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Short Communication

Molecular Cloning and Characterization of a cDNA Encoding Caltractin from *Dunaliella salina*

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We cloned a cDNA encoding caltractin, a 20 kDa calcium-binding protein, from *Dunaliella salina* (DSCALT). The Ca^{2+}-bound mobility shift detected in *Chlamydomonas* caltractin was hardly detectable in DSCALT. Also, some differences were found in the electrophoretic mobility between the native DSCALT and the bacterial-expressed DSCALT. This difference may have resulted from the posttranslational modification. Immunoblot analysis revealed that this protein might be localized mainly in the basal body complex, the major microtubule organizing center (MTOC) in *D. salina* and the functional homologue of the centrosome in the animal cell.

Key words: Basal body — Caltractin — cDNA cloning — *Dunaliella salina*.

Caltractin, also known as centrin, is considered to be a ubiquitous component of the basal body/centriole complex in eukaryotic cells (Baron et al. 1992, Melkonian et al. 1992). It is a 20 kDa calcium-modulated EF-hand protein structurally homologous to calmodulin (Salisbury et al. 1984, Huang et al. 1988, Lee et al. 1991, Lee and Huang 1993, Ogawa and Shimizu 1993, Errabolu et al. 1994). Based on a comparative analysis of protein sequences, algal and mammalian caltractins, together with the yeast *Saccharomyces cerevisiae CDC31* gene product which is known as a cell-division-cycle-related protein, appear to be representatives of a separate branch in the evolution of EF-hand calcium-binding proteins (Nakayama et al. 1992, Bhattacharya et al. 1993, Errabolu et al. 1994). There is also evidence that these proteins are related at the level of cell function. They have been found to be localized to the major microtubule organizing center (MTOC) in their respective cells, i.e., the basal body complex in flagellated green algae (Huang et al. 1988), the centrosome in human and other mammalian cells (Lee and Huang 1993, Errabolu et al. 1994), and the spindle pole body in yeast (Spang et al. 1993).

In the flagellate green algae, caltractin is prominently localized to calcium-sensitive contractile fibers, the nucleus basal body connector (NBBC) (Lee and Huang 1990). The NBBC is a calcium-modulated, contractile organelle (Salisbury and Floyd 1978, Salisbury et al. 1987). Contraction of the NBBC can be elicited by elevated calcium concentrations and is characterized by supercoiling of caltractin-containing fine (3–8 nm) filaments (Salisbury et al. 1984). There is substantial evidence that the NBBC in green algae is required for correct basal body duplication and segregation during the cell cycle (Wright et al. 1985, 1989, Taillon et al. 1992).

In the present study, we have cloned a cDNA encoding caltractin from the eukaryotic unicellular green algae, *Dunaliella salina* (Volvocales, Chlorophyceae). *D. salina*, known as a super halotolerant, can adapt to a very wide range of salt concentrations (0.5–5.5 M NaCl), and has several peculiarities compared to other unicellular green alga (Brown and Borowitzka 1974, Pick and Chitlaru 1991). Through the study of cloning and characterization, we found some distinct features in the *D. salina* caltractin (DSCALT) compared to previously reported caltractins, especially that in *Chlamydomonas* and thus the possible significance is discussed herein.

D. salina (Dunal) Teod. was grown vegetatively in the media containing 1.7 M NaCl according to the method of Pick and Chitlaru (1991). Cultures were grown at 26°C under continuous illumination with white fluorescent lamps at 3,800 Lux with axenic air bubbling and maintained at the logarithmic growth phase. A cDNA library in UniZAP XR vector (Stratagene) was constructed using poly(A)$^+$ RNA isolated from *D. salina* cultured for two weeks. A pair of degenerate oligonucleotides, primer A (5′-YTNGGNTTYGARCCNAAKAA-3′) and primer B (5′-TTYTCNCCNARYTCMTTNGC-3′), were designed based on the conserved regions among previously reported caltractin protein sequences for RT-PCR. The resulting PCR product was used as a probe for cDNA library screening after random-primed labeling. Approximately 6×10^4 recombinant phages from the cDNA library were screened and one positive clone was isolated. The *DSCALT* cDNA is 1,020 nucleotides in length consisting of a 510-nucleotide open reading frame (ORF), a 73-nucleotide 5′-untranslated region (UTR), and a 437-nucleotide

The nucleotide sequence reported in this paper has been submitted to GenBank under accession number U53812.

