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Dictyostelium discoideum contains a family of calmodulin-related EF-hand proteins that are developmentally regulated

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Abstract A full-length genomic DNA fragment that codes for a novel EF-hand protein from *Dictyostelium discoideum* was cloned and sequenced. The protein is composed of 168 amino acids and contains four consensus sequences that are typical for Ca^{2+}-binding EF-hand domains. The protein sequence exhibits only minor similarities to other calmodulin-type proteins from *Dictyostelium*. The genomic DNA harbors two short introns; their positions suggest that the gene is unrelated to the EF-hand proteins from the calmodulin group. Northern blot analysis showed that the mRNA level was significantly increased during development. Polyclonal antibodies raised against the recombinant protein recognized in Western blots a protein of about 20 kDa. Like the mRNA, also the protein was more abundant in developing cells. Overlay experiments with $^{45}Ca^{2+}$ indicated that the EF-hands in fact have Ca^{2+}-binding activity. The recent description of CBP1, another calmodulin-type *Dictyostelium* protein that is upregulated during development [Coukell et al. (1995) FEBS Lett. 362, 342–346], suggests that *D. discoideum* contains a family of EF-hand proteins that have specific functions during distinct steps of development. We therefore designate the protein described in this report as CBP2.

Key words: Ca^{2+}-binding protein; Development; *Dictyostelium*; EF-hand protein

1. Introduction

The control of numerous cellular reactions by Ca^{2+} occurs with high spatial and temporal precision and requires a finely tuned modulation of Ca^{2+} concentrations in the cytoplasm. Many of the effects of Ca^{2+} are exerted via proteins containing EF-hand domains for Ca^{2+} binding as a common structural motif [1,2]. Proteins like α-actinin or spectrin harbor intramolecular EF-regions as regulatory domains [3,4], others act either as monomeric proteins (e.g. calmodulin, S100) that transiently interact with their targets or as permanently bound regulatory subunits [5–7].

In *Dictyostelium* cells Ca^{2+} plays a role in chemotactic signalling as well as during development and differentiation [8–10]. Several EF-hand type proteins have been identified in this organism and most of them are rather evenly expressed throughout development: α-actinin is an antiparallel homodimer that crosslinks actin filaments in a Ca^{2+}-regulated fashion. This regulation is mediated by two perfect EF-hand regions in the C-terminus of the subunits [4,11]. The concentrations of the small acidic Ca^{2+}-binding proteins calmodulin [12] and calfumirin [13] are also essentially unaltered during growth and development.

Recently CBP1, a third small calcium-binding protein of the EF-hand type, was described as being developmentally regulated [14]. The protein is nearly absent during growth and strongly up-regulated at the onset of aggregation. We describe here the isolation of a cDNA clone that codes for a new EF-hand protein in *Dictyostelium*. The sequence shows only a moderate homology to the previously described EF-hand proteins. However, specific features of the nucleotide-derived protein sequence as well as changes in the amounts of mRNA or protein during development are similar to CBP1. This suggests that *Dictyostelium* contains a family of calmodulin-related proteins whose function appears to be required for development. We propose to number these developmentally regulated Ca^{2+}-binding proteins and consequently describe this new member of the family as CBP2.

2. Materials and methods

2.1. *Growth of* Dictyostelium *cells*

D. discoideum wild-type strain AX2 was axenically grown at 21°C essentially as described [15]. For development cells were harvested at a density of $2–4\times10^6$ cells/ml, washed twice with 17 mM phosphate buffer (pH 6.0), deposited on Millipore filters [16], and the cells were collected at different stages of development.

2.2. *Cloning and sequencing of CBP2 cDNA*

During a search for small cytoskeletal proteins we isolated a cDNA clone whose derived amino acid sequence showed similarities to EF-hands but was different from the corresponding regions in *Dictyostelium* α-actinin [11] and calmodulin [12]. By using this DNA fragment as a probe for screening a *Dictyostelium* cDNA library [17] we isolated a 455 bp internal fragment that spanned the coding region between nucleotides 86–705 (Fig. 1); the missing 5'-end was found with this fragment in another cDNA-library [18]. For completion of the 3'-end a RACE approach was used [19]. With the aid of these separate sequences a full-length clone was isolated by RT-PCR with primers from the 5'- and the 3'-end of the sequence. The PCR product was cloned into the pUC19 vector, and the sequence verified. Under the same conditions a 700 bp genomic clone was isolated from *Dictyostelium* DNA and sequenced.

