protein of 423 residues (Fig. 1A) with a molecular mass of 46.7 kDa and with a low isoelectric point of 4.5. TBlastN searches of the NCBI databases showed that the most closely related proteins to this leech sequence were those of the protein phosphatase 4 regulatory subunit 2 family. PP4-R2 was first identified in mammals as a 453 amino acid protein with a molecular mass of 50.4 kDa that forms complexes with PP4 [17]. Mammalian PP4-R2 has a characteristic 43 residue conserved region in the NH_2-terminal half of the protein that

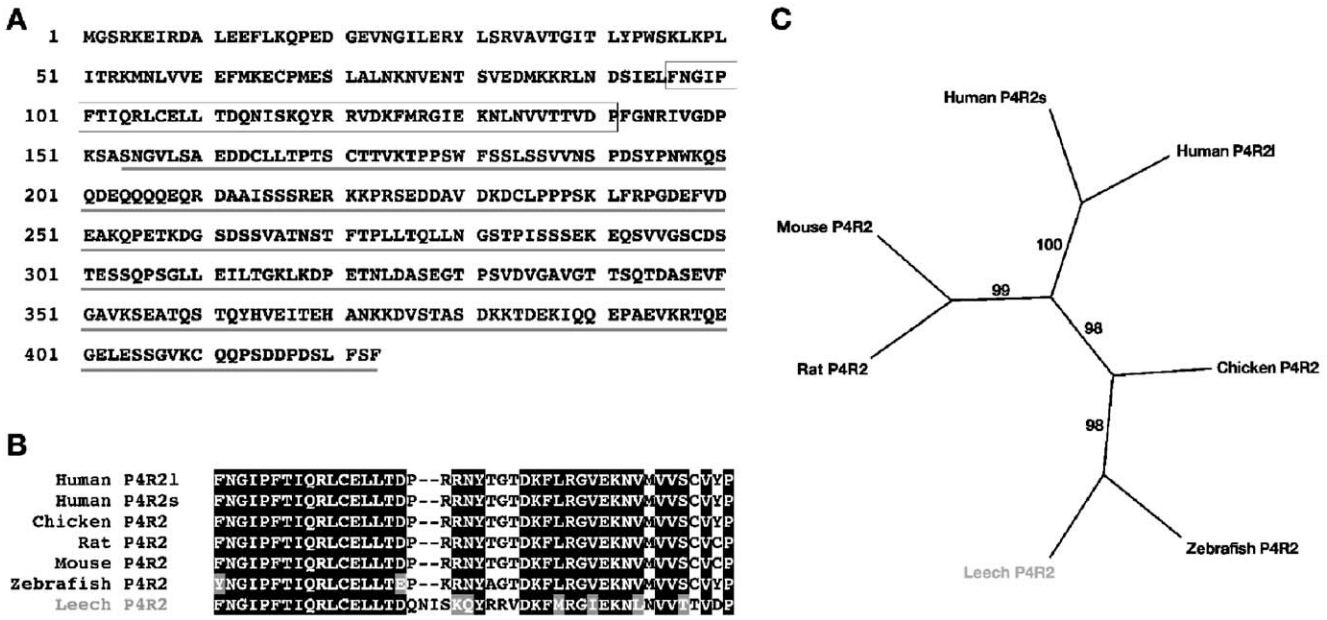

Fig. 1. The predicted protein sequence and phylogenetic relationship of leech PP4-R2. (A) The complete 1269 nt open reading frame derived from the leech PP4-R2 cDNA translates into a 423 aa protein with a predicted molecular mass of 46.7 kDa. The COOH-terminal sequence identified by its interaction with Calsensin in the screen is underlined. (B) Sequence alignment of leech PP4-R2 with vertebrate members of the PP4-R2 family: human PP4-R2long (CAB93534), human PP4-R2short (NP_777567), chicken (NP_001006142), rat (XP_216225), mouse (BAC38523) and zebrafish (AAH60912). The position of this sequence in leech PP4-R2 is indicated by the boxed region in panel A. Identical residues are highlighted in black while similar residues are shown in grey. (C) Phylogenetic relationship of leech PP4-R2 with the vertebrate PP4-R2 family members listed in panel B. The consensus maximum parsimony tree was derived from an alignment of the entire sequences. The tree is unrooted and is depicted with the associated bootstrap support values from 1000 iterations.

