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Calcium-binding calmyrin forms stable covalent dimers *in vitro*, but *in vivo* is found in monomeric form✪★

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The EF-hand Ca^{2+}-binding protein calmyrin is expressed in many tissues and can interact with multiple effector proteins, probably as a sensor transferring Ca^{2+} signals. As oligomerization may represent one of Ca^{2+}-signal transduction mechanisms, we characterised recombinant calmyrin forms using non-reducing SDS/PAGE, analytical ultracentrifugation and gel filtration. We also aimed at identification of biologically active calmyrin forms. Non-reducing SDS/PAGE showed that *in vitro* apo- and Ca^{2+}-bound calmyrin oligomerizes forming stable intermolecular disulfide bridges. Ultracentrifugation indicated that at a 220 μM initial protein concentration apo-calmyrin existed in an equilibrium of a 21.9 kDa monomer and a 43.8 kDa dimer (trimeric or tetrameric species were not detected). The dimerization constant was calculated as $K_a = 1.78 \times 10^3$ M^{-1} at 6°C. Gel filtration of apo- and Ca^{2+}-bound calmyrin at a 100 μM protein concentration confirmed an equilibrium of a monomer and a covalent dimer state. Importantly, both monomer and dimer underwent significant conformational changes in response to binding of Ca^{2+}. However, when calmyrin forms were analyzed under non-reducing conditions in cell extracts by Western blotting, only monomeric calmyrin was detected in human platelets and lymphocytes, and in rat brain. Moreover, in contrast to recombinant calmyrin, crosslinking did not preserve any dimeric species of calmyrin regardless of Ca^{2+} concentrations. In summary, our data indicate that although calmyrin forms stable covalent dimers *in vitro*, it most probably functions as a monomer *in vivo*.

Keywords: EF-hand calcium-binding proteins, calmyrin monomer, covalent dimer, human lymphocytes

Ca^{2+} signals play a pivotal role in the regulation of many fundamental cellular processes ranging from rapid modulations of effector proteins to changes in gene expression (Berridge, 1998). The precise transduction of Ca^{2+} signals is mediated by a multitude of Ca^{2+}-binding proteins (CaBP), of which numerous belong to the EF-hand superfamily (Heizmann & Hunziker, 1991; Nakayama & Kretsinger, 1994). These proteins are characterized by the presence of EF-hand motifs where Ca^{2+} is bound at increasing cellular Ca^{2+} concentrations, what in turn changes their conformation and triggers interactions with cellular targets, resulting in modulation of enzymes, ion channels or cell surface receptors (Ikura, 1996).

Calmyrin, also termed CIB (Naik *et al.*, 1997) or KIP (Wu & Lieber, 1997), contains four EF-hand motives, two of which, located in the C-terminal half of the protein, are canonical and have been shown to bind Ca^{2+} with high affinities (Yamniuk *et al.*, 2004; Gentry *et al.*, 2004). Calmyrin amino-acid sequence shows highest similarity to calcineurin B and calmodulin (58% and 56%, respectively), two typical ubiquitous EF-hand Ca^{2+}-sensor proteins (Naik *et al.*, 1997). In addition, the structural features of calmyrin are similar to the group of EF-hand neuronal calcium sensor (NCS) proteins known also as recoverin family (Burgoyne & Weiss, 2001). In accord with structural similarities to the NCS proteins, we have recently described in details

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Abbreviations: BS3, bis-(sulfosuccinimidyl)suberate; BSA, bovine serum albumin; CaBP, Ca^{2+}-binding proteins; DTT, dithiothreitol; KChIP, potassium channel inhibitory protein or calsenilin; 2-ME, 2-mercaptoethanol; NCS, neuronal calcium sensor; PBS, phosphate buffered saline; TCEP, Tris[2-carboxyethyl]phosphine.