2.3. *Expression of CBP2 in* Escherichia coli *and isolation of polyclonal antibodies*

A 455 bp fragment was cloned into the pIMS-expression vector [20] and expressed in *E. coli*. The soluble homogenate was loaded onto a DEAE column (2.5×4 cm; DE52, Whatman) in TEDABP buffer (10 mM Tris-HCl, pH 8.0; 1 mM EGTA, 1 mM DTT, 0.02% NaN_3, 1 mM benzamidine, 0.5 mM PMSF), and bound proteins were eluted with a linear salt gradient (2×150 ml, 0–300 mM NaCl in TEDABP). Elution of the recombinant protein was analyzed by SDS-PAGE and Coomassie blue staining, the corresponding fractions were pooled, dialyzed against TEDABP containing 3 mM $CaCl_2$, and chromatographed on a second DEAE column in the presence of excess Ca^{2+}. Under these conditions only the elution of the recombinant protein was shifted towards lower salt concentrations. Two rabbits were immunized at 14 day intervals with several subcutaneous injections of 100 μg protein and 10 μg adjuvant (Gerbu Biotechnik, Gaiberg, Germany). For Ca^{2+}-binding studies the complete cDNA was cloned via

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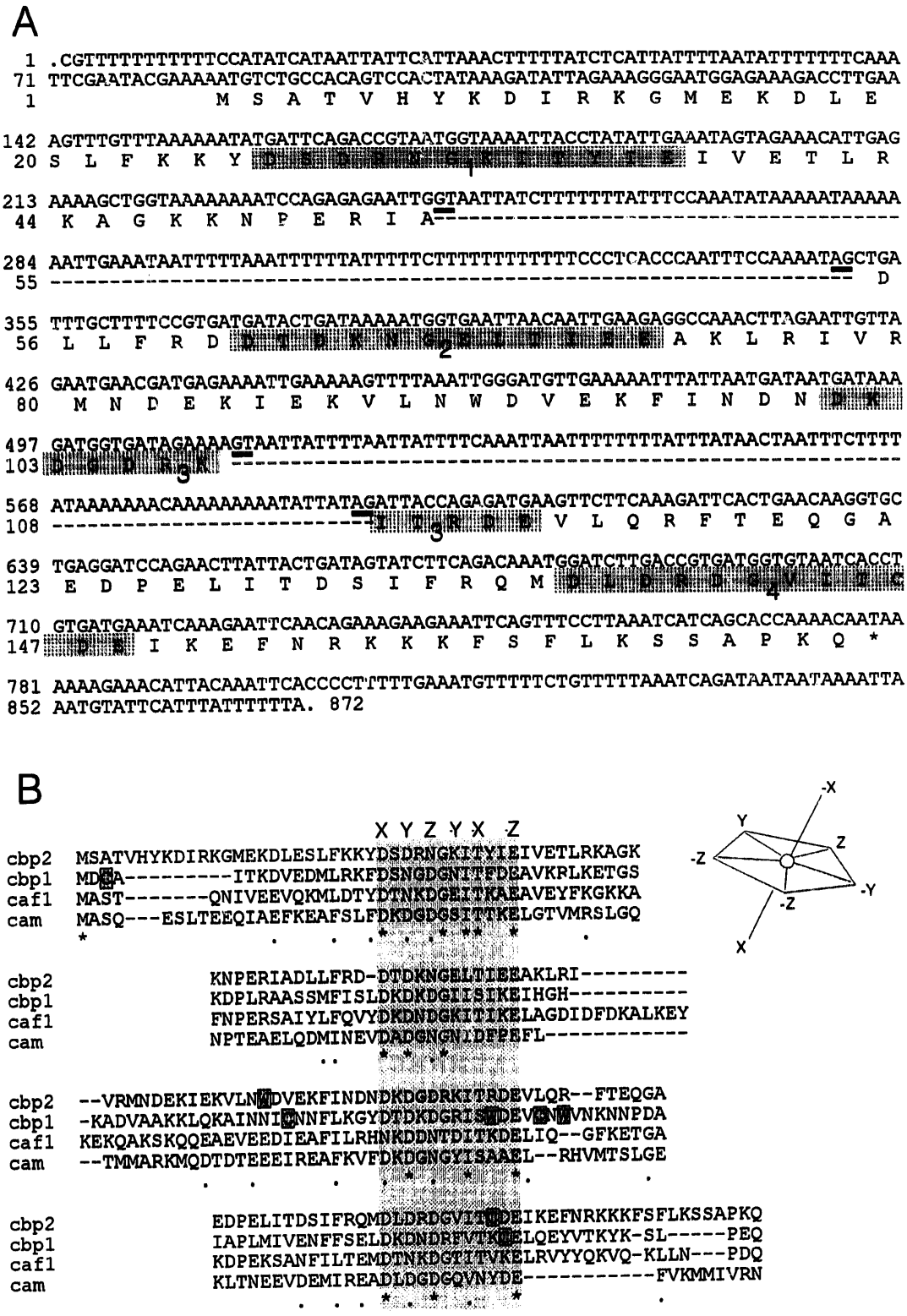

