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Calcium-Induced Exposure of a Hydrophobic Surface of Mouse ALG-2, Which Is a Member of the Penta-EF-Hand Protein Family[1]

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ALG-2 is a 22 kDa EF-hand type Ca^{2+}-binding protein associated with lymphocyte apoptosis. Comparison of the primary structure of ALG-2 with those of EF-hand type proteins revealed that it belongs to the penta-EF-hand (PEF) protein family including the small subunit of calpain. We established a convenient method for the purification of the recombinant mouse ALG-2 expressed in *Escherichia coli*. The recombinant protein was first pelleted from a lysate in the absence of a Ca^{2+}-chelator, and then extracted with buffer containing EDTA/EGTA followed by purification by conventional column chromatographies. Estimation of the molecular mass by gel filtration suggested that the recombinant ALG-2 occurred as a monomeric form. Ca^{2+}-dependent precipitation was blocked by inclusion of non-ionic detergent Triton X-100, suggesting hydrophobic self-aggregation at high concentrations of the protein. The N-terminal deletion mutant lacking the hydrophobic non-PEF region was found to be more soluble than the wild type in the presence of Ca^{2+}. Analysis using a fluorescent hydrophobicity probe indicated that ALG-2 exposed a hydrophobic surface in a Ca^{2+}-concentration dependent manner, the half-maximal effect occurring at approximately 6 μM. Mg^{2+} was not effective for the conformational change. On Western blotting, ALG-2 was detected in particulate fractions from cultured mammalian cells, suggesting the association of the protein with macromolecules in the cells.

Key words: ALG-2, apoptosis, calcium, calcium-binding protein, EF-hand.

ALG-2 is a 22 kDa EF-hand type Ca^{2+}-binding protein expressed in various mouse tissues and cells (*1*). ALG-2 depletion through expression of its anti-sense mRNA protects mouse T-cell hybridoma 3DO from cell death induced by several stimuli, such as glucocorticoids, T-cell receptors, and Fas triggering. Because caspases are normally activated upon stimulation in ALG-2 depleted cells, the Ca^{2+}-binding protein has been suggested to work downstream of the caspase action (*2*). Comparison of the primary structure of mouse ALG-2 with those of EF-hand type proteins revealed that it belongs to the penta-EF-hand (PEF) protein family including sorcin, grancalcin, and yeast hypothetical protein YG-25, as well as the large and small subunits of calpains (*3*). PEF proteins possess five EF-hand motifs (EF-1 to EF-5), where only some of EF-hands actually bind Ca^{2+}. The EF-5s of all PEF proteins have a two residue-insertion in the Ca^{2+}-binding loop and deviate from the canonical EF-hand motif (*4*). X-ray crystallographic analyses revealed that Ca^{2+}-unbound EF-5 of the calpain small subunit pairs up with EF-5 of the counter subunit to form a dimer (*5-7*). Since sorcin and grancalcin are also known to exist as homodimers (*8, 9*), the EF-5s of these proteins may be involved in dimerization. Among the PEF proteins, clusters of Gly and hydrophobic residues are found in the N-terminal regions of ALG-2, the calpain small subunit, sorcin and grancalcin (*3*). Ca^{2+}-induced translocation from the cytoplasm to membranes is another common feature of this subfamily, whereas such translocation has not been reported yet in the case of ALG-2 (*10-12*). Despite the intriguing physiological function of ALG-2, the biochemical properties of the Ca^{2+}-binding protein have not been well characterized yet. In this paper, we report for the first time the purification of the recombinant ALG-2 expressed in *Escherichia coli* and provide evidence that Ca^{2+} induces a conformational change to expose a hydrophobic surface(s) on the ALG-2 molecule.

MATERIALS AND METHODS

Materials—Most of the molecular biological reagents were purchased from Takara Shuzo (Kyoto), Toyobo (Osaka), or New England Biolabs (Beverly, MA, USA). Proteinase inhibitor Pefabloc was from Boehringer Mann-

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Abbreviations: CaM, calmodulin; CBB, Coomassie Brilliant Blue R250; FBS, fetal bovine serum; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.2); PCR, polymerase chain reaction; PEF, penta-EF-hand; PMSF, phenylmethylsulfonylfluoride; TBS, Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl); TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate.

heim. Other reagent grade chemicals were obtained from Nacalai Tesque (Kyoto) or Wako Pure Chemicals (Osaka). *Escherichia coli* expression vector pET-3d and host strain BL21(DE3)pLysS were from Novagen (Madison, WI, USA). Mammalian expression vector pCXN2 was a kind gift from Dr. Miyazaki.

RT-PCR Cloning of Mouse ALG-2 cDNA—After total mouse liver RNA had been transcribed with RAV-2 reverse-transcriptase using a mouse ALG-2 specific primer, 5′-GATTTGGCATCTTCAGTC-3′, the reaction mixture was boiled for 5 min and then chilled on ice. After centrifugation, 2 μl of the supernatant was used as the template for PCR in a total reaction mixture volume of 100 μl with *Ex Taq* polymerase (Takara, Kyoto) for 25 cycles. The primers used were: 5′-ACCATGGCTGCCTACTCCTACC-3′ [the nucleotide at the −3 position around the translation initiation codon was changed from C to A in order to match Kozak's preferred initiation sequence (*13*)] and 5′-CTGG-TTATACAATGCTGAAGACCA-3′ (containing the complementary sequence of the translation termination codon). The DNA fragment in a 580 bp band was recovered from a 1% agarose gel with QIAEX II (QIAGEN, California, USA). After blunt-ending with T4 DNA polymerase, the fragment was inserted into the *Sma*I site of enforcement cloning vector pKF3 (Takara). The nucleotide sequences of the isolated clones were confirmed with an automated fluorescent sequencer, ABI PRISM 310 (PE Applied Biosystems). One of the clones, designated as pKF-ALG-2, was used for further subcloning.