calmyrin expression in certain areas of human brain (Berstein *et al.*, 2005). However, in contrast to the NCS proteins, calmyrin expression is not limited to neurons, but has been found in a variety of human tissues (Naik *et al.*, 1997; Wu *et al.*, 1997, Stabler *et al.*, 1999; Kauselmann *et al.*, 1999). This broad expression pattern suggests an involvement of calmyrin in a variety of cellular functions, and indeed calmyrin has been shown to interact with a broad spectrum of proteins that are involved in diverse cellular processes. The list of calmyrin targets includes integrin alpha IIb (Naik *et al.*, 1997), the polo-like kinases Snk/Fnk (Kauselmann *et al.*, 1999; Ma *et al.*, 2003), a protein coded by the gene *NBR1* located next to *BRCA1* (Whitehouse, 2002), DNA-dependent protein kinase (Wu & Lieber, 1997), ubiquitin-protein ligase EDD (Henderson *et al.*, 2002), human telomerase reverse transcriptase (Lee *et al.*, 2004), coagulation factor VIII (Fang *et al.*, 2001), caspase-2s (Ito *et al.*, 2000), Pax3 (Hollenbach *et al.*, 2002), focal adhesion kinase (Naik & Naik, 2003) and presenilin 2 (Stabler *et al.*, 1999). The mechanisms used by this one protein to transmit Ca^{2+} signals to such a variety of target molecules remain to be elucidated.

One of the possible Ca^{2+}-induced mechanisms of target recognition and binding involves exposition of hydrophobic pockets in an EF-hand protein that bind α-helices of a protein ligand. Such pockets have been described in the crystal structure of calmyrin molecule in an area located on the opposite side from the functional Ca^{2+}-binding EF-hands 3 and 4 (Gentry *et al.*, 2004). A second type of Ca^{2+} signalling was described as a Ca^{2+}-myristoyl switch for some myristoylated NCS proteins (Meyer & York, 1999). As shown for recoverin (Ames *et al.*, 1997), Ca^{2+}-binding causes extrusion of an N-terminal myristoyl group from a hydrophobic cavity in the protein molecule, providing a mechanism of anchoring Ca^{2+}-binding proteins to intracellular and plasma membranes in response to elevated Ca^{2+} levels. Calmyrin has been shown to be myristoylated at its N-terminus (Stabler *et al.*, 1999), but so far no Ca^{2+}-dependent translocation to intracellular membranes has been demonstrated. A third mechanism of Ca^{2+} signalling seems related to protein oligomerization, but no simple common paradigm exists. Ca^{2+}-binding regulates oligomerization of some calmyrin homologs from the NCS protein family. Neurocalcin forms a dimer upon Ca^{2+}-binding (Vijay-Kumar & Kumar, 1999), while its Ca^{2+}-free form is monomeric (Olshevskaya *et al.*, 1999). An opposite way of Ca^{2+}-dependent dimerization was described for guanylate cyclase-activating protein 2, which forms dimers only in its apo-form (Olshevskaya *et al.*, 1999). An involvement of other factors in Ca^{2+}-dependent dimerization has also been shown for some NCS proteins. Potassium channel inhibitory protein 3 (KChIP3, also known as calsenilin) in its Ca^{2+}-free state exists as a tetramer, while Ca^{2+}-bound calsenilin is dimeric, but only at low protein concentrations (Osawa *et al.*, 2001). KChIP1 dimerization occurs in the presence of its ligand (Zhou *et al.*, 2004). Recently Gentry *et al.* (2004) described the crystal structure of calmyrin obtained at 2.0 Å resolution. The asymmetric crystal unit contained two calmyrin molecules. The authors showed however, that *in vitro* calmyrin was largerly monomeric, as evidenced by analytical ultracentrifugation and gel filtration over a dynamic range of concentrations, in the presence or absence of Ca^{2+}. Nevertheless, the experimental conditions used did not allow addressing the calmyrin's ability to form covalent, disulfide-bound oligomers; such a possibility is demonstrated in the present study. As one of the mechanisms of Ca^{2+} signalling and differential target recognition may involve formation of various quaternary protein structures in the cell, we also attempted detecting the calmyrin forms in cell extracts.