Fig. 1. Sequence of CBP2 and comparison with other EF-hand proteins from *Dictyostelium*. (A) Genomic DNA and deduced amino acid sequence. The four EF-hand regions are shadowed and numbered, the introns are indicated by dashed lines, the nucleotides that contribute to the splicing consensus are underlined. (B) Comparison of the CBP2 protein sequence with related *Dictyostelium* proteins CBP1 [14], CAF1 [13] and calmodulin [12]. The EF-hand loop regions are shadowed, homologous and identical residues are indicated by dots and stars, respectively. Only CBP1 and CBP2 contain tryptophan and cysteine residues (shadowed). The Ca^{2+} ligating coordinates are written on top of the first EF-hand; a schematic representation is shown in the upper right corner.

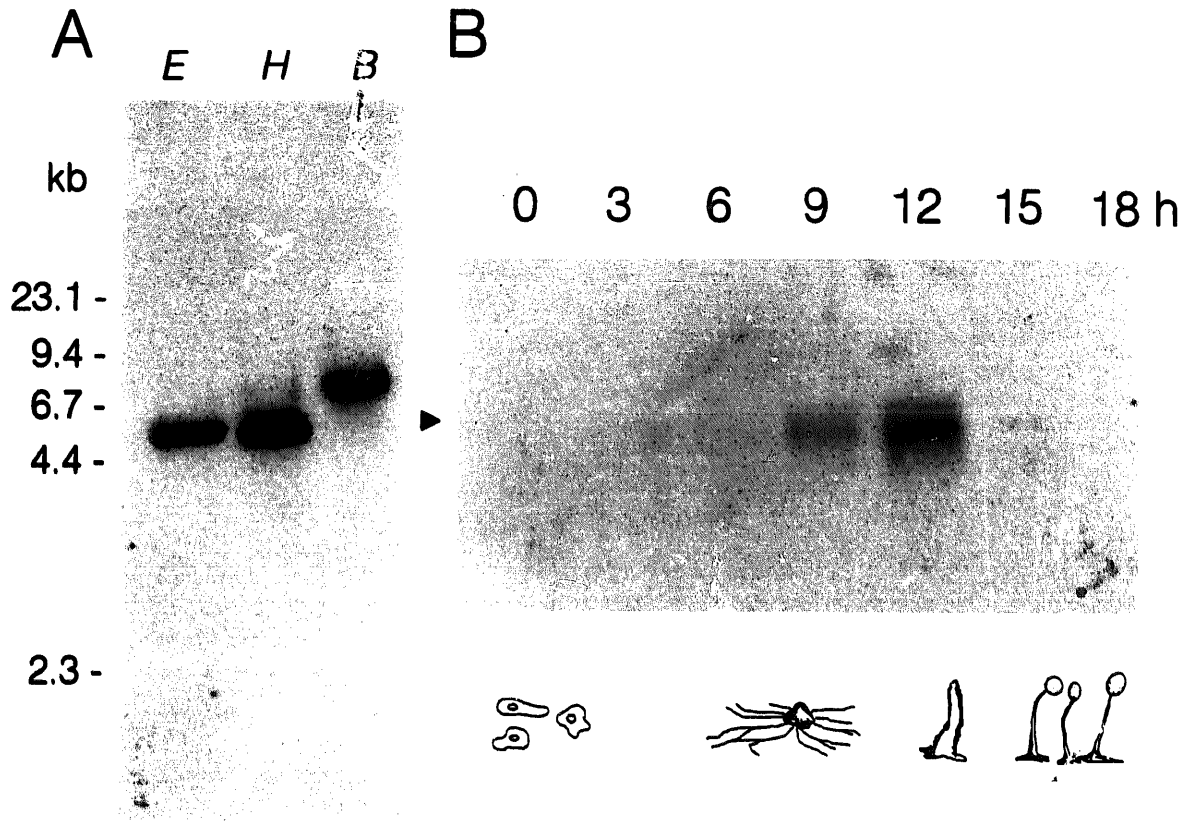

Fig. 2. Southern and Northern blot analysis. (A) Genomic *Dictyostelium* DNA was digested with *Eco*RI (E), *Hin*dIII (H) and *Bgl*II (B) and probed with a CBP2 cDNA fragment. The appearance of single bands suggests that the genome habours a single CBP2 gene. (B) Expression of CBP2 mRNA (0.5 kb; arrowhead) during development of *Dictyostelium*. The developmental stages are indicated in hours (top) and as schematic drawings (bottom).

*Nde*I and *Hin*dIII restrictions sites into pT7-7 [21] and the full-length protein expressed essentially as described [22]. A crucial step for purification was anion exchange chromatography (DE52, Whatman) at different Ca^{2+} concentrations. The protein remained in the flow-through in the presence of 2 mM $CaCl_2$, but bound to the resin in the presence of 2 mM EGTA.

2.4. Miscellaneous methods