was predicted to form an α-helical structure [17]. Fig. 1B shows an alignment of this region from representative vertebrate PP4-R2 proteins with the corresponding leech sequence which shows a high level of conservation (70% amino acid identity). Furthermore, we used alignments covering the entire amino acid sequence of these proteins to construct maximum parsimony phylogenetic trees. Fig. 1C shows that the leech sequence, which has 63% overall sequence similarity to human PP4-R2, forms a monophylectic clade with zebrafish PP4-R2 with 98% bootstrap support. Interestingly, the acidic nature of the leech protein is also shared by mammalian PP4-R2 which has a similarly low isoelectric point of 4.7 [17]. Taken together, these data strongly suggest that the identified leech protein is a homolog of vertebrate PP4-R2 proteins and in the following it will therefore be referred to as leech PP4-R2. Other potential invertebrate homologs of PP4-R2 previously have been identified in flies and worms [17].

In order to verify the interaction between Calsensin and leech PP4-R2 we performed pull-down assays with biotinylated full-length Calsensin (biot-Calsensin) and carboxyl-terminal (aa 153–423) leech PP4-R2 GST-fusion protein (GST-PP4-R2) in the presence (1 mM) and absence of calcium (Fig. 2A). The biot-Calsensin fusion protein was coupled to streptavidin beads, incubated with GST-PP4-R2 fusion protein, washed,