MATERIALS AND METHODS

Purification of recombinant calmyrin and analysis of protein disulfide bridges by non-reducing SDS/PAGE. Recombinant calmyrin was obtained as described previously (Bernstein *et al.*, 2005). Briefly, a full length human calmyrin cDNA (GenBank accession No. U82226.1) was obtained by RT-PCR using human lymphocyte mRNA as the starting material. The amplification product was cloned into bacterial pET28a vector containing N-terminal His-tag (Novagen) and expressed in *Escherichia coli*. Calmyrin was purified from bacterial lysate using Ni^{2+}-nitriloacetic acid-agarose resin according to manufacturer's instructions (Qiagen). Protein eluted at 100 mM imidazole was concentrated on Amicon YM 10 membrane (Millipore), dialyzed 3 × 2 h against 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, and cleaved overnight with thrombin (Sigma) to remove the His-Tag. The protein was further purified by FPLC anion-exchanger chromatography on a MonoQ column connected to the Akta Purifier system (Amersham) and resin-bound calmyrin was eluted with a continuous gradient of 150–250 mM NaCl. This fraction was concentrated to 18 mg/ml using Vivaspin centrifugal concentrators, cut off 10 kDa (Sigma), and 200 µl samples were applied on a gel filtration Superose 12 column (Amersham) equilibrated in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl. After elution at 0.3 ml/min in this buffer, protein peaks were analyzed by non-reducing 12% SDS/PAGE, i.e. omitting reducing agents from the sample buffer. Reduction of calmyrin disulfides was investigated using 2-mercaptoethanol (2-ME), dithiothreitol (DTT), and Tris[2-carboxyethyl]phosphine (TCEP) from Pierce (Germany). The reducing agents were added to pro-

tein samples preincubated for 30 min with 0.2 mM $CaCl_2$ or 2 mM EGTA at a 2.5 mg/ml protein concentration. The protein samples were then subjected to non-reducing SDS/PAGE as described above.

Analytical ultracentrifugation. A Beckmam, Inc. Optima XL-I analytical ultracentrifuge equipped with a four-place An-Ti rotor was used for sedimentation equilibrium experiments at 6.0°C. The density of the AUC buffer (20 mM Tris/HCl, 150 mM NaCl, 1 mM EGTA, 2 mM 2-ME, pH 7.5) was 1.00563 g/ml at 20.00 ± 0.01°C, as determined with an Anton Paar Model DMA-58 densitometer. A partial specific volume of 0.717 ml/g was calculated for calmyrin from the amino-acid composition and the values of Zamyatnin (1984). For interference optics the calibration value of 3.191 ± 0.005 fringes $(mg/ml)^{-1}$ (Nosworthy *et al.*, 1998) was used. For sedimentation equilibrium runs, 0.110 ml calmyrin (4.84 mg/ml protein) dialyzed overnight against the AUC buffer was loaded into the right channel of a double sector cell (1.2 cm path length) equipped with sapphire windows and a carbon-filled epoxy centerpiece, and 0.110 ml of dialysate was placed in the reference channel. The rotor was accelerated to 3000 r.p.m. until optical calibrations and initial scans were performed and then maintained at 42000 r.p.m. for 26 h with interference scans made at 10 min intervals. The last 25 interference scans overlaped entirely and showed that the meniscus was completely cleared from the protein (meniscus depletion method). Subsequently the rotor speed was reduced to 18000 r.p.m. and maintained at that speed for 75 h. During that time programmed absorbance autoscans at 280 nm (7 averages, 0.001 cm steps in the step-mode) were made at 2 h intervals. At the end of the run the protein was removed from the assembled cell and the right channel was flushed repeatedly before reloading with dialysate. Interference and absorbance baselines for subtraction from the final scans were obtained at rotor speeds of 42000 r.p.m. and 18000 r.p.m., respectively, after thermally equilibrating the rotor at 6°C. Data analysis was performed with the software provided by Allen P. Minton (NIDDK, NIH).