Construction of an ALG-2 Expression Plasmid—A T7 RNA polymerase system involving the *E. coli* host strain of BL21(DE3)pLysS was employed (*14*). Since the mouse ALG-2 cDNA contained *Nco*I sites at the translation initiation site and the C-terminal region, respectively, the full-length cDNA was inserted into pET-3d stepwisely. First, a synthetic oligonucleotide block corresponding to the C-terminal region was inserted between the *Nco*I and *Bam*HI sites of pET-3d to prepare the intermediate construct, pALG-2-Cterm. The oligonucleotide block contained a stop codon (underlined) overlapping an *Eco*RI site, and overhanging residues of *Nco*I and *Bam*HI sites:

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5′-CATGGTCTTCAGCATTGTATGAATTCG      -3′
3′-      CAGAAGTCGTAACATACTTAAGCCTAG-5′
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Then, the 560 bp *Nco*I fragment corresponding to the remaining ALG-2 cDNA was inserted into the *Nco*I site of pALG-2-Cterm to creat the full-length construct, pET-ALG-2. A deletion mutant lacking the hydrophobic N-terminal residues, ALG-2-ΔN23, was obtained by inserting a fragment obtained on PCR using the upper primer containing an *Nco*I site at the new N-terminal site.

Purification of Recombinant ALG-2—E. coli host strain BL21(DE3)pLysS was transformed with pET-ALG-2. The transformant was cultured at 30°C overnight in ZB-broth (NZ Amine A 10 g/liter, NaCl 5 g/liter) containing 50 μg/ml ampicillin and 12.5 μg/ml chloramphenicol, diluted with ZYG medium (NZ Amine A 10 g/liter, yeast extract 5 g/liter, NaCl 5 g/liter, 0.4% glucose) supplemented with the antibiotics, and then cultured until the mid-log phase at 37°C. Induction was performed by adding IPTG to 0.2 mM, and then cultivation was continued for 2 h.

For large scale purification of ALG-2, 5 mM $CaCl_2$ was added to the culture medium to ensure precipitation of the protein. All purification procedures were performed at 4°C. Harvested cells from one liter-culture were frozen at −20°C, thawed, and then suspended in 50 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF, 0.1 mM Pefabloc, 5 mM benzamidine). Lysis was performed by sonication for 7 min with 20% pulses (Shimadzu USP-300 ultrasonic processor). After centrifugation at 12,000 rpm for 15 min, the pellets were washed once with lysis buffer containing 0.1 mM $CaCl_2$, suspended in lysis buffer supplemented with 5 mM EDTA and 5 mM EGTA, and then mixed for 15 min. After centrifugation, the supernatant was saved and combined with the re-extracted solution. Ammonium sulfate precipitation was performed at 20–35% saturation for the wild type ALG-2 and at 20–45% saturation for the N-terminal deletion mutant, ALG-2-ΔN23, and the pellets were dissolved in 10 ml of buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol) and then dialyzed against the buffer. The dialysate was centrifuged to remove insoluble material. Anion exchange chromatography was performed using HiTrapQ (Pharmacia) with a linear gradient of 50–250 mM NaCl. The eluted sample was further purified by gel filtration chromatography on Superdex75 (Pharmacia) in buffer A containing 150 mM NaCl. The following modified purification procedure was found to be effective. To the dialysates of ammonium sulfate-precipitated solutions was added $CaCl_2$ to 2 mM, the wild type and mutant ALG-2 being precipitated. The pellets were dissolved in buffer A supplemented with 5 mM each of EDTA and EGTA, and then subjected to anion exchange chromatography. Molar extinction coefficients were calculated from the amino acid compositions with the program, ProtParam (http://www.expasy.ch/sprot/protparam.html), and the following values were used for the purified ALG-2 and its N-terminal deletion mutant, respectively: $\varepsilon = 3.9 \times 10^4\ M^{-1} \cdot cm^{-1}$ (ALG-2); $3.6 \times 10^4\ M^{-1} \cdot cm^{-1}$ (ALG-2-ΔN23).

Fluorescence Measurements—All fluorescence measurements were performed with a Shimadzu RF-5300C fluorescence spectrophotometer thermostated at 25°C. Spectra were corrected for the background signal. The purified ALG-2 was extensively dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM 2-mercaptoethanol). A stock solution of 100 mM TNS was dissolved in DMSO, and then stored at −20°C. A diluted solution of 1 mM TNS in 10% DMSO was used for the assay. Fluorescence spectra were obtained after preparation of samples of 1 μM ALG-2, containing 10 μM TNS in buffer B, with $CaCl_2$ added in 600 μl. Excitation was performed at 340 nm with a band width of 5 nm and emission spectra were collected with a band width of 5 nm. The concentration of a trace of Ca^{2+} which might contaminate buffer B was determined to be below 0.5 μM by the dual excitation ratio method using fura-2 (*15*).