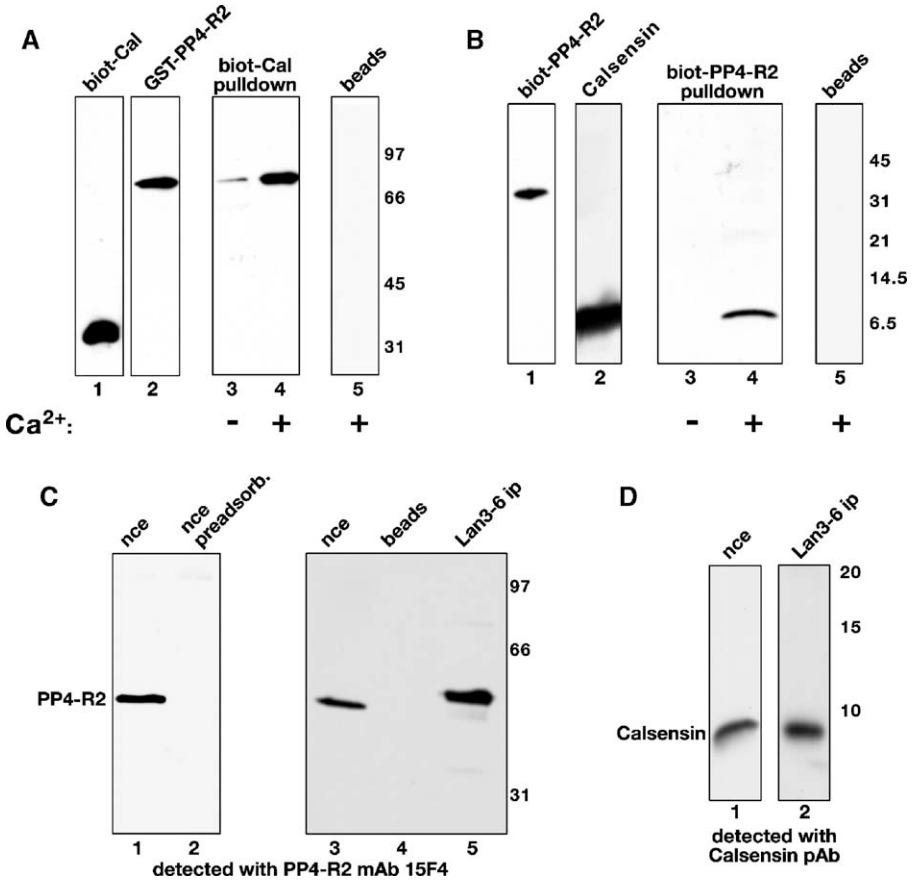

Fig. 2. Calsensin interacts with leech PP4-R2 in pulldown and immunoprecipitation assays. (A) Leech COOH-terminal GST-PP4-R2 fusion protein (aa 153–423) incubated with biotinylated Calsensin (biot-Cal) or with a beads only control was pelleted with streptavidin beads in the presence or absence of 1 mM calcium and the interacting protein fractionated by SDS-PAGE, immunoblotted, and probed with GST-antibody. The input biot-Cal protein detected with biotin-antibody is shown in lane 1 whereas the input GST-PP4-R2 protein detected with GST-antibody is shown in lane 2. Biotinylated-Calsensin was able to pull-down GST-PP4-R2 in the presence of calcium as detected by the GST-antibody (lane 4); however, the pull-down activity by biot-Calsensin was greatly reduced in the absence of calcium (lane 3). The streptavidin beads-only controls showed no pull-down activity (lane 5). (B) Leech COOH-terminal biotinylated-PP4-R2 (biot-PP4-R2) fusion protein (aa 153–423) incubated with purified recombinant Calsensin or with a beads only control was pelleted with streptavidin beads in the presence or absence of 1 mM calcium and the interacting protein fractionated by SDS-PAGE, immunoblotted, and probed with the Calsensin specific mAb Lan3–6. The input biot-PP4-R2 protein detected with biotin-antibody is shown in lane 1 whereas the input Calsensin protein detected with the mAb Lan3–6 is shown in lane 2. Biotinylated-PP4-R2 was able to pull-down Calsensin in the presence of calcium as detected by the mAb Lan3–6 (lane 4); however, there was no pull-down activity by biot-PP4-R2 in the absence of calcium (lane 3). The streptavidin beads-only controls showed no pull-down activity (lane 5). (C) Immunoblot analysis of protein extracts from leech nerve cords (nce) shows that mAb 15F4 recognizes leech PP4-R2 as a 51 kDa band (lane 1) and that this labeling is abolished by preincubation with biot-PP4-R2 fusion protein (lane 2). Immunoprecipitation (ip) of leech nerve cord extracts were performed using Calsensin antibody (mAb Lan3–6, lane 5) or with immunobeads only as a control (lane 4). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using PP4-R2 mAb 15F4 for detection. PP4-R2 antibody staining of nerve cord extract is shown in lane 3. PP4-R2 is detected in the ip sample as a 51 kDa band (lane 5) but not in the control sample (lane 4). The ratio of total lysate loaded to that used for the immunoprecipitation experiments was 1:10. (D) Immunoblot analysis of protein extracts from leech nerve cords (nce) shows that mAb Lan3–6 ips Calsensin as a 9 kDa band (lane 2) also present in the nerve cord extracts (lane 1) as detected by the Calsensin-specific antiserum, Frigg. The relative migration of molecular weight markers is indicated to the right of the immunoblots in kDa.

fractionated by SDS-PAGE, and analyzed by immunoblot analysis using GST-antibody (Fig. 2A). Whereas the streptavidin beads-only controls showed no pull-down activity, biot-Calsensin was able to pull-down GST-PP4-R2 in the presence of calcium as detected by the GST-antibody. However, the pull-down activity by biot-Calsensin was greatly reduced in the