Gel filtration. A Superose 12 column (Amersham) attached to the Akta Purifier system (Amersham) was equilibrated and calibrated in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA or 0.2 mM $CaCl_2$, at a 0.3 ml/min flow. Low Molecular Weight Gel Filtration Calibration Kit (Amersham) was used for a broad calibration range and next precise calibration within 20–60 kDa was performed using purified globular proteins of known structure: UbcH5a (17 kDa), staphopain B (20 kDa), murein endopeptidase (28 kDa), prostaphopain B (40 kDa), and BSA (60 kDa). Protein samples were applied in max. 100 µl volume at 2.2 mg/ml. Calmyrin samples were loaded on this column and analyzed under the same conditions, protein peaks were collected and elution of reduced (–SH) or S–S calmyrin was confirmed by 12% SDS/PAGE under non reducing conditions. K_{av} = Ve–Vo/Vt–Vo values were calculated using Vo and Vt provided by the column manufacturer.

Cell culture and fractionation. Human lymphocytes were immortalized using Epstein-Barr virus as described earlier (Miller, 1974) and grown in RPMI supplemented with 10% FBS. Lymphocytes and human platelets were homogenized in 50 mM Mes, 5 mM $CaCl_2$ or 2 mM EGTA, 5 mM $MgCl_2$, 150 mM NaCl, and protease inhibitor cocktail (Roche) with a Dounce (teflon-glass) homogenizer and centrifuged at 1000 × *g* for 15 min at 4°C to pellet cell nuclei/debris. The supernatant was used for Western blotting with antibodies (see below) against calmyrin. Part of the lymphocyte supernatant was centrifuged again at 100000 × *g* for 1 h at 4°C. The pellet containing crude lymphocyte membrane fraction was solubilized in 3% Triton X-100. After 30 min of incubation at 4°C, the fraction was centrifuged at 11000 × *g* for 30 min, and the supernatant was taken for a cross-linking experiment. Crude membrane fraction of rat brain (pellet after 100000 × *g*) was prepared similarly as of human lymphocytes and was next analyzed by Western blotting.

Crosslinking and Western blotting. 7 µg of purified recombinant calmyrin in PBS or 30 µg of solubilized membrane fraction with or without addition of 100 ng of purified calmyrin were incubated in 0.1 mM $CaCl_2$ or 2 mM EGTA for 30 min at 4°C. After this incubation BS3 (bis-(sulfosuccinimidyl)suberate, Pierce) was added to 4 mM final concentration and the reaction mixture was incubated for 30 min at room temperature. The reaction was quenched by addition of a buffer containing Tris. Cellular protein fractions were separated on 12% SDS/PAGE gels, blotted onto nitrocellulose, blocked with 5% non-fat dry milk and incubated overnight with anti-calmyrin polyclonal antibodies diluted 1:20000. These antibodies were generated (Innovagen, Sweden) against our purified recombinant human calmyrin protein and their specificity was initially confirmed by immunoblotting of the recombinant protein. Detection was performed with HRP-conjugated anti-rabbit secondary antibody (Sigma) and the ECL detection kit (Amersham Biosciences).

RESULTS

Calmyrin covalent dimers are stable *in vitro* and change conformation in response to increased Ca^{2+} concentration

Human calmyrin contains three cysteine residues which may participate in the formation of intra- and intermolecular disulfide bridges. To detect possible covalent oligomers, recombinant calmyrin

purified to homogeneity was analyzed on an SDS-gel under non-reducing conditions (Fig. 1). This analysis revealed that the calmyrin sample was composed of monomers and dimers. The ratio of these two calmyrin forms was similar in samples containing 0.2 mM Ca^{2+} or 2 mM a Ca^{2+} chelator (EGTA), suggesting that Ca^{2+} concentration does not regulate calmyrin dimerization. Incubation of calmyrin for 30 min in the presence of several reducing agents of varying redox potential proved insufficient for full reduction of the disulfides stabilizing the calmyrin dimer (Fig. 1a). The dimers were fully converted to monomers only after long-term incubation at strongly reducing conditions (Fig. 1b), what suggested that calmyrin dimerization occurred by formation of stable intermolecular disulfide bridges. Again, no significant changes in susceptibility of the covalent dimers to reducing agents were observed comparing calmyrin samples containing Ca^{2+} or EGTA (Fig. 1). While non-reducing SDS/PAGE method allowed the detection of calmyrin covalent dimers, monomers containing intramolecular disulfide bridges were difficult to be distinguished from those containing reduced sulfhydryls.