Transient Expression of ALG-2—A 0.58 kb *Bam*HI/*Bgl*II fragment from pKF-ALG-2 was inserted into the *Bgl*II site of the eukaryotic expression vector, pCXN2 (*16*), and then plasmids with the sense (pCXN2-ALG-2-S) and antisense (pCXN2-ALG-2-AS) orientations were prepared using a plasmid purification kit from QIAGEN. COS-1 cells (ATCC CRL 1650), an African green monkey kidney derived fibroblast-like cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with

10% heat-inactivated FBS, 4 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C under humidified air containing 5% CO_2. Plasmid DNA, 6 μg, was introduced into COS-1 cells (approximately 1×10^6 cells/10-cm dish) using lipofectin (GIBCO-BRL) in the absence of FBS according to the instruction manual provided, and cultured as described above for 40 h.

After washing with PBS, the cells were scraped off and collected in 1.5 ml tubes. Rough subcellular fractionation was performed by lysing the cells by sonication in the presence of 1 mM $CaCl_2$ or 5 mM EGTA, in 50 μl of low salt buffer (20 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$) containing protease inhibitors (0.1 mM Pefabloc, 25 μM leupeptin, 10 μM E-64, and 1 μM pepstatin), followed by centrifugation at either $100,000\times g$ (Beckman TLA 100 rotor: 60,000 rpm) for 30 min or at $10,000\times g$ (Sakuma M-150 rotor: 12,000 rpm) for 5 min at 4°C.

Western Blotting Analysis—Proteins were separated by SDS-PAGE using 12.5% polyacrylamide gels, and then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) using a semi-dry blotter in the transfer buffer (24 mM Tris, 192 mM glycine, 15% methanol, 0.01% SDS, pH 8.3). Polyclonal antibodies against the purified mouse ALG-2 were raised in rabbits by the conventional method. The antiserum (5 ml) was loaded onto an affinity column of ALG-2 which had been immobilized to a HiTrap NHS-activated column (1 ml, Pharmacia). After washing the column successively with TBS, the washing buffer (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 1% Triton X-100), TBS, and 0.15 M NaCl, antibodies were eluted with 0.1 M glycine-HCl buffer, pH 2.5, and then the eluate was neutralized with Tris. The affinity-purified antibody fraction (approximately 0.2 mg/ml) was diluted 1,000-fold with PBS containing 0.1% Tween 20 and then used as the primary antibody. Peroxidase-conjugated goat anti-rabbit IgG (H+L), purchased from Jackson Immuno-Research Laboratories (West Grove, PA, USA), was used as the secondary antibody. Immuno-signals were detected by the color development method using diaminobenzidine (DAB).

Protein Analyses—Protein concentrations were determined by the method of Bradford (*17*) using a kit from Bio-Rad. The hydrophobicity profile was obtained with the program, ProtScale (http://expasy.hcuge.ch/cgi-bin/protscale.pl), using the normalized consensus hydrophobicity scale (*18*). A window of 9 residues was selected.

RESULTS

Repetitive Domain Structure—As illustrated in Fig. 1A, mouse ALG-2 contains five repetitive sequences, among which EF-3 best matches the canonical sequence of the EF-hand structure proposed by Kretsinger (*4*). Although EF-5 well matches the sequence, it contains a two-residue insertion in its Ca^{2+}-binding loop. From the results of deletion mutant analysis, Vito *et al.* suggested that both EF-1 and EF-3 were necessary for Ca^{2+}-binding in the ^{45}Ca overlay assay involving the glutathione-S-transferase-ALG-2 fusion protein (*1*). Since the Ca^{2+}-coordinating positions, *z*, are occupied by basic residues, EF-2 and EF-4 may not bind Ca^{2+} at all or may bind only weakly. The hydrophobicity profile depicts five hydrophilic troughs where the Ca^{2+}-binding loops are partially overlapped (Fig. 1B).

Expression of Recombinant ALG-2 in E. coli—The addition of IPTG to the culture medium induced ALG-2 efficiently as a major protein in *E. coli* (Fig. 2A). ALG-2 was recovered in the pellet when the lysis buffer containing no chelating agents was used, but most was recovered in the supernatant when the buffer contained EGTA and EDTA (Fig. 2B). Similar results were obtained for the deletion mutant of ALG-2 lacking the hydrophobic 23 N-terminal residues (Fig. 2C). In the cases of the PEF domains of calpain subunits, Ca^{2+} had no effect on the solubility even at 10 mM $CaCl_2$ (Fig. 2, D, E, and data not shown). The presence of 10 mM Mg^{2+} was not effective for precipitation of ALG-2 when the buffer contained 5 mM EGTA to chelate potentially contaminating Ca^{2+} (data not shown). The addition of 1% of non-ionic detergent Triton X-100 to the lysis buffer containing no or 0.1 mM $CaCl_2$ resulted in solubilization of the recombinant ALG-2 and ALG-2-ΔN23