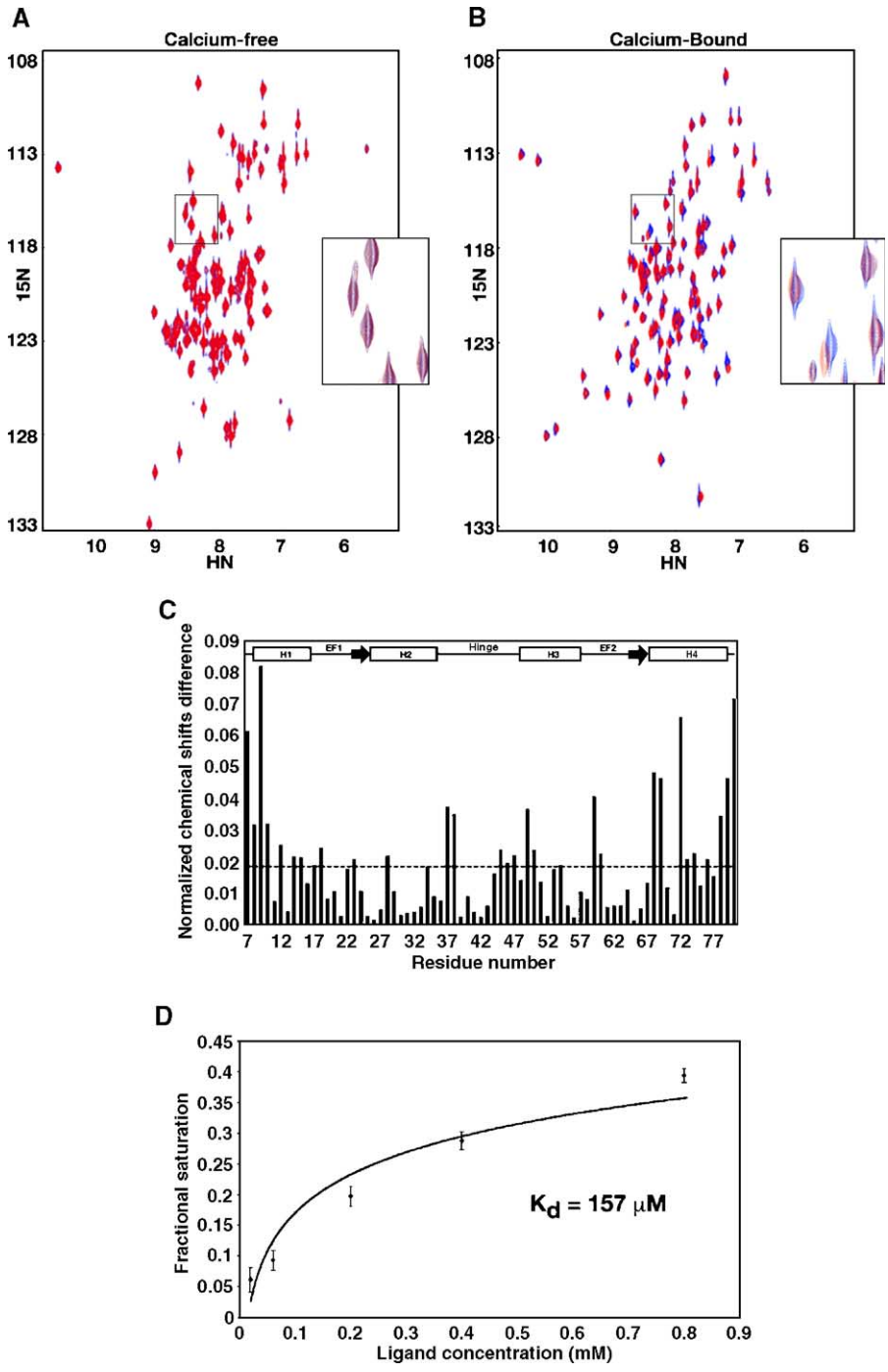

Fig. 3. Chemical shift mapping of the binding of carboxyl-terminal leech PP4-R2 to Calsensin. (A) Superimposed $^{1}H–^{15}N$ HSQC spectra of 0.8 mM ^{15}N-labeled Calsensin in calcium-free buffer with (red spectrum) and without (blue spectrum) 0.8 mM of unlabeled leech carboxyl-terminal PP4-R2 protein. (B) Superimposed $^{1}H–^{15}N$ HSQC spectra of 0.8 mM ^{15}N-labeled fully calcium-bound Calsensin with (red spectrum) and without (blue spectrum) 0.8 mM of unlabeled leech COOH-terminal PP4-R2 protein. The inserts in panels A and B show expanded views of the boxed regions. (C) Normalized chemical shift differences in ppm of ^{1}H and ^{15}N resonances of free Calsensin and Calsensin bound to COOH-terminal PP4-R2 protein obtained from $^{1}H–^{15}N$ HSQC spectra. The chemical shifts are from fully calcium-bound Calsensin and plotted as a function of residue number. The secondary structure of Calsensin is depicted at the top and the dotted line indicates the average chemical shift perturbation per residue (0.017 ppm). (D) Plot of the chemical shift changes of the ^{15}N-resonances of seven different residues of Calsensin as a function of the concentration of added leech PP4-R2 protein. The concentration of ^{15}N-labeled Calsensin in the calcium-bound holo form was 0.8 mM. The continuous curve represent the best fit theoretical binding curve with a equilibrium dissociation constant of 157±70 μM (n=5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

absence of calcium (Fig. 2A, lane 3). In the reciprocal pull-down experiments a carboxyl-terminal (aa 153–423) leech PP4-R2 biotinylated fusion protein (biot-PP4-R2) coupled to streptavidin beads was incubated with purified full-length recombinant Calsensin and analyzed as described above (Fig. 2B). Whereas the streptavidin