The molecular mass of calmyrin monomer determined by non-reducing SDS/PAGE corresponded to 21.0 kDa and that for S–S stabilized calmyrin dimmer to 38.2 kDa. Since SDS can denature non-covalent calmyrin oligomers, we applied analytical ultracentrifugation and gel filtration to determine

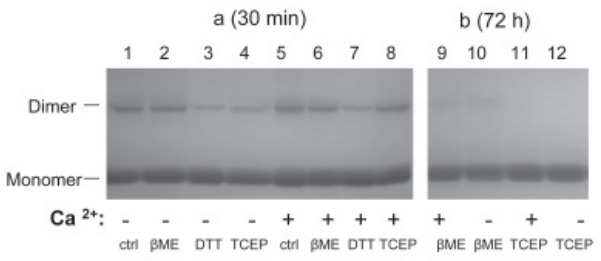

Figure 1. Calmyrin forms stable intermolecular disulfide bridges in the presence or absence of Ca^{2+}.

Non-reducing 12% SDS/PAGE of purified calmyrin in 2 mM EGTA (1–4, 10, 12) or in 0.1 mM $CaCl_2$ (5–8, 9, 11) before (1, 5) and after incubation at 4°C for: a) 30 min, b) 72 h with reducing agents: 2 mM 2-ME (2, 6), 30 mM DTT (3, 7), 20 mM 2-ME (9, 10) and 2 mM TCEP (4, 8, 11, 12).

the molecular mass of recombinant calmyrin forms. The ultracentrifugation results obtained for sedimentation equilibrium at 42000 r.p.m. at a 220 μM initial protein concentration allowed detection of apo-calmyrin monomers with the molecular mass of 21.9 kDa (Fig. 2A, a). This value is similar to the molecular mass of 23.6 kDa determined by Gentry *et al.* (2004), and close to the predicted 21.7 kDa. The high rotational speeds required by the meniscus depletion method prevented detection of oligomers under these conditions. To enable detection of calmyrin oligomers, sedimentation equilibrium at 18000 r.p.m.

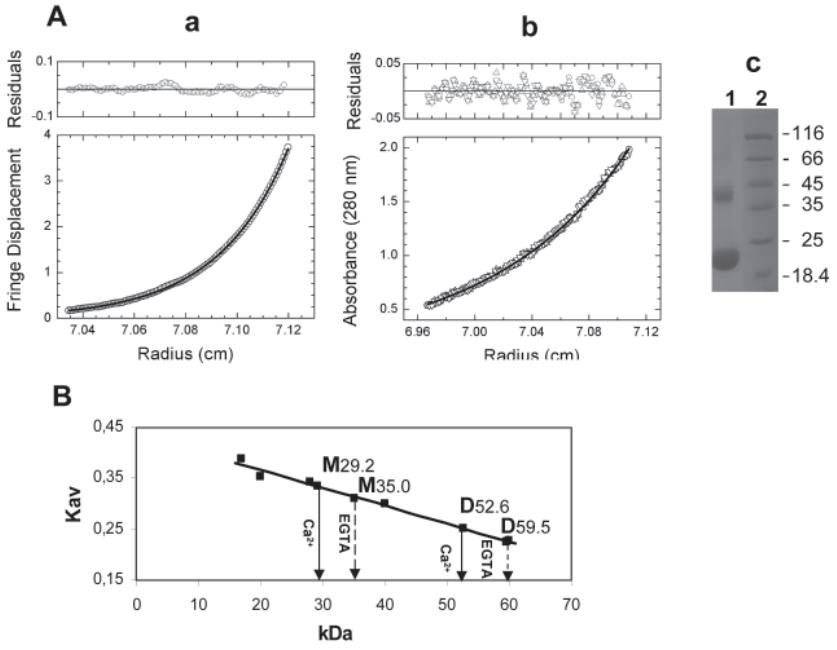

Figure 2. Calmyrin exists as monomer and covalent dimer *in vitro* and both forms change conformation in response to Ca^{2+}.