DNA and RNA were prepared as described [23]. Hybridizations were performed at 37°C for 15 h in 2× SSC containing 50% formamide. The Southern blots were washed at 37°C for 3×5 min in 2× SSC, 0.1%SDS, and then for 1 h in 2× SSC, 50% formamide. The sequences were analyzed with software from the University of Wisconsin Genetic Computer Group (UWGCG [24]). SDS-polyacrylamide gel electrophoresis [25], immunoblotting [26], and blot overlays with $^{45}Ca^{2+}$ [27] were performed according to standard protocols. Protein concentrations were measured following the protocol of Bradford [28] with BSA as a standard.

3. Results

3.1. Cloning of CBP2 and analysis of the sequence

To obtain a complete CPB2 clone it was necessary to employ a series of different approaches that included screening of two cDNA libraries, 3′ RACE, and RT-PCR. To exclude sequence errors that might result from using these techniques, we isolated and sequenced in addition a genomic clone. This allowed verification of the sequence and revealed the presence and positions of two introns (Fig. 1A). As is typical for most *Dictyostelium* genes, the ATG start codon is preceded by three adenosines [29]. Two putative polyadenylation signals are present in the 3′-untranslated region. The genomic clone spans 696 bp and the open reading frame is interrupted by two A/T-rich introns with a length of 105 and 83 bp, respectively. Splicing follows the 5′-GT- - -3′-AG rule [30].

The gene codes for a small acidic protein of 168 amino acids with a total molecular mass of 19.8 kDa; the calculated isoelectric point is 5.34. Fig. 1B shows an alignment of all small EF-proteins so far described from *D. discoideum*, the Ca^{2+}-ligating loops of the EF-hands are marked. In contrast to calmodulin and calfumirin, CBP1 and CBP2 contain tryptophan and cysteine residues that are however not in homologous positions. The overall identities of the protein sequences between CBP1 and CBP2, CAF1, and CAM are calculated as 32, 31, and 23%, respectively, which indicates that the proteins are only distantly related and that other features like expression during development might be more important to classify these proteins as belonging to different groups.

