Biochemical Characterization of Two Analogues of the Apoptosis-linked Gene 2 Protein in Dictyostelium discoideum and Interaction with a Physiological Partner in Mammals, Murine Alix*

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Two homologues, Dd-ALG-2a and Dd-ALG-2b, of the mammalian calcium-binding protein ALG-2 (apoptosis-linked gene 2) have been characterized in the cellular slime mold Dictyostelium discoideum. Fluorescence titrations showed that both proteins bind calcium ions with affinities (Ca²⁺)₀.₅ of 30 and 450 μM, respectively, at sites specific to calcium. Calcium ion binding resulted in changes of conformation associated with the unmasking of hydrophobic regions of the proteins. Surface plasmon resonance analysis showed that Dd-ALG-2a homodimers formed (K₅ of 1 μM) at calcium ion concentrations similar to those necessary for Ca²⁺-induced conformational changes. Deletion of the hydrophobic N-terminal sequence or EF-hand 5 of Dd-ALG-2a prevented dimerization. The Dd-ALG-2b homodimer was not detected, and the Dd-ALG-2a/Dd-ALG-2b heterodimer formed only when Dd-ALG-2b was the immobilized partner. Murine Alix formed a heterodimer (K₅ = 0.6 μM) with Dd-ALG-2a but not with Dd-ALG-2b, and the interaction strictly depended upon calcium ions. The N-terminal construct of Dd-ALG-2a lost its interaction capacity with mouse Alix. The genes encoding both proteins, Dd-alg2a and Dd-alg2b, were expressed in growing cells. The levels of mRNA were at a maximum during aggregation (4–8 h) and decreased rapidly thereafter. In contrast, the levels of proteins remained fairly stable. Dd-ALG-2a and Dd-ALG-2b were found to be dispensable for growth and development, based on the finding that single Dd-alg2a⁻ or Dd-alg2b⁻ and double Dd-alg2a⁻/Dd-alg2b⁻ mutant cell lines showed normal growth in axenic medium or on bacterial lawns and exhibited unaltered development.

Programmed cell death (PCD), a physiological suicide process, is implicated in the development and maintenance of an organism’s integrity. Its most common phenotype, apoptosis, is generally considered as the paradigm of PCD in multicellular organisms. However, PCD is no longer confined to situations initially termed as apoptosis. This is particularly so in unicellular organisms where morphological diversity and multiple mechanisms of PCD have been observed. The nature of executors involved in PCD has also broadened, with the discovery of caspase-independent pathways and autophagic processes (1).

In the cellular slime mold, Dictyostelium discoideum, the developmental cycle includes a multicellular phase that involves both differentiation and morphogenesis (2, 3). Within the multicellular structure that forms from the initial aggregation of starving individual cells, prestalk cells undergo a PCD process that leads to the formation of a stalk comprising dead vacuolated cells (4) supporting a mass of viable spores. In an in vitro system that permits differentiation without morphogenesis, cells committed to PCD were unable to re-grow and showed specific morphological features, including massive vacuolization and cytoplasmic and focal chromatin condensations (5, 6). Study of this type of developmentally regulated cell death in eukaryotes, which diverged early in the development of life, might reveal underlying mechanisms of PCD that are conserved as such, or slightly modified, in more complex organisms. The sequencing of the genome of D. discoideum is almost completed (7). Investigating the amebal genome shows that only a limited number of the proteins involved in mammalian PCD have homologues in D. discoideum. In addition to the “apoptosis inducing factor” or AIF and a putative paracaspase (8, 9), we have identified D. discoideum homologues of the apoptosis-linked gene 2 (ALG-2) and of its binding partner, ALG2-interacting protein X, Alix (2). In mammalian cells, both proteins have been determined as being components of the apoptotic machinery (10–12). The molecule, ALG-2, is a calcium-binding protein of the penta-EF-hand family, which includes sorcin, peflin, granalcadin, and calpain (13–17).