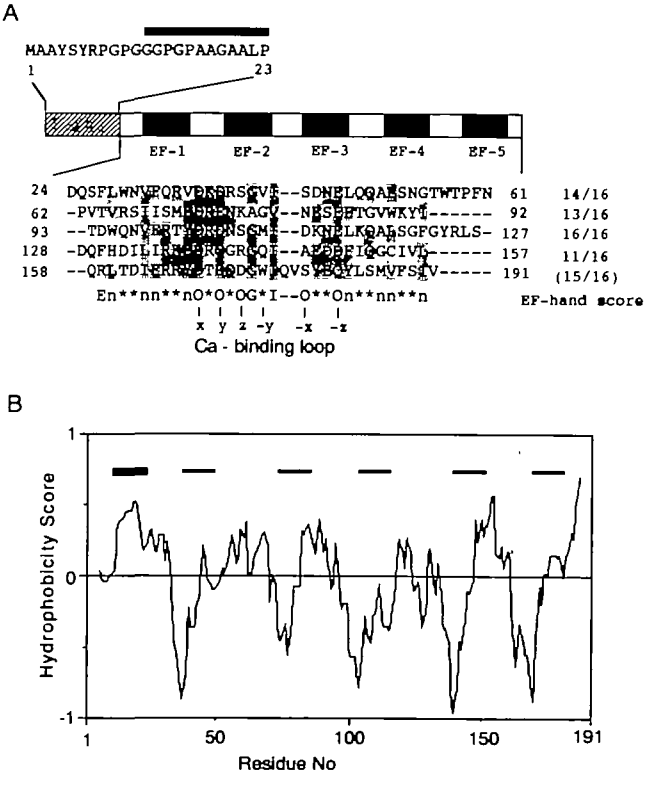

Fig. 1. **Repetitive EF-hand motifs in ALG-2.** A: Five repetitive EF-hand like sequences (EF-1–EF-5). Identical or similar residues in at least three repeats are stippled. The cross-hatched bar above the N-terminal non-repetitive sequence (diagonally hatched region) indicates a relatively hydrophobic region based on the hydrophobicity score shown in panel B. The canonical EF-hand sequence (*4*) contains 16 preferred residues: E, acidic; n, hydrophobic; O, oxygen-containing; G, glycine; I, aliphatic side chains (Ile, Leu, Val, and Met); asterisks, variable residues (often hydrophilic); hyphens, gaps. EF-hand score: number of matched residues. Calcium-coordinating positions are indicated by x, y, z, −y, −x, and −z, where the oxygen atoms of side chains (x, y, z, −z), carbonyls (−y), and water molecules (−x) are usually ligands. B: Hydrophobicity profile. Positive values are relatively hydrophobic. The regions of potential calcium-binding loops are indicated by closed bars. Cross-hatched bar, the N-terminal hydrophobic region.

(data not shown). Taking advantage of the Ca^{2+}-dependent precipitation, we established a convenient method for purification of the recombinant ALG-2. The recombinant protein was first pelleted from the lysate in the absence of a Ca^{2+}-chelator. ALG-2 was solubilized by extraction with buffer containing EDTA/EGTA, this process resulting in efficient purification of the protein (Fig. 3, lane 4). ALG-2 was further purified to homogeneity by ammonium sulfate precipitation (20-35% saturation), anion exchange column chromatography, and gel filtration chromatography. The molecular mass estimated with Superdex75 was about 28 kDa (Fig. 4), suggesting that the purified protein occurred as a monomeric form.

Ca^{2+}-Dependent Precipitation and Inhibition by Triton X-100—The addition of Ca^{2+} to a solution containing 25 μM ALG-2 caused aggregation, and wild type ALG-2 was recovered in the pellet fraction (Fig. 5A). At a lower concentration of ALG-2 (1 μM), however, about half of the ALG-2 remained in the supernatant. The degree of aggregation was dependent on the Ca^{2+} concentration (Fig. 5B). The N-terminal deletion mutant, ALG-2-ΔN23, was more soluble than the wild type ALG-2. By including 1% Triton-X100 in the mixture containing Ca^{2+}, most ALG-2 was recovered in the supernatant even at a higher concentration (25 μM), suggesting a hydrophobic interaction for the precipitation (Fig. 5A). At a much lower concentration (0.1 μM), ALG-2 was adsorbed to plastic tubes even in the absence of Ca^{2+} when the protein was incubated without a non-ionic detergent or carrier protein (bovine serum albumin) (data not shown).

Ca^{2+}-Dependent TNS-Binding—TNS is a commonly used hydrophobic probe which shows significant fluorescence upon binding to hydrophobic sites in proteins (*19*). In the present study, excitation was performed at 340 nm instead of at 365 nm to avoid the Raman effect around 415