3.2. Genomic organization of the CBP2 gene; transcript levels during development of Dictyostelium

Dictyostelium DNA was digested with *Eco*RI, *Hin*dIII and *Bgl*II and probed under high stringency conditions with the 455 bp cDNA fragment that spans almost the entire coding region. The appearance of only one band in all three cases suggested that a single gene codes for CBP2 (Fig. 2A). The CBP2 specific mRNA has a size of approx. 0.5 kb. It was barely detectable in RNA isolated from growing cells, but

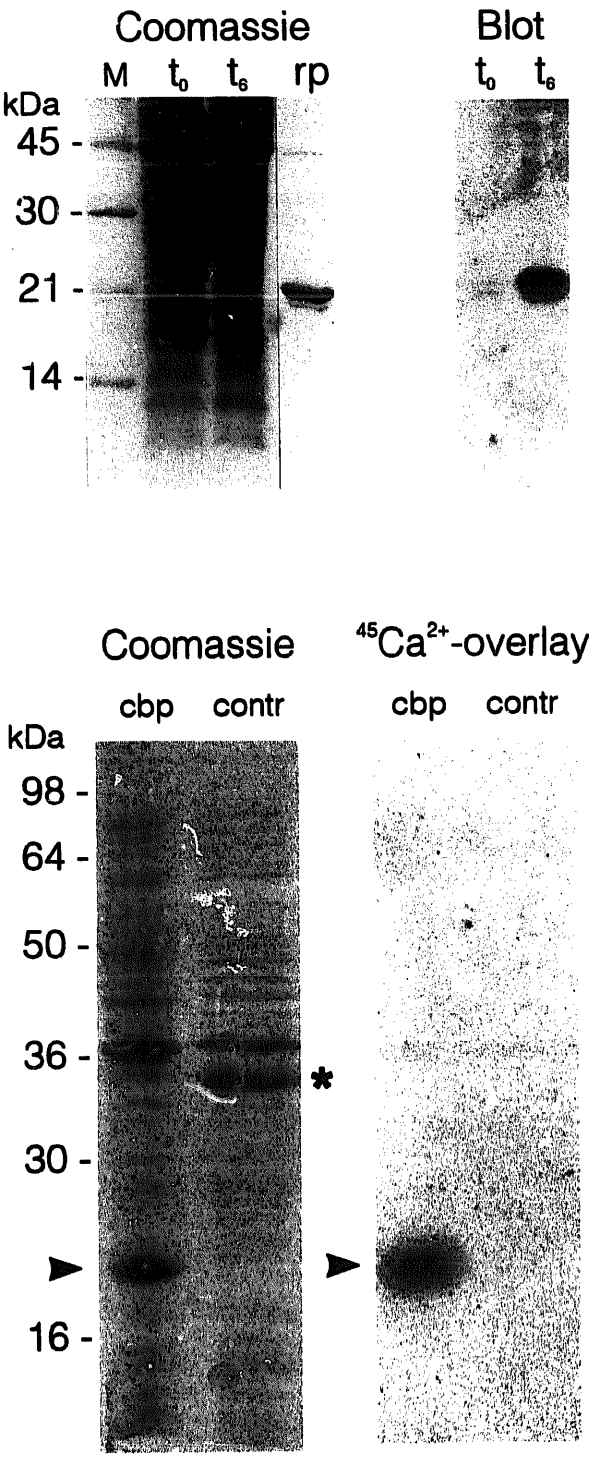

Fig. 3. Western blot analysis (upper panels) and $^{45}Ca^{2+}$ overlay (lower panels). Equal numbers of cells from growing cultures of strain AX2 (t_0) and after 6 h of starvation in shaking suspension (t_6) were analyzed on SDS-polyacrylamide gels (15% acrylamide), and the proteins stained with Coomassie blue (upper left) or blotted onto nitrocellulose and incubated with polyclonal antiserum against CBP2 (upper right). Recombinant CBP2 protein (rp) from another gel is shown in the upper left panel for comparison. The lower panels show *E. coli* homogenates that contain either recombinant CBP2 (arrow head) or the core domain of *Dictyostelium* annexin VII (star). After transfer onto nitrocellulose and overlay with $^{45}Ca^{2+}$ only CBP2 exhibited Ca^{2+}-binding activity.

accumulated to appreciable levels during aggregation in shaking suspension. Fig. 2B shows a more detailed investigation of CBP2 mRNA levels during the whole development of *D. discoideum*. The CBP2 mRNA peaked after 9 h of development on filters and decreased again after 12 h. Only after long exposure times was CBP2 mRNA also detectable at low abundance in the other developmental stages.