During the development stage induced by starvation in D. discoideum, calcium ions have been described as regulating cell type differentiation (18–20). Even though a number of calcium-binding proteins have been characterized in this organism, among which are CBP1–4, CAF1, CaM, and CalB (21–27), type differentiation (18–20). Even though a number of calcium-binding proteins have been characterized in this organism, among which are CBP1–4, CAF1, CaM, and CalB (21–27), none so far has been assigned a specific role as a mediator of 6-sulfonate; ALG-2, apoptosis-linked gene 2; Alix, apoptosis-linked gene 2-interacting protein X; MBP, maltose-binding protein; MBP-ALG-2a, maltose-binding protein-tagged Dd-ALG-2a; MBP-ALG-2b, maltose-binding protein-tagged Dd-ALG-2b; His₅-ALG-2b, polyhistidine-tagged Dd-ALG-2b; PBS, phosphate-buffered saline; DIG, digoxigenin; MES, 4-morpholineethanesulfonic acid.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF358913 and AF358911 for Dd-alg2a and AF358914 and AF358912 for Dd-alg2b.

The abbreviations used are: PCD, programmed cell death; IPTG, isopropyl-β-D-thiogalactopyranoside; TNS, 2-π-toluidino)naphthalene-6-sulfonate; ALG-2, apoptosis-linked gene 2; Alix, apoptosis-linked gene 2-interacting protein X; MBP, maltose-binding protein; MBP-ALG-2a, maltose-binding protein-tagged Dd-ALG-2a; MBP-ALG-2b, maltose-binding protein-tagged Dd-ALG-2b; His₅-ALG-2b, polyhistidine-tagged Dd-ALG-2b; PBS, phosphate-buffered saline; DIG, digoxigenin; MES, 4-morpholineethanesulfonic acid.

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calcium ion signaling during this phase of differentiation. In view of the proposed involvement of Dd-ALG-2 in apoptosis in higher eukaryotes, we decided to examine whether D. discoideum multicellular development was dependent upon this calcium-binding protein.

In this paper, we describe the calcium-dependent biochemical properties of the two D. discoideum homologues of mammalian ALG-2 that we have identified and their interaction with mammalian Alix. While this work was in progress, the two ALG-2 homologues were independently identified (28). Our report provides original and complementary biochemical data pertaining to their physiological role with respect to Alix and describes ALG-2 knock-outs and their characterization during vegetative life and the developmental cycle.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phenylnleucylsulfonyl fluoride, leupeptin, pepstatin, aprotinin, and IPTG were from Roche Diagnostics. TNS was from Sigma-Aldrich. pMAM-C2, pQE-30, and pGEX-6P-2 expression vectors were from New England Biolabs, Qiagen, and Amersham Biosciences, respectively.

**Cell Culture Conditions and Differentiation**—All experiments were done using wild-type developing D. discoideum KAX-3 and KJ10, a thymidine auxotroph (29), as parental strains. Cells were grown at 21°C in cultures shaken at 180 rpm in HL5 medium supplemented when necessary with 7.5 μg/ml blasticidin, 20 μg/ml G418, or 100 μg/ml thymidine. Developmental phenotypes were studied after plating cells on non-nutritive Na,KP-buffered agar plates (30).

**Cloning and Sequencing the D. discoideum ALG-2 genes**—For both alg2a and alg2b genes, the sequencing of the coding DNAs was performed on non-nutritive agar plates including introns were obtained after PCR experiments with forward primers (5'-TCAAATGGTGCTCAGTCATGTAACA and 5'-TGGAAATGATGCCGATGGAATTTG) and reverse primers (5'-TGGAAATGATGCCGATGGAATTTG and 5'-TGGAAATGATGCCGATGGAATTTG) (from Oligo Express, Paris, France) for Dd-ALG-2a and Dd-ALG-2b, respectively. Primers were designed on the basis of relevant clones in the Dictyostelium cDNA data base (www.dipteranet.de/pcDNAproject.html). The genomic and cDNA sequences have been deposited at GenBank® under accession nos. AF358913 and AF358911 for Dd-ALG-2a and AF358914 and AF358912 for Dd-ALG-2b, respectively.