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3′-UTR. The ORF of 169 amino acids corresponds to a protein with a molecular mass of 19.4 kDa and a theoretical isoelectric point of 4.6 (Fig. 1A). The identity of amino acid sequence of DSCALT with that of *Chlamydomonas* caltractin, plant calmodulin, and troponin C was 87, 51, and 46%, respectively. The deduced amino acid sequence of *DSCALT* cDNA was compared to those of caltractins from *Chlamydomonas reinhardtii* (Huang et al. 1988), *Scherffelia dubia* (Bhattacharya et al. 1993), *Naegleria gruberi* (Levy et al. 1996), *Atriplex nummularia* (Zhu et al. 1992), mouse (Ogawa and Shimizu 1993), human (Lee and Huang 1993) and yeast CDC31 (Baum et al. 1986) using the PALIGN program of PC/GENE sequence analysis software (IntelliGenetics Inc. Switzerland) (Fig. 1B). The

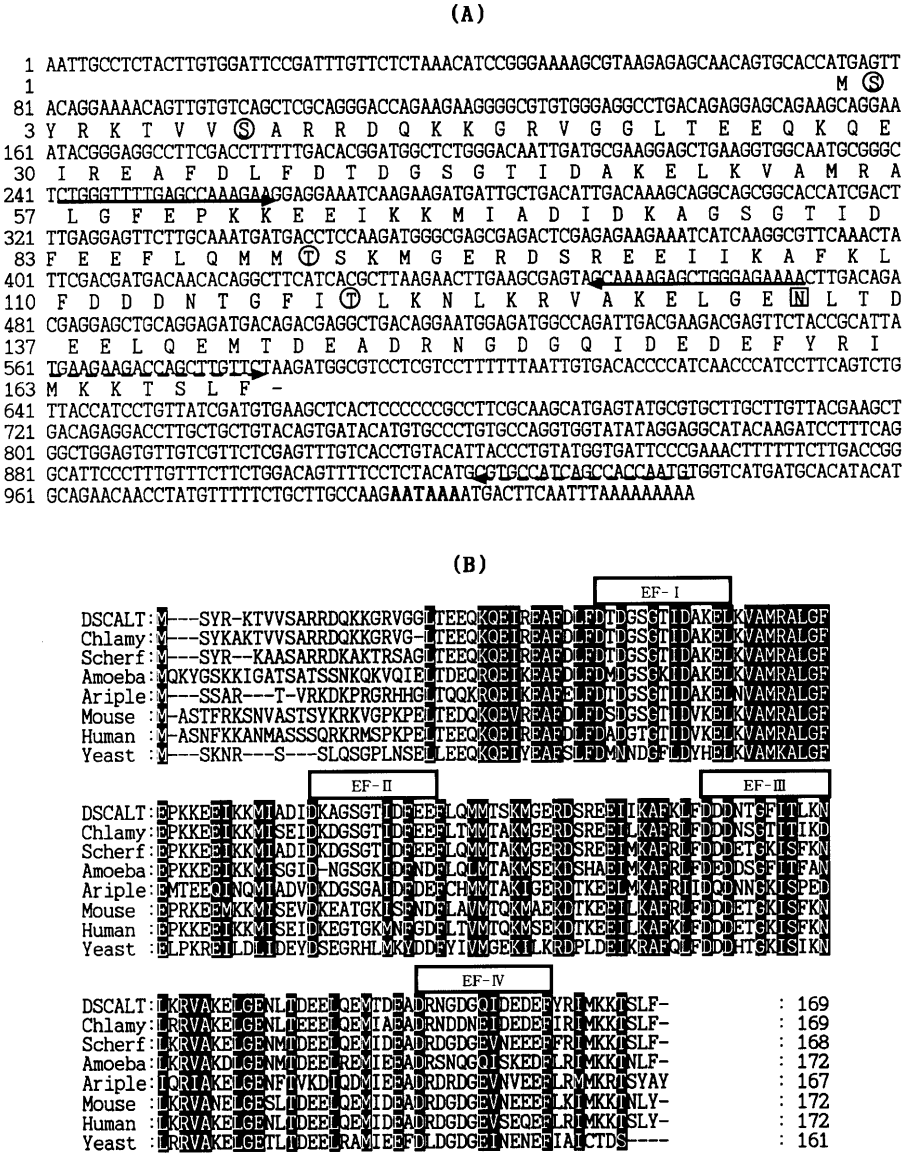

Fig. 1 Analysis of the nucleotide and amino acid sequence of *DSCALT* cDNA. (A) Nucleotide sequence and deduced amino acid sequence. A pair of solid arrows and broken arrows indicate oligonucleotide primers for cDNA cloning and gene-specific probe, respectively. Circled amino acids are the putative phosphorylation site by protein kinase C and the squared amino acid is the putative *N*-glycosylation site. Bold letters in the last line show the putative polyadenylation signal sequence (AATAAA). The numbering is given on the left side. (B) Alignment and comparison of the deduced amino acid sequences of DSCALT with caltractins from different species. Caltractin amino acid sequences of *Chlamydomonas reinhardtii* (Chlamy) (GenBank No. X12634), *Scherffelia dubia* (Scherf) (GenBank No. X69220), *Naegleria gruberi* (Amoeba) (GenBank No. U21725), *Atriplex nummularia* (Atripl) (GenBank No. M90970), mouse (GenBank No. D16301), human (GenBank No. X72964), and yeast CDC31 (Yeast) (GenBank No. M14078) are aligned to maximize the similarity with DSCALT. Dashes indicate gaps introduced to give the best alignment. Four open bars on top of the sequence are conserved calcium-binding domains (EF-hands).