3.3. Detection of CBP2 protein in immunoblots; Ca^{2+}-binding activity

The 455 bp cDNA fragment was cloned into the pIMS ATG-expression vector [20] and the partially purified protein used to raise polyclonal antibodies in rabbits. The antibodies recognized a protein of approx. 20 kDa. Similar to the mRNA levels, CBP2 protein was only faintly detectable in whole cell homogenates from growing *Dictyostelium* cultures but its concentration was strongly increased in aggregation competent cells after 6 h of starvation (Fig. 3, upper panels).

A Ca^{2+}-dependent mobility shift in SDS gel electrophoresis which is characteristic for many calmodulins [31] was neither observed with recombinant full-length CBP2 nor with native protein from *Dictyostelium* whole cell homogenates. This raised some uncertainties about the Ca^{2+}-binding activity of CBP2. The fact that the recombinant protein bound during purification only in the presence of EGTA to DEAE resin but remained in the flowthrough in the presence of free Ca^{2+} indicated Ca^{2+}-binding activity. To prove the presence of functional EF-hands we used $^{45}Ca^{2+}$ in overlay experiments. Fig. 3 (lower panels) shows that recombinant CBP2 bound radioactive Ca^{2+} whereas contaminating *E. coli* proteins and recombinant annexin VII from *D. discoideum* [32] did not. Positive controls were performed with chicken gizzard calmodulin and *Dictyostelium* α-actinin (data not shown).

4. Discussion

We report here the cloning and first characterization of a new Ca^{2+}-binding protein in *Dictyostelium*. Based on the protein sequence this small, acidic EF-hand protein resembles calmodulin. However, CBP2 is not an isoform of either calmodulin [12], calfumirin [13], or CBP1 [14]. The CBP2 gene contains two introns, one in the linker region between the first and the second EF-hand, the second intron in the third EF-hand. S100 proteins have only two EF-hands and harbor usually an intron in the linker region [6]. The lack of a corresponding intron in CBP2 between EF-hands 3 and 4, however, suggests that CBP2 did not evolve from a S100-like precursor by gene duplication. In *Dictyostelium* calmodulin the introns were found right after the start ATG (intron I), in the first EF-hand (intron II) and in the linker region between the second and the third EF-hand (intron III) [12]. These locations are completely different from the location of introns in CBP2. Also, in a comparison to the *Dictyostelium* α-actinin gene that contains an intron in the first EF-hand [33] CBP2 can not be classified as homologous. Although the positions of the introns are both in the first helix of the helix-loop-helix motif, the exact locations differ.

The protein sequences of the loop regions indicate that all four EF-hands have the potential to bind Ca^{2+}. Based on a large body of structural data [1] we suggest that four corners of the central pentagonal plate are occupied in the Y, Z, and −Z positions by D, N/D, and the bidentate coordination from

the E side chain, respectively. The −Y coordination comes from the main chain carbonyl oxygen and therefore accepts any amino acid. Interestingly this position in EF-hands 1 and 2 is filled by K and E. Taking into consideration that the side chains of these two amino acids extend into the solvent and that pairs of EF-hands are usually arranged perpendicular to each other, one could speculate that the first EF-hands are tightly connected by a salt bridge between K and E. The pyramidal coordinations would be guaranteed by the invariable D in the X position, and in analogy to the majority of EF-hand proteins by a water molecule in the −X position because the side chain of the invariant T is too short to directly coordinate Ca^{2+}. Consistent with these structural homologies we found a Ca^{2+}-binding activity of CBP2, the exact molar stoichiometry will be subject of further studies.

The regulated expression of CBP2 during development raises the questions for which cellular reactions this protein might be required. The role of Ca^{2+} concentrations during *Dictyostelium* development is still poorly understood. It could be shown, however, that zones of high Ca^{2+} concentrations in *Dictyostelium* aggregates are roughly coincident with those regions that later differentiate into stalk cells [8], and that treatment of developing cells with the chemoattractant cAMP elicited an increased Ca^{2+} uptake [10,34,35]. To better understand the function of developmentally regulated EF-hand proteins in this organism, our studies in the future will focus on distribution of these proteins, their major targets, and changes in behavior of the cells after inactivating the genes via homologous recombination.

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