**Generation of Mutants and Plasmid Constructs**—The single Dd-alg2a and Dd-alg2b null mutants were made in KAX-3 strain by inserting the blasticidin S resistance cassette bar (31) at position 294 (in Dd-alg2a) and 295 (in Dd-alg2b) of the genomic DNA. Two independent double knock-out mutant, a Dd-alg2a-deficient strain was first generated in the KJ10 strain by disruption of the coding sequence with the THY1 gene-containing cassette (29). This was then used to introduce the Dd-alg2a gene knock-out construct containing the bar cassette. Transformants were cloned by plating cells on SM-agar plates in the presence of Klebsiella aerogenes. Potential gene knock-out clones were first analyzed by Western blot and confirmed by Southern blot.

The cDNA sequences (except for the internal methionine) coding for full-length or truncated proteins ALG-2a, ALG-2aNT (amino acids 28–197), ALG-2aEF5 (amino acids 3–175), ALG-2b, and ALG-2bEF5 (amino acids 2–183) were subcloned in-frame with the maltose-binding protein (MBP), into the pMAL-c2 expression vector (MBP-ALG-2a and -2b) or with the amino-terminal polyhistidine tag contained in the pQE-30 expression vector (His6-ALG-2b). In all cases, PCR amplification was used to create the appropriate subcloning sites and the constructs were then verified by sequencing. Recombinant proteins were expressed in Escherichia coli strain BL21 cells.

**Purification of Recombinant Proteins**—BL21 bacteria expressing the various recombinant Dd-ALG-2 proteins were grown overnight at 37°C in Luria Broth, diluted to 1/50 in Terrific Broth, and grown at 37°C to A600 of 0.6. IPTG was then added at a final concentration of 1 mM and cell growth was monitored for 3 h. Cells were then harvested by centrifugation at 10,000 × g for 15 min, and the bacterial pellets frozen at –20°C use. The MBP-fused proteins were expressed at high levels (about 50 mg/liter of bacterial suspension) and in soluble forms. In contrast, His6-ALG-2b was expressed at a high level but always as inclusion bodies. To purify recombinant MBP-tagged proteins, the bacterial pellets were thawed in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA (buffer A) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 20 μg/ml pepstatin and leupeptin, and 0.5% Triton X-100. All steps were carried out at 4°C. Suspensions were sonicated for 3 min and centrifuged for 1 h at 200,000 × g. Supernatants were mixed with amyllose beads washed in buffer A and rotated on a wheel overnight at 4°C. After extensive washing with buffer A, MBP-tagged proteins were eluted with buffer B containing 10 mM maltose and 1 mM EDTA. The recombinant proteins were >98% pure (see Fig. 2) without any additional purification step.

**Mouse Alix**—N-terminal GST (glutathione S-transferase)-tagged mouse Alix, cloned into vector pGEX-6P-2, was expressed in BL21 bacteria and induced with 1 mM IPTG, then grown for another 15 h at 20°C. After harvesting cells, fusion proteins on glutathione-Sepharose 4B, the GST tag was cleaved with PreScission protease (Amersham Biosciences) to obtain Alix. The protein appeared as a single band on SDS-PAGE.

**Antibodies and Western Blots**—His6-ALG-2b was expressed in BL21 bacterial strain (see above). Purified inclusion bodies were dissolved in 6 M urea, purified on nickel-nitrioltriacetic acid-agarose (Qiagen) and extensively dialyzed against 10 mM Tris-Cl, pH 8.0. The preparation was checked for homogeneity by SDS-PAGE, lyophilized, and injected into rabbits to raise polyclonal antibodies. Western blot analysis showed that the immune serum recognized both Dd-ALG-2a and -2b. To follow the expression of the endogenous Dd-ALG-2 throughout development, protein samples were prepared from developing amebae of the wild-type strain, KAX-3, or single knock-out strains, and done using non-nutritive Na,KP-buffered agar plates for 4, 8, 12, 16, 20, or 24 h. At each time, 6 μg of total RNAs were separated by electrophoresis on formaldehyde- containing agarose gel, transferred to a nylon blotting membrane, and hybridized with a specific digoxigenin (DIG)-labeled RNA probe. DIG-labeled RNA probes were generated according to the manufacturer’s instructions (Roche Molecular Biochemicals). The RNA probes for Dd- alg2a and -2b corresponded to their full-length sequence. Hybridization was performed in DIG-Easy buffer at 50°C. The mRNAs were detected after binding of anti-DIG antibodies coupled to alkaline phosphatase with CDP-Star® as substrate.