amino acid sequence of DSCALT was significantly homologous to caltractin genes from various sources including yeast CDC31, i.e., 94% similarity with *C. reinhardtii*, 92% with *S. dubia*, 80% with *N. gruberi*, 81% with *A. nummularia*, 83% with mouse, 83% with human and 62% with yeast CDC31. These proteins are highly conserved over the region of the four EF-hand domains, but DSCALT has only two potential calcium-binding site sequences (EF-I and EF-IV) which is compatible with a functional helix-loop-helix EF-hand structure (Moncrief et al. 1990).

We performed a genomic Southern blot analysis with the *DSCALT* gene to determine the number of copies in the *D. salina* genome. Intact genomic DNA was extracted from the vegetatively grown *D. salina* (the condition of haploid cell) using the method of Long et al. (1989). For a gene-specific probe, 3′-UTR was generated by PCR and used (Fig. 2A). Even under high-stringency conditions, two or more strongly hybridizing bands were found in each restriction digestion (except the digestion of *Xba*I), suggesting that *DSCALT* is encoded by a small multi-copy gene (Fig. 2B). This result is in contrast to the report that other algal caltractins are encoded by a single copy gene (Huang et al. 1988, Bhattacharya et al. 1993). Recently, Madeddu et al. (1996) suggested that a number of genes code for closely related centrin molecules in *Paramecium*. Also, Zhu et al. (1996) reported that the *H6* gene from *A. nummularia* shared high sequence homology with algal caltractin and belonged to a multigene family.

Caltractin has been proposed to function in the regulation of MTOC duplication during cell division. Consistent with this proposal, caltractin shares the highest sequence similarity with the 19 kDa yeast *CDC31* gene product. The CDC31 protein is required for the duplication of spindle pole body, the MTOC of yeast (Baum et al. 1986, Biggins and Rose 1994). A defect in CDC31 results in the failure of MTOC duplication and uncoupling of cell cycle control from MTOC formation (Baum et al. 1986). Therefore, we investigated whether the DSCALT is localized in the basal body complex. The basal body complex was isolated using the method of Geimer et al. (1997). Concentrated cells were washed, and lysed by the addition of 2% Triton X-100 in TE buffer (10 mM Tris-HCl, 2 mM EDTA, pH 7.4). The cytoskeleton fraction