beads-only control and biot-PP4-R2 in the absence of calcium showed no pull-down activity, biot-PP4-R2 was able to pull-down Calsensin in the presence of calcium as detected by the Calsensin specific mAb Lan3–6. These results strongly suggest that Calsensin and leech PP4-R2 are present in the same protein complex and that this interaction is calcium-dependent.

In order to identify leech PP4-R2 on immunoblots we generated a mAb, 15F4, to the GST-PP4-R2 fusion protein. On immunoblots of leech nerve cord extracts this antibody recognizes a single band migrating at approximately 51 kDa which is close to the predicted molecular mass of leech PP4-R2 of 46.7 kDa (Fig. 2C). The 51 kDa band immunoreactivity was specifically competed away if the antibody was preadsorbed with biot-PP4-R2 fusion protein (Fig. 2C, lane 2). These results strongly indicate that mAb 15F4 specifically recognizes leech PP4-R2 on immunoblots. Unfortunately, in subsequent experiments mAb 15F4 proved not to be suitable for immunoprecipitation or immunocytology assays. However, in order to test for an in vivo interaction between PP4-R2 and Calsensin we extracted leech nerve cord proteins and immunoprecipitated with Calsensin mAb Lan3–6, fractionated the immunoprecipitated proteins on SDS-PAGE, immunoblotted, and probed with the PP4-R2 mAb 15F4. Fig. 2C shows such an immunoprecipitation experiment where the co-immunoprecipitate of the Calsensin mAb is detected as a 51 kDa band also present in the nerve cord extracts. This band was not present in lanes where immunobeads-only were used for the immunoprecipitation. Fig. 2D confirms that mAb Lan3–6 was able to immunoprecipitate Calsensin from the nerve cord extracts. These results provide evidence that leech PP4-R2 and Calsensin are present in the same protein complex in leech nerve cords in vivo.