**Surface Plasmon Resonance Measurements and Data Evaluation**—Real-time analysis of the Ca2+-dependent interactions between the two Dd-ALG-2 isoforms and mouse Alix was performed at 25°C on a BIAcore instrument. Proteins were diluted to 10 μg/ml in 10 mM sodium acetate, pH 5.0, and coupled to the carboxymethylated dextran surface of a CM5 sensor chip using a N-hydroxysuccimide/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide coupling mixture. The amount of immobilized protein was determined using 3000 and 4000 resonance units. Running buffer for protein interaction contained 150 mM NaCl, 10 mM HEPES, pH 7, 0.05% Tween 20, and varying amounts of Ca2+ and 3 μM TNS was included to exclude any Ca2+ signals. The dissociation rate constant, determined for samples of 5 μM. To obtain blank sensorgrams, for subtraction of bulk refractive index background, equal volumes of each protein were injected over a surface with immobilized MBP alone. Regeneration of the surfaces was carried out by injecting 5 μl of 8 M urea after checking that binding isotherms were unaffected by such a denaturation step.

Sensorgrams were analyzed by nonlinear least square curve fitting. A single-site binding model was used for kinetic analysis of interactions. The equation \( R_c = R_{max}(1 - \exp(-kR(t - t_0))) \) was used for the association phase; and \( k = k_C + k_0 \), where \( C \) is the concentration of injected analyte. The rate constants \( k_C \) and \( k_0 \) were determined from the slope of \( k_C \) versus \( C \). The \( k_0 \) constant was also obtained from the analysis of the dissociation phase using the equation \( R_c = R_{off} \exp(-k_0(t - t_0)) \). The parameter \( R_{off} \) is the amount of ligand bound at time \( t \) and \( R_{off} \) is the amount of ligand bound at time \( t_0 \) corresponding to the beginning of the dissociation phase. The mean value obtained from a series of injections was taken as dissociation constant \( k_0 \). The values of \( k_C \) derived directly from dissociation curves or from secondary association plots were in agreement. The apparent dissociation constant, \( k_{app} \), calculated from the two-site model \( k_{app} = k_C/k_0 \).

In the case where interactions approached equilibrium, the dissociation constant \( K_D \) was also determined directly from equilibrium levels of the analyte binding using the equation \( R_c/C = (R_{max} - R)/K_D \). The parameter, \( R_{max} \), was the maximal binding capacity of the immobilized ligand and \( K_D \) was derived from the slope of the \( R_c/C \) versus \( R_{max} \) plot.
MAXC (version 2.10) program (33) available at www.stanford.edu/~cappton/webmaxx2.htm.

Fluorescence Measurements—Two types of fluorescence experiments were performed to follow changes of protein conformation: intrinsic fluorescence alteration and 2-(p-toluidino)naphthalene-6-sulfonate (TNS) fluorescence enhancement. All fluorescence measurements were recorded at 21 °C with a Spex Fluoromax spectrophotofluorometer in a buffer made of 150 mM NaCl, 25 mM HEPES, pH 7.5. Intrinsic fluorescence of the MBP-ALG2 isosforms (40 μg/ml, −0.6 μM) was measured using excitation and emission wavelengths set at 295 and 348 nm, respectively. In control experiments, it was checked that the addition of Ca²⁺ or EGTA had no effect on the shape of the fluorescence spectra of the proteins and solely affected the fluorescence intensity. TNS fluorescence was measured in 2.5 ml of buffer containing 100 μg of the protein of interest to which 10 μM TNS was added. Excitation and emission wavelengths were set at 325 and 427 nm, respectively. Fluorescence variations were expressed as ΔFF/F₀ in percentages.