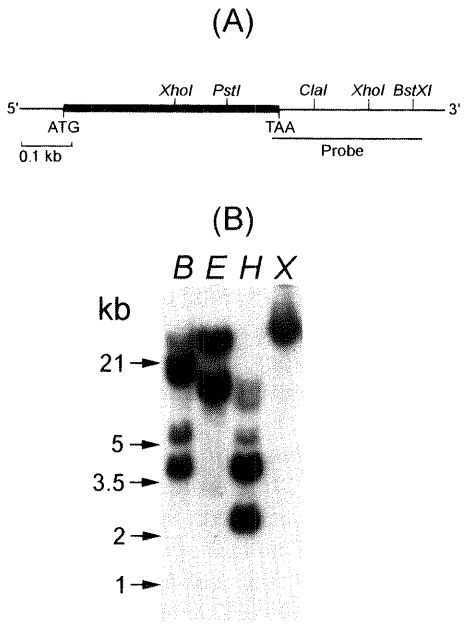

Fig. 2 Genomic Southern blot analysis of *DSCALT* gene. (A) Partial restriction map of *DSCALT* gene. Lower bar indicates the PCR-generated gene-specific probe. (B) Genomic Southern blot using gene-specific cDNA probe. Isolated genomic DNA from *D. salina* (10 μg) was digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H) and *Xba*I (X). Each DNA sample was then separated on an 0.6% agarose gel blotting onto a nylon membrane (Hybond-N, Amersham), and hybridized to a gene-specific probe prepared by random priming. The membrane was hybridized at 42°C in 50% (v/v) formamide, 0.25 M phosphate buffer (pH 7.0), 0.25 M NaCl, 7% (w/v) SDS, 1 mM EDTA and 1.0 mg ml^{-1} denatured salmon sperm DNA.

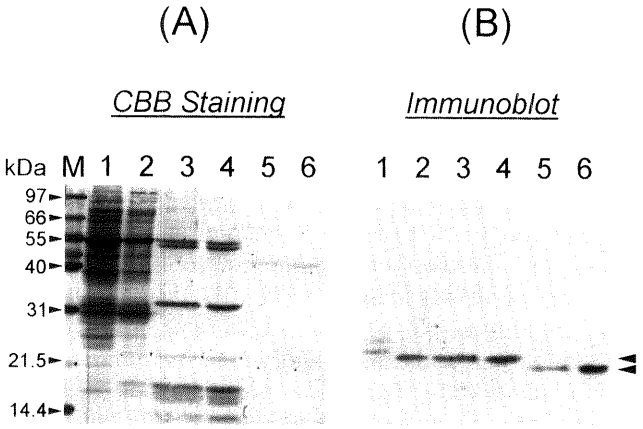

Fig. 3 Subcellular localization and Ca^{2+}-bound mobility shift assay of DSCALT. (A) Isolation of the basal body complex from *D. salina*. Fractions from the isolation step were analyzed by SDS-PAGE (12.5%) and stained with Coomassie brilliant blue. Lane 1, supernatant of the crude extract; Lane 2, pellet of the crude extract; Lane 3, basal body fraction from the sucrose step gradient plus 5 mM EGTA; Lane 4, basal body fraction plus 5 mM $CaCl_2$; Lane 5, bacterial-expressed DSCALT digested with factor Xa (NEB, U.K.) plus 5 mM EGTA; Lane 6, bacterial-expressed DSCALT digested with factor Xa plus 5 mM $CaCl_2$. M: molecular size marker. (B) Immunoblotting analysis of a SDS-PAGE loaded as in panel (A). Immunoblotting analysis was carried out using the anti-DSCALT antibody as the primary antibody and the horseradish peroxidase-conjugated anti-mouse IgG antibody as the secondary antibody. Blots were developed using the ECL detection system (Amersham, England). Upper and lower arrowheads on the right side indicate the DSCALT and the bacterial-expressed DSCALT, respectively.