To further characterize the binding of the COOH-terminal tail of leech PP4-R2 to Calsensin we performed chemical shift mapping using $^{1}H–^{15}N$ HSQC experiments. Resonance assignments and a high-resolution 3D solution structure of calcium-bound Calsensin were previously obtained [4]. Fig. 3A shows a $^{1}H–^{15}N$ HSQC spectrum (in blue) of 0.8 mM ^{15}N-labeled purified recombinant Calsensin in calcium-free buffer (50 mM

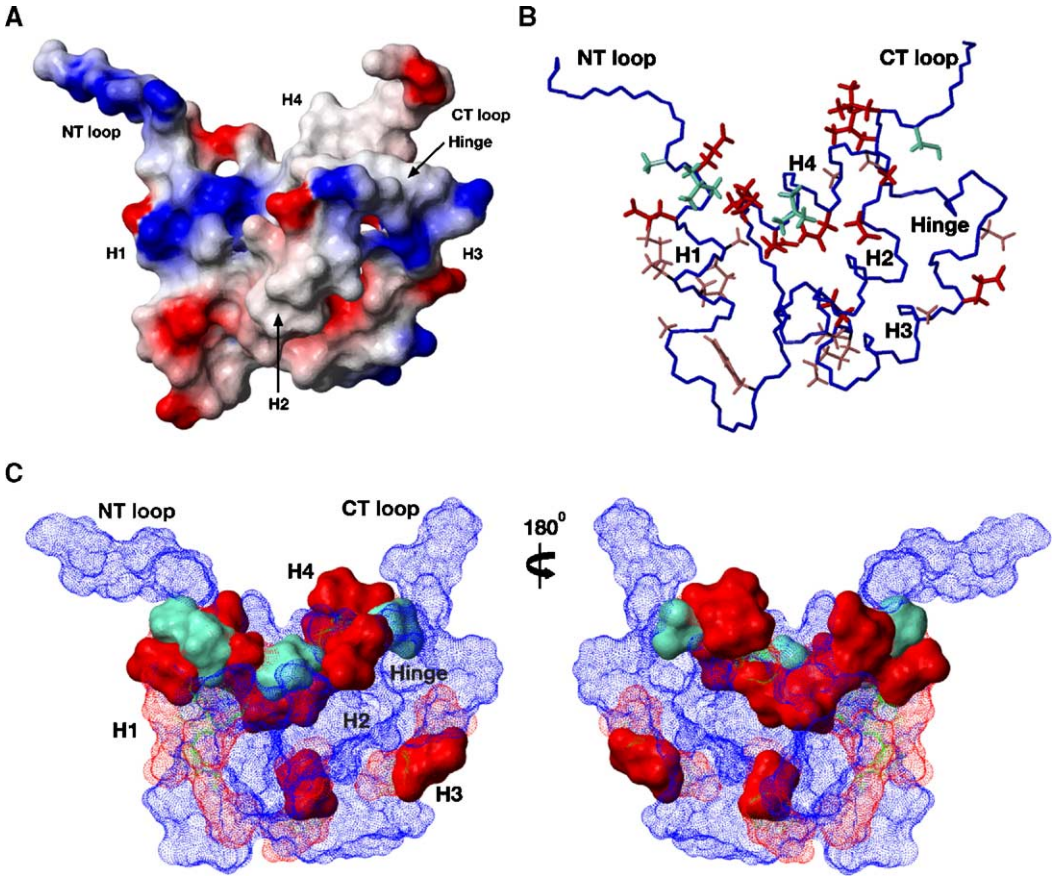

Fig. 4. Mapping of residues exhibiting significant chemical shift change upon complexation with leech carboxyl-terminal PP4-R2 protein on the solution structure of calcium-bound Calsensin. (A) Surface plot of Calsensin. The regions coloured in blue have positive electrostatic potential, those coloured in red have negative electrostatic potential, and the regions in white are neutral. The hydrophobic residues in helices 2, 3, and 4 as well as in the hinge region are exposed to the surface. The backbone structure of Calsensin is shown in panel B, whereas (C) shows two orthogonal views of molecular surface representations. Residues coloured in green, red, and brown have normalized chemical shift values >0.06 ppm, >0.03 pm, and >0.02 ppm, respectively. The blue surface indicate that that there is no significant $^{1}H/^{15}N$ shift perturbation. All plots were rendered using MOLMOL [33]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Na_2PO_4, 75 mM NaCl, 2 mM DTT, 8 mM EDTA, 0.02% NaN_3, pH 6.0) corresponding to the apo form of Calsensin. After adding up to 0.8 mM of unlabeled purified carboxyl-terminal PP4-R2 fusion protein (aa 153–423) (higher concentrations lead to precipitation) there were no significant chemical shifts in the $^{1}H–^{15}N$ HSQC spectrum (Fig. 3A, spectrum in red) suggesting that there is no measurable interaction between Calsensin and COOH-terminal PP4-R2 under these conditions. Fig. 3B shows a $^{1}H–^{15}N$ HSQC spectrum of ^{15}N-labeled Calsensin (spectrum in blue) in the fully calcium-bound holo form with 32 equivalents of calcium added to the buffer. Under these conditions in the presence of 0.8 mM purified carboxyl-terminal PP4-R2, significant chemical shift changes were observed for a subset of residues (Fig. 3B, spectrum in red). The normalized chemical shifts calculated from $\Delta_{av}=[(\Delta_{HN})^2+(\Delta_N/5)^2]^{1/2}$ [29] and plotted as a function of residue number is shown in Fig. 3C. In addition, the residues with the largest chemical shift perturbations (> 0.02 ppm) are mapped onto the calcium-bound 3D solution structure of Calsensin [4] in Fig. 4B and C. Calsensin has a potential shallow, binding groove formed primarily by hydrophobic residues located in helix 1, the hinge region, and helix 4 [4] (Fig. 4A). As illustrated in Fig. 4B and C, almost all of the