A. Sedimentation equilibrium of calmyrin analyzed at 6°C at: a) 42000 r.p.m. for 26 h and (b) 18000 r.p.m. for 75 h. Molecular mass values were determined based on absorbance scans as described in Materials and Methods. Ultracentrifuged calmyrin in 20 mM Tris/HCl, 150 mM NaCl, 1 mM EGTA, 2 mM 2-ME, pH 7.5, was also analyzed by 12% non-reducing SDS/PAGE (c). **B**. Gel filtration on Superose 12 column. Linear regression fit $R^2 = 0.9831$. M, monomer; D, dimer.

was performed and the data were analyzed using a global fit of three scans taken in 30 min intervals. A representative sedimentation equilibrium absorbance scan for calmyrin after 26 h at 18000 r.p.m. is shown in Fig. 2B, b, together with the fit of this data to a model of reversible self-association of a monomer to a dimer. Residuals of the fit showed random distribution around zero with less than 0.03 absorbance deviations. The calculated dimerization constant is $K_A^N = 1.78 \times 10^3\ M^{-1}$ at 6°C. Trimeric or tetrameric species were not detected. Under these conditions, approx. 40% of the protein was present in the form of a 43.8 kDa dimer. A similar dimer content (31%) was determined in a sample subjected to ultracentrifugation at 18000 r.p.m. by densitometry of calmyrin forms separated in non-reducing SDS-gels (Fig. 2A, c). This suggests that calmyrin exists *in vitro* in an equilibrium of monomers and dimers stabilized by disulfide bridges. Formation of intramolecular sulfhydryl bonds cannot be excluded.

We further analyzed the forms of recombinant calmyrin by gel filtration under non-reducing conditions in the presence or absence of Ca^{2+} (Fig. 2B). Under both conditions two protein peaks were observed in the elution profiles. The protein peak identified by non-reducing SDS/PAGE as a monomer was eluted at a volume corresponding to 29.2 kDa in Ca^{2+} presence and to 35.0 kDa in EGTA presence (Fig. 2B). These molecular mass values of calmyrin monomer in the presence or absence of Ca^{2+} are similar to the results published earlier (30.6 kDa Ca^{2+}-monomer, 32.5 kDa apo-monomer) (Gentry *et al.*, 2004). These values are higher than those predicted by amino-acid sequence or ones determined by analytical ultracentrifugation, but gel filtration may reflect the shape difference between the dumbbell-like EF-hand protein and the typical globular proteins used for calibration of gel filtration columns. Some other EF-hand proteins also elute from size exclusion chromatography resins at volumes corresponding to a higher than predicted molecular mass (e.g. Olshevskaya *et al.*, 1999). We also observed a difference in the molecular mass of apo- and Ca^{2+}-bound calmyrin monomer, in agreement with NMR data showing a conformational change in calmyrin into a more compact structure caused by Ca^{2+} binding (Yamniuk *et al.*, 2004). Our gel filtration data demonstrated separation of calmyrin monomers from dimers stabilized by S–S bridges (Fig. 2B). The protein peak containing calmyrin dimers eluted at a volume corresponding to 52.6 kDa in Ca^{2+} presence, and to 59.5 kDa in EGTA presence. The molecular mass values were slightly less than double of those determined for calmyrin monomers, which seems to reflect a more compact conformation of apo- and Ca^{2+}-dimers compared to monomers. Importantly, the difference in the apparent molecular masses of dimers under different Ca^{2+} concentrations points to a significant conformational change in dimers upon Ca^{2+} binding (Fig. 2B). It suggests that both calmyrin