was collected by centrifugation (800 × *g*, 30 min, 4°C), and loaded onto a sucrose step gradient (6 ml 60%, 9 ml 50%, 9 ml 40%, and 6 ml 20% sucrose in TE buffer). After centrifugation (12,500 × *g*, 1 h, 4°C), the basal body complex was isolated from a milky band below the 20% to 40% sucrose interphase. Fractions from the isolation step were analyzed by SDS-PAGE (Fig. 3A). In lanes 1 and 2, 50 μg of proteins are loaded but 5 μg in lanes 3 and 4, respectively. The protein composition of the isolated basal body complex is shown in Fig. 3A, lane 3 and 4. In addition to DSCALT (20 kDa), SF-assemblin (appeared at 31 kDa) and tubulin (appeared at 55 kDa) were enriched and this pattern is in good agreement with the report of Geimer et al. (1997). The entire ORF was expressed in *E. coli* using the pMAL-c2 vector (NEB, U.K.) to prepare an antibody for DSCALT. The construct was then transformed to *E. coli* DH5α and the encoded protein was induced and isolated according to the manufacturer's protocol (NEB, U.K.). The recombinant protein was digested with factor Xa (NEB, U.K.) to divide maltose binding protein (MBP), the carrier protein, and DSCALT. Further purification was performed by SDS-PAGE and injected into a mouse to raise antibodies (Harlow and Lane 1988). By immunoblot analysis with the anti-DSCALT antibody, the DSCALT was not detected in the supernatant but in the pellet of the crude extract (cytoskeleton fraction) (Fig. 3B, lane 1 and 2), and further, mainly in the fraction of the basal body complex (Fig. 3B, lane 3 and 4). This result was coincided with the immunofluorescence data that caltractin homologues are found in every eukaryotic lineage investigated in association with a variety of flagellar or basal body-associated structures (Melkonian et al. 1992). However, there were another two immunoreactive polypeptides in the supernatant fraction (Fig. 3B, lane 1). Though the nature of these polypeptides is unclear at present, they have a larger molecular mass and weaker immunoreactivity than DSCALT. Thus, the possibility of being a caltractin isologue would be rather low and further study would be needed.

Polypeptides possessing EF-hands are characterized by a high affinity binding of Ca^{2+} with dissociation constants around 1 μM (Klee and Vanaman 1982, Roberts et al. 1986). One characteristic of these proteins is that they bind Ca^{2+} in the presence of SDS. This property alters their conformations and causes their mobility to be accelerated in SDS-PAGE (Burgess 1982). However, Ca^{2+}-bound mobility shift detected in *C. reinhardtii* caltractin (Weber et al. 1994) was not clearly detected in DSCALT (Fig. 3B, lane 3 and 4). The reason for this difference may be due to the fact that DSCALT has only two of the functional EF-hand domains. Recombinant human caltractin has been found to bind 2 mol of calcium per mol of protein under conditions that 4 mol of calcium are bound per mol of *C. reinhardtii* caltractin (Weber et al. 1994). Sequence analysis has also indicated that the yeast CDC31 may bind only 2 mol of calcium per mol of protein. The second and third potential calcium-binding loops do not possess all the proper residues at positions required for calcium binding (Baum et al. 1986, Moncrief et al. 1990, Nakayama et al. 1992). Further studies on the structure and biochemistry of the individual proteins are required to understand how the potential differences in calcium binding properties among members of the caltractin branch of calcium-binding proteins contribute to cellular functions.

Fig. 3 also shows the electrophoretic mobility of the native DSCALT from *D. salina* and the that of bacterial-expressed DSCALT. DSCALT is barely seen in Fig. 3A (lane 5 and 6), but reacted strongly with anti-DSCALT (Fig. 3B, lane 5 and 6). A protein of 42 kDa, seen in Fig. 3A (lane 5 and 6) is the MBP that is the product of factor Xa digestion. Interestingly, the electrophoretic mobility of native DSCALT was more retarded than the bacterial-expressed protein (Fig. 3B, lane 3, 4 and 5, 6). The reason for this result is not certain, but the requirement of some posttranslational modification for eukaryotes is expected. Using PC/GENE sequence analysis software, we found that the sequence of the DSCALT has several putative posttranslational modification sites, including the sites of phosphorylation and *N*-glycosylation. DSCALT also has a conserved serine residue at position 167, which is a potential cAMP-dependent phosphorylation site as in other caltractins (Huang et al. 1988).

Genetic data indicate that caltractin in *C. reinhardtii* and the CDC31 in yeast are required for the normal duplication and segregation of the MTOC in the respective cells. Furthermore, as a calcium-binding component of the NBBC that presumably transduces the changes of cytosolic calcium concentrations, caltractin may function in a signal transduction pathway that affects cell division (Zhu et al. 1992).

D. salina and *C. reinhardtii* have been known to be very close genealogically. Interestingly, we discovered several differences in the caltractin between them as described above. Thus, the molecular cloning of the caltractin from *D. salina* and the distinct characteristics, as reported here, have provided a defined target as well as a tool for studying the nature of caltractin and in future, the mechanism of cell division.

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