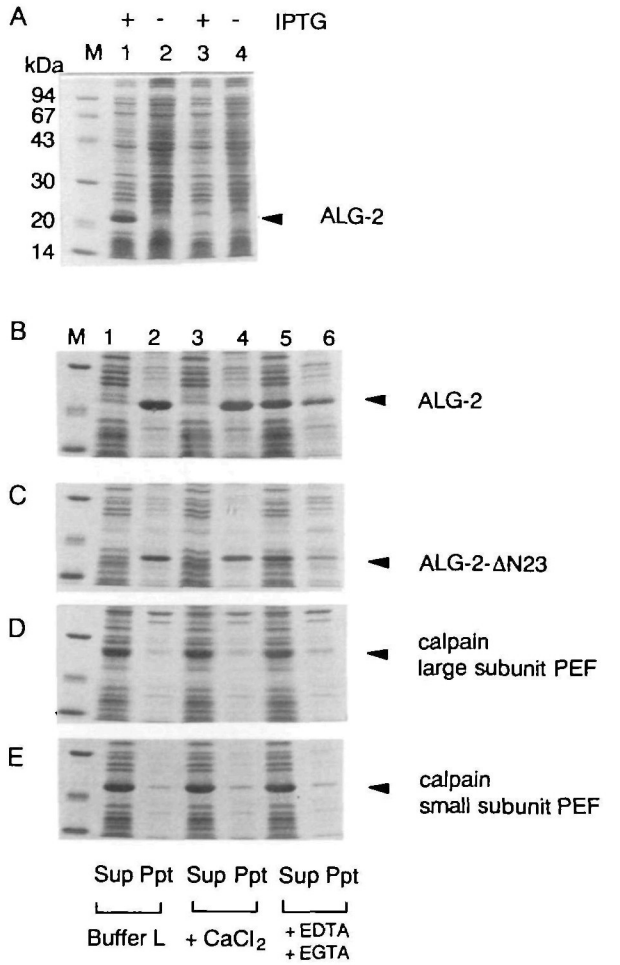

Fig. 2. **Expression of mouse ALG-2 in *E. coli*.** A: Induction of ALG-2 with IPTG in *E. coli* BL21(DE3)pLysS cells harboring expression plasmids was analyzed by SDS-PAGE, followed by CBB staining. Lanes 1 and 2, pET-ALG-2; 3 and 4, pET-3d. B, C, D, and E: Effects of divalent metal ions in the lysis buffer on the solubility of ALG-2 and other PEF proteins. ALG-2 (B), N-terminal deletion mutant ALG-2-ΔN23 (C), the μ-calpain large subunit PEF domain (D), and the calpain small subunit PEF domain (E) were expressed in *E. coli* as described in A and previously (*25, 28*), and the crude lysates were centrifuged at 13,000 rpm for 5 min. Samples of supernatants (Sup) and pellets (Ppt) were analyzed by SDS-PAGE. The lysis buffers used were: buffer L (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) (lanes 1 and 2); buffer L containing 0.1 mM $CaCl_2$ (lanes 3 and 4); buffer L containing 5 mM each of EDTA and EGTA (lanes 5 and 6).

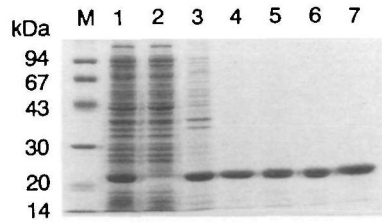

Fig. 3. **Purification of the recombinant ALG-2.** Samples from each purification step were analyzed by SDS-PAGE. Lane 1, total cell lysate; 2, cell lysate supernatant; 3, cell lysate pellet; 4, EDTA/EGTA extract; 5, ammonium sulfate precipitate (20-35% saturation); 6, anion exchange chromatography (HiTrapQ) fraction; 7, Superdex 75 fraction.

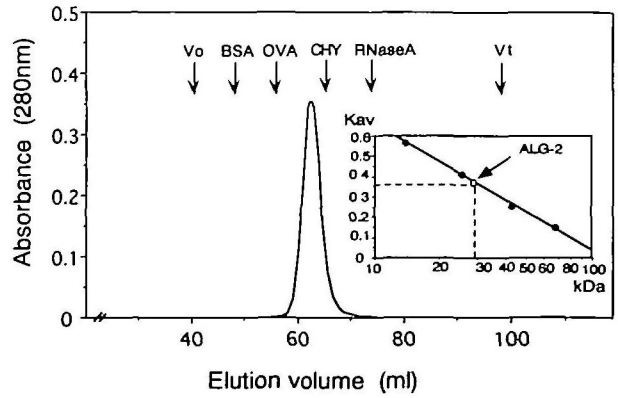

Fig. 4. **Gel filtration chromatography of the recombinant ALG-2.** ALG-2 (0.5 ml) purified by anion exchange chromatography was applied to a gel filtration column (1.6 cm × 60 cm, Superdex 75 HiLoad 16/60) equilibrated with 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 5 mM 2-mercaptoethanol using a HiLoad PSLC3 system (Pharmacia) at the flow rate of 0.8 ml/min. Molecular mass calibration was performed using bovine serum albumin (BSA: 67,000), ovalbumin (OVA: 43,000), chymotrypsinogen A (CHY: 25,000), and RNaseA (13,700). The elution volumes (V_e) of the standard proteins are indicated by arrows. The V_o (exclusion volume) and V_t (total volume) values were obtained by applying blue dextran 2000 and acetone, respectively. Inset: The K_{av} value was calculated as follows: $K_{av} = (V_e - V_o)/(V_t - V_o)$.

nm. In the absence of a protein, TNS showed a very low level of fluorescence background (Fig. 6A). The presence of ALG-2 affected the fluorescence slightly in the absence of Ca^{2+}, and the degree of the enhancement was greater than in the case of CaM (data not shown). Inclusion of Ca^{2+} in the assay mixture caused significant enhancement of the fluorescence intensity, but not in the case of Mg^{2+}. Quite similar spectra were obtained for the N-terminal deletion mutant, ALG-2-⊿N23 (data not shown). The Ca^{2+}-concentration dependency on the fluorescence enhancement was examined by monitoring the emission at 435 nm (Fig. 6B). The effect was observed slightly even at 2 μM and reached the maximal level at 20–50 μM for both the wild type and mutant ALG-2. The addition of EGTA to the mixture containing Ca^{2+} returned the fluorescence to the basal level, suggesting that the Ca^{2+}-dependent conformational change was reversible (data not shown).