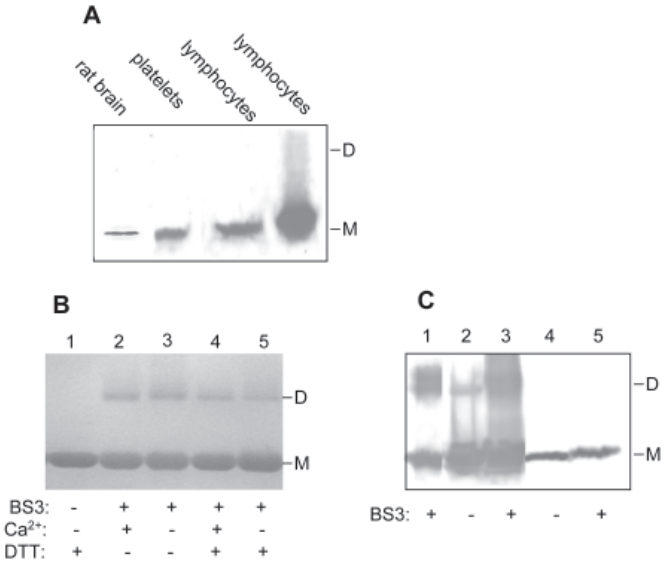

Figure 3. Calmyrin in cell extracts exists only in monomeric form.

A. Western blot developed with anti-calmyrin antibodies of protein fractions maintained at non-reducing conditions: Lines from the left: (1) rat brain solubilized membranes, (2–4) human cell extracts after removal of cell debris/nuclei by centrifugation at 1000 × *g*. Equal amounts of protein (30 μg) were loaded in lanes 1–3 and 60 μg in lane 4. **B**. 12% SDS/PAGE analysis of : (1) recombinant calmyrin, (2–5) calmyrin crosslinked with BS3 in the absence (2, 3) or presence (4, 5) of 2 mM DTT and 0.1 mM $CaCl_2$ (2, 4) or 2 mM EGTA (3, 5). Equal amounts of protein (7 μg) were loaded in each lane. **C**. Western blot developed with anti-calmyrin antibodies (1) 50 ng of purified recombinant calmyrin crosslinked with BS3, (2–5) solubilized lymphocyte membranes: (2, 3) containing 100 ng of added recombinant calmyrin, (4, 5) no exogenous calmyrin, (2, 4) before, and (3, 5) after crosslinking with BS3. Equal amounts of membrane proteins (30 μg) were loaded in each lane. M, monomer, D, dimer.

monomers and covalent dimers are able to bind Ca^{2+} and change their conformation. Therefore, it seems possible that calmyrin dimers may function in Ca^{2+}-signalling to target molecules in cells.

Calmyrin in cell extracts does not form dimers and is present only as a monomer

We aimed at verification of the hypothesis that covalent dimers may represent the Ca^{2+}-sensing calmyrin form *in vivo*. We earlier found that calmyrin protein levels are rather low in human brain, even in membrane fractions where calmyrin was enriched (Bernstein *et al.*, 2005). Therefore, in this report we investigated the forms of endogenous calmyrin in rat brain membranes as well as in cell extracts from human lymphocytes and platelets, which contain higher amounts of calmyrin (Fig. 3A). Immunoblotting of cell extracts maintained under non-reducing conditions demonstrated the presence of calmyrin monomers and a lack of dimeric species in neuronal and non-neuronal cells (Fig. 3A). Even doubling the protein amount in the lymphocyte extract did not allow detection of calmyrin dimers, while monomer immunoreactivity was very strong (Fig. 3A).

We decided to aid the preservation of possible dimeric species in the extracts by crosslinking them before analysis. As visualized by SDS/PAGE in Fig. 3B, dimers formed by recombinant calmyrin *in vitro* can be efficiently crosslinked even in the presence of reducing agents and irrespective of Ca^{2+} concentration. This data confirmed our previous observations that calmyrin dimers are stable, relatively resistant to reduction, and that they are formed by calmyrin in the apo- or Ca^{2+}-bound state. Similar results were obtained when the crosslinking was performed in lymphocyte extracts containing exogenous calmyrin (Fig. 3C), proving that crosslinker concentration under the applied conditions was sufficient for preservation of dimers formed by the exogenous protein. However, no dimers were detected after crosslinking of cell extracts without exogenous calmyrin, confirming the lack of endogenous dimeric calmyrin forms in the cell extracts. The only detected calmyrin form *in vivo* was the monomeric one when either 2 mM EGTA or 0.2 mM Ca^{2+} were added to the cell extracts (not shown).