residues with the largest chemical shift perturbations were located in this region of the protein. These chemical shift perturbations were similar to those obtained for the residues determining the interaction between the third extracellular domain of the interleukin-6 receptor and its ligand [34] and for the residues responsible for DNA-binding to the *E. coli* cell division activator protein CedA [35]. To estimate the dissociation constant of the Calsensin/PP4-R2 interaction, we titrated uniformly labeled 0.8 mM ^{15}N Calsensin with increasing amounts of purified unlabeled carboxyl-terminal PP4-R2 and monitored the changes by recording $^{1}H–^{15}N$ HSQC spectra. The system is in fast exchange on the NMR time scale. Thus the normalized chemical shifts values are a measure of the ligand occupancy of Calsensin. Unfortunately, due to precipitation of the PP4-R2 carboxyl-terminal fragment at concentrations higher than 0.8 mM, we could not saturate the binding interaction. However, from the dependence of normalized chemical shift on ligand concentration that was obtained, we estimated a dissociation constant K_d, of 157 ± 70 μM [27,30] for the interaction between Calsensin and the carboxyl-terminal fragment of PP4-R2 (Fig. 3D). These data strongly suggest that the binding of leech PP4-R2 maps to a well-defined binding surface of Calsensin and that especially residues A7, L9, L72 and C80 are involved in this interaction.

Immunolocalization studies of PP4 in rat have shown a widespread distribution in all tissues examined with particularly high levels in brain and testes [36]. Within cells, both PP4 and PP4-R2 were found in an overlapping pattern throughout the cytoplasm and nucleus but with more intense staining in the nucleus and at centrosomes [17,36,37]. In addition, many protein phosphatase complexes are located in distinct regions of the cell and are targeted to these subcellular locations by their regulatory subunits [17]. Thus, specific phosphatase functions may be conferred by the regulatory subunits of the holoenzyme in a tissue and cell context dependent manner [5]. Calsensin is expressed in only a small subset of neurons many of which fasciculate in a single axon tract [2,3]. The calcium-dependent interaction between Calsensin and leech PP4-R2 demonstrated in this study therefore has the potential to regulate phosphatase activity in these neurons and modulate specific signal transduction processes governing the functional properties of these neurons. Furthermore, the findings raise the possibility that calcium-dependent modulation of phosphatase complexes through interactions with small calcium-binding proteins such as the S100 family may be a general mechanism for regulation of signal transduction pathways.

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