Subcellular Localization of ALG-2 Expressed in Mammalian Cells—Transiently expressed ALG-2 in COS-1 cells was detected by Western blotting analysis using anti-ALG-2 antibodies (Fig. 7). The level of the immunoreactive 22 kDa protein was higher in the lysate of pCXN2-ALG-2-S (sense orientation)-transfected cells than in that of pCXN2-ALG-2AS (antisense orientation)-transfected cells, indicating that the 22 kDa band corresponded to ALG-2. When the lysates of the pCXN2-ALG-2-S transfected cells were centrifuged at 100,000×g for 30 min, ALG-2 was detected in the pellets (Fig. 7, lanes 4 and 5), regardless of the absence (lanes 4 and 6) or presence (lanes 5 and 7) of Ca^{2+} in the buffer. To eliminate artifacts due to overexpression of the protein in the transfected cells,

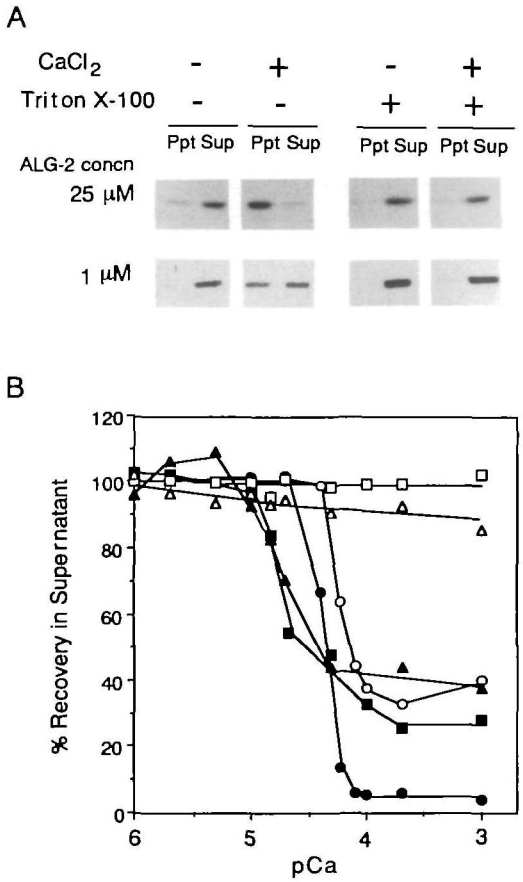

Fig. 5. **Ca^{2+}-dependent precipitation of ALG-2.** A: To the purified ALG-2 at different concentrations (25 μM and 1 μM, 50 μl each), was added $CaCl_2$ to 1 mM in the presence or absence of 1% Triton X-100, followed by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatants and pellets were analyzed by SDS-PAGE, and the protein bands were detected by CBB-staining (25 μM) or Western blotting (1 μM). B: The purified wild type ALG-2 (closed symbols) and the N-terminal deletion mutant, ALG-2-⊿N23 (open symbols), were incubated at 25°C for 10 min with various concentrations of $CaCl_2$, followed by centrifugation. Protein concentrations in the supernatants were determined by measuring the absorbance at 280 nm (●, ○, ■, □) or by the method of Bradford (▲, △). The protein concentrations used were: 1 μM (▲, △), 5 μM (■, □), 25 μM (●, ○).

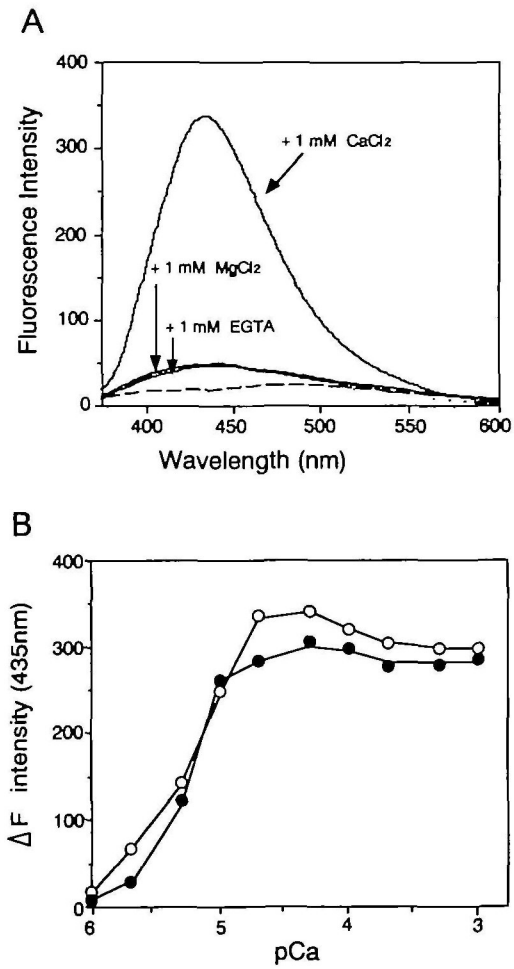

Fig. 6. **Fluorescence analysis of ALG-2 using a hydrophobic probe.** A: Fluorescence emission spectra of TNS affected by ALG-2 and divalent ions. ALG-2 purified by gel filtration chromatography was extensively dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM 2-mercaptoethanol). The assay mixtures contained 10 μM TNS, 1 μM ALG-2, and divalent metal ions or EGTA, as indicated (solid lines). A control spectrum was obtained in the presence of 10 μM TNS and 1 mM $CaCl_2$ without ALG-2 (broken line). Spectra were corrected by subtracting the background spectrum of buffer B without TNS from the spectra of samples containing TNS. Excitation, 340 nm. Ordinate, arbitrary unit of fluorescence intensity. B: Ca^{2+}-concentration dependency of fluorescence enhancement. The increase in the TNS fluorescence intensity at 435 nm was measured at various Ca^{2+} concentrations (pCa = $-\log[Ca^{2+}]$). The intensity at zero Ca^{2+} in the presence of EGTA was subtracted. Wild type ALG-2 (●); N-terminal deletion mutant ALG-2-⊿N23 (○).