DISCUSSION

Multiple studies evidenced that most EF-hand proteins from the S100 family exhibit biological activity not as monomers, but as homo- or heterodimers (Wojda & Kuznicki, 1994; Donato, 2001; Maler *et al.*, 2002; Moroz *et al.*, 2003; Koltzscher et al, 2003), and proved that the mechanism of dimer formation is also functionally relevant. For instance, S100B exhibits the activity of neurite extension factor only as a covalent dimer stabilized by disulfide bridges (Winningham-Major *et al.*, 1989; Haglid *et al.*, 1997), while a non-covalent homodimer represents physiological form of S100A6, despite the ability of S100A6 to form disulfide-bound dimers (Wojda & Kuznicki, 1993; 1994; Potts *et al.*, 1995). Posttranslational maturation of proteins includes formation of disulfide bridges where it is required for stability and function, while reduced sulfhydryls are maintained in proteins designed to function without intermolecular covalent bonds (Wendland *et al.*, 1991). While the S100 proteins in the apo- and Ca^{2+}-bound state exist as dimers, some EF-hand NCS proteins exhibit Ca^{2+}-dependent dimerization or oligomerization (Vijay-Kumar & Kumar, 1999; Olshevskaya *et al.*, 1999; Osawa *et al.*, 2001; Zhou *et al.*, 2004). Recently, an NCS homolog, the EF-hand-type Ca^{2+}-binding recombinant calmyrin was shown to exist under reducing conditions mainly in the monomeric state, with some marginal ability to dimerize (Gentry *et al.*, 2004). Here we demonstrated that calmyrin can dimerize *in vitro via* disulfide bridges and that this dimerization is not Ca^{2+}-dependent. Both in the presence and absence of Ca^{2+} calmyrin can exist in an equilibrium of monomers and dimers undergoing reversible self-association. Moreover, our data show that although calmyrin forms dimers irrespective of Ca^{2+}-binding, covalent dimers change conformation in response to increasing Ca^{2+} concentrations. This suggests that, at least in theory, dimers may independently function in Ca^{2+}-signalling *in vivo* and may potentially transduce Ca^{2+} signals to different protein targets than monomers. Accordingly, the cysteine residues in calmyrin seem to be conserved in mammals. However, no covalent dimers were detected under non-reducing conditions in rat brain membrane preparations and in human lymphocytes and platelets, where calmyrin is highly expressed. Also, no covalent or non-covalent calmyrin dimers were preserved by crosslinking in cell extracts, implicating maintenance of calmyrin in the monomeric conformation in the cell *in vivo*.

Homology-based models of calmyrin assumed Ca^{2+}-dependent exposure of hydrophobic residues in the C-terminal domain of calmyrin (Hwang & Vogel, 2000). Such exposure is typical for calmodulin and other calcium sensor proteins enabling subsequent binding to α-helical structures of their cellular targets. Indeed, a hydrophobic pocket has been described in the crystal structure of calmyrin molecule on the opposite side from EF-hands 3 and 4 in the C-terminal part of the protein (Gentry *et al.*, 2004). The above mechanism of Ca^{2+} signalling seems to be involved in calmyrin binding to the α-helical cytoplasmic tail of platelet integrin αIIbβ3 (Barry *et al.*, 2002). However, the broad spectrum of calmyrin protein targets ranging from the nuclear DNA-de-

pendent kinase (Wu *et al.*, 1997) to the membraneous presenilin 2 (Stabler *et al.*, 1999) challenges the uniform mechanism of target binding. Moreover, there are also examples of EF-hand proteins which bind some targets in a Ca^{2+}-independent manner employing acidic residues of their EF-hand structures (see for instance Atkinson *et al.*, 2001; Seidenbecher *et al.*, 2004). The data presented here show that calmyrin dimers or oligomers seem not to be present in cells and thus cannot account for differential target recognition and binding. Also, since calmyrin interaction with its cellular targets was originally identified in a yeast-two-hybrid screen, where the N-terminal myristoylation was not involved, a Ca^{2+}-myristoyl switch mechanism for these interactions is hard to conceive. In this light elucidation of the structural basis for the specificity of monomeric calmyrin interactions with its various cellular targets awaits further investigations.

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