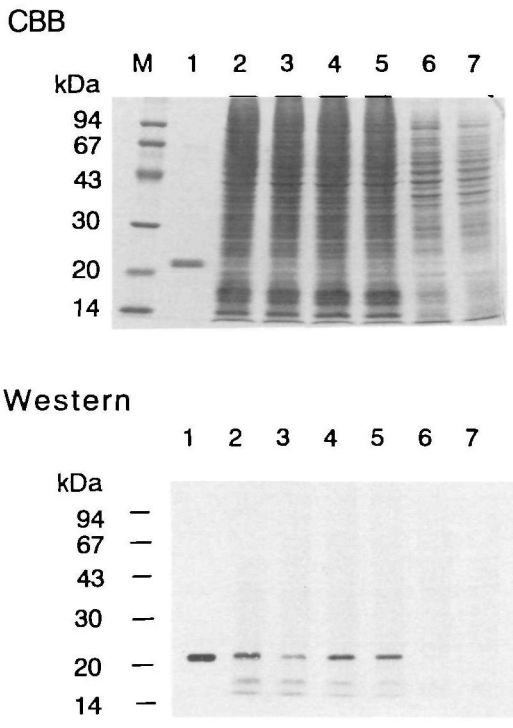

Fig. 7. **Subcellular localization of ALG-2 expressed in COS-1 cells.** COS-1 cells were transfected with ALG-2 expressing plasmids and then cultured for 40 h. The proteins in total cell lysates (lanes 2 and 3), 100,000×*g* pellets (lanes 4 and 5), and 100,000×*g* supernatants (lanes 6 and 7) were separated by SDS-PAGE on a 12.5% gel and then stained with CBB or anti-ALG-2 antibodies (Western). Cell lysates were prepared in the presence of either 1 mM $CaCl_2$ (lanes 5 and 7) or 5 mM EGTA (lanes 4 and 6). Lane 1, recombinant ALG-2 (500 ng for CBB; 50 ng for Western); 2 and 4-7, pCXN2-ALG-2-S (sense)-transfected cells; 3, pCXN2-ALG-2-AS (antisense)-transfected cells.

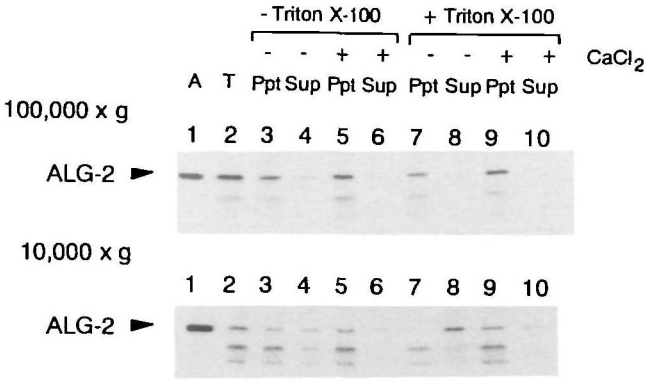

Fig. 8. **Effects of Triton X-100 on the precipitation of ALG-2.** Lysates were prepared from untransfected COS-1 cells as described in the legend to Fig. 7 in the absence (lanes 3-6) or presence (lanes 7-10) of 1% Triton X-100, and then centrifuged at different speeds (100,000×*g* and 10,000×*g*), as indicated. ALG-2 was detected by Western blotting. Lane 1, authentic recombinant ALG-2 (A); 2, total cell lysate (T); odd numbers, precipitates (Ppt); even numbers, supernatants (Sup).

untransfected cells were also used for examination of the subcellular localization (Fig. 8). ALG-2 was recovered in the particulate fractions (100,000×*g* pellets) even in the presence of 1% Triton X-100. A difference in the effect of Ca^{2+} was observed on centrifugation at lower gravity (10,000×*g*) using lysates containing the detergent. While ALG-2 was recovered in the supernatant in the absence of Ca^{2+} (lane 8), more than half of the ALG-2 was recovered in the pellet in the presence of Ca^{2+} (lane 9). Without Triton X-100, only a portion of ALG-2 appeared in the supernatant in the absence of Ca^{2+} (lane 4).

DISCUSSION

Ca^{2+} is one of the key second messengers and is involved in various cellular functions. Ca^{2+} signaling is mediated by a variety of Ca^{2+}-activated enzymes and Ca^{2+}-binding proteins (*20*). The EF-hand motif, *i.e.* the Ca^{2+}-binding helix-loop-helix structure, has been identified in numerous Ca^{2+}-binding proteins (*4, 21*). The number of repetitive EF-hand motifs in protein molecules, regardless of whether or not they are capable of Ca^{2+} binding, ranges from two to eight (*4*). PEF proteins are unique in possessing five potential EF-hand motifs (EF-1-5) (*3*). Although all of the EF-5s of the identified members of the PEF protein family contain two-residue insertions in their potential Ca^{2+}-binding loops and do not match the canonical EF-hand sequence exactly, they may form helix-loop-helix structures, as revealed on X-ray crystallographic analyses of calpain small subunits (*5-7*). The EF-5s of the calpain subunits function as dimerization sites. Although the dimerization sites have not been clarified yet, two other PEF protein family members, sorcin and grancalcin, have been shown to form homodimers (*8, 9*). In the present study, we investigated the properties of the newly discovered PEF protein family member, ALG-2, which was shown to be involved in apoptosis (*1, 2*).

Gel filtration analysis of the recombinant mouse ALG-2 suggested that it occurred as a monomeric form under the conditions used (Fig. 4). The native form of calpain is known to be composed of heterodimers of the large and small subunits with mutually homologous PEF domains (previously called CaM-like domains). Thus, there remains the possibility that ALG-2 forms a heterodimer with an as yet unknown cellular protein with a similar EF-5 sequence.

At high protein concentrations, ALG-2 was precipitated by Ca^{2+} whether or not it was in an unpurified or purified form (Figs. 2 and 5). The precipitation was not due to divalent ion bridges through Ca^{2+} but probably due to Ca^{2+}-induced hydrophobic interactions among ALG-2 molecules: (i) non-ionic detergent Triton X-100 blocked the precipitation (Fig. 5), and (ii) Mg^{2+}, which was not effective in inducing exposure of the hydrophobic surface(s), did not cause precipitation (Fig. 6 and data not shown). The adsorption of ALG-2 to plastic tubes in the presence of Ca^{2+} may also account for the detection of the unnegligible amount of ALG-2 in the pellet fraction at low ALG-2 concentrations (Fig. 5). Since the deletion mutant, ALG-2-ΔN23, was more soluble in the presence of high Ca^{2+}, the N-terminal hydrophobic region may enhance Ca^{2+}-dependent aggregation. At least two molar excess concentrations of Ca^{2+} are required to precipitate ALG-2 (Fig. 5B). The apparently lower concentration of Ca^{2+} derived from cells and the lysis buffer, however, was sufficient to precipitate ALG-2 in the crude *E. coli* cell lysate (30-60 μM ALG-2 in the lysate, estimated from the amount of the expression

product). ALG-2 might interact with membrane proteins or membranes which reduce the required concentration of Ca^{2+} for hydrophobic interactions.

EF-hand proteins have been shown to expose hydrophobic surfaces in a Ca^{2+}-dependent manner (*22, 23*). TNS is a good hydrophobic probe for studying such Ca^{2+}-induced conformational changes of EF-hand proteins (*24, 25*). As revealed in the present study, ALG-2 also showed Ca^{2+}-dependent exposure of a hydrophobic surface(s) (Fig. 6). The similarity in the profiles of the Ca^{2+}-dependent TNS fluorescence enhancement between the wild type and mutant ALG-2 suggests that the PEF domain itself contains the TNS-interacting hydrophobic site whose formation is mediated by Ca^{2+}. The observed Ca^{2+}-dependent conformational change, however, did not exactly correlate with the Ca^{2+}-dependent precipitation. The half maximal effect values of the Ca^{2+} concentrations required for the TNS-monitored conformational change (6 μM) and the precipitation (20 μM) were different for 1 μM ALG-2, and no precipitation was observed for mutant ALG-2-ΔN23. Moreover, the addition of TNS at least up to 1 mM did not inhibit Ca^{2+}-dependent precipitation (data not shown). Thus, Ca^{2+}-dependent hydrophobic sites (low-affinity TNS-binding, not observed under the present conditions) in addition to the N-terminal region might be important for the precipitation.

In the transfected COS-1 cells, ALG-2 was recovered in the particulate fraction (100,000 $\times g$ pellet) regardless of the presence or absence of Ca^{2+} (Fig. 7), even in the presence of Triton X-100 (Fig. 8). In contrast, ALG-2 was recovered in the supernatant of the detergent-containing lysate on centrifugation at lower gravity (10,000 $\times g$) in the absence of Ca^{2+} (Fig. 8, lane 8), but recovered mostly in the pellet in the presence of Ca^{2+} (lane 9). Thus, the Ca^{2+}-binding protein may associate with Triton X-100 resistant macromolecules which aggregate in the presence of Ca^{2+}. Alternatively, ALG-2 associated with 100,000 $\times g$ precipitable macromolecules may bind to 10,000 $\times g$ precipitable macromolecules in a Ca^{2+}-dependent manner. At present, we do not know what the Triton X-100 resistant macromolecules are, but they might be cytoskeletal proteins or associated proteins. A portion of ALG-2 was also detected in the precipitate at 600 $\times g$ in the presence of Ca^{2+} but less in the absence of the ion (data not shown). In order to determine the exact subcellular localization of ALG-2 in untransfected normal cells, specific antibodies good enough for immunocytochemical studies are required. Unfortunately, unnegligible cross-reactions with cellular proteins occurred with the anti-mouse ALG-2 polyclonal antibodies prepared in the present study. The low titer of the antiserum may be due to the high degree of homology among mammals (only two amino acid residues are different in the ALG-2 sequence between mouse and human) (Kawai *et al.*, unpublished observation). Preparation of high-affinity specific polyclonal antibodies or mouse monoclonal antibodies against human ALG-2 would aid clarification of the subcellular localization of ALG-2.

EF-hand proteins are known to interact with cellular proteins in a Ca^{2+}-dependent manner (*20, 21*). The target proteins of calmodulin form basic amphiphilic α-helices (*26*). In contrast, the PEF domains (previously termed CaM-like domains) of calpain subunits interact with the endogenous inhibitor protein calpastatins (*27, 28*), which form potentially acidic amphiphilic α-helices (*29*). While sorcin has been shown to interact with the N-terminal domain of synexin (annexin VII) in a Ca^{2+}-dependent manner (*30*), it also associates with the cardiac ryanodine receptor in the absence of Ca^{2+} (*31, 32*), suggesting that the protein plays a role in Ca^{2+} mobilization as well as contributing to the multi-drug resistance of cancer cells (*33*). The purified recombinant ALG-2 will facilitate the finding of target cellular proteins of ALG-2 to elucidate its physiological function. The recombinant protein should also be useful for screening anti-apoptotic drugs which inhibit the Ca^{2+}-dependent hydrophobic exposure *in vitro*.

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