Subcellular Fractionation—For subcellular fractionation, 1.5 × 10⁸ cells were homogenized with a cell cracker in 3 ml of buffer: 0.25 M sucrose, 0.5 mM EDTA, 25 mM MES-KOH, pH 6.5, and a mixture of protease inhibitors. Unbroken cells and nuclei were recovered by first centrifuging the homogenates at 1,000 × g for 5 min. The postnuclear supernatant was then loaded onto a 21-ml 24% (v/v) self-forming Percoll gradient in 0.11 M KCl, 0.5 mM EDTA, 10 mM HEPES, pH 7.5; spun for 1 h at 18,000 rpm in a Kontron TFT70 rotor; and eluted in 2-ml fractions.

Immunofluorescence—Immunofluorescence observations were made from D. discoideum cells carrying C-terminal e-Myc-tagged constructs of ALG-2a and -2b under the control of Act15 promoter (pEXP4- vector). Approximately 2 × 10⁹ amebae were allowed to adhere onto coverslips for 10 min and fixed in a solution of 40 mM MES-Na, pH 6.5, 4% paraformaldehyde for 10 min at 20 °C. They were then permeabilized in 0.2% Triton X-100–40 mM MES-Na, pH 6.5, for 2 min. After several washes in PBS, 0.5% bovine serum albumin, cells were incubated with the primary anti-e-Myc antibody (Roche Molecular Biochemicals, 4 mg/ml stock, dilution 1/500) for 1 h. The secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse antibody, Jackson Immunoresearch) was added at a dilution of 1/200 after three washes in PBS plus 0.1% Tween 20. Finally the samples were washed extensively in PBS-Tween 20, mounted on glass slides, and observed in a Zeiss Axiosplan microscope.

RESULTS

D. discoideum Possesses Two Homologues of Apoptosis-linked Gene 2 (alg-2).—The ALG-2 protein, first described by Vito et al. (11), belongs to the family of calcium-binding proteins with penta-EF-hands. The D. discoideum expressed sequence tag data base was searched for homology with murine ALG-2. We found several clones corresponding to two distinct cDNA sequences. There were 10 members in a first group and 5 members in a second group with significant probability scores. The full sequences of the two D. discoideum gene, Dd-ALG-2a (GenBank™ accession nos. AF358914 and AF358912; this work) and Dd-ALG-2b (GenBank™ accession nos. AF358914 and AF358912; this work). Identities between the two proteins are boxed, and homologies are indicated by a gray background. Secondary structure helices a₁—a₈ are placed according to the three-dimensional structure (41), and the five putative EF-hands are indicated as text between the bars. B and C, homology models for Dd-ALG-2a (B) and Dd-ALG-2b (C) were calculated by Swiss Model and visualized with Deep View Swiss-PDB Viewer (40) using the published structure of mouse ALG-2 (1HQV) as a template (41). Structures were colored according to secondary structure succession, with a transition from blue to red from the most N-terminal helix to the C-terminal end.

Expression and Purification of MBP-ALG-2a/2b Constructs—All the constructs used to examine protein-protein interactions (Dd-ALG-2a, Dd-ALG-2aΔEF5, Dd-ALG-2aNter, Dd-ALG-2b, and Dd-ALG-2bΔNter) were expressed in E. coli with an MBP tag, a strategy that allowed us to obtain soluble proteins with a high yield. Proteins purified on amylose columns by affinity were always more than 98% pure in one step,
and they were used without any further purification (Fig. 2, lanes 1–5). For immunization purposes, a polyhistidine (His$_6$) tag was preferred to the MBP tag. The protein was produced as inclusion bodies. His$_6$-ALG-2b was solubilized in 8 M urea, purified on a nickel-nitrilotriacetic acid-agarose column (Fig. 2, lane 6), and lyophilized before injection into rabbits.

**Ca**$^{2+}$-Induced Conformational Changes of MBP-ALG-2a and -2b—To detect conformational changes because of calcium binding to Dd-ALG-2a and -2b, the intrinsic fluorescence spectra of both MBP-tagged proteins were recorded in the presence of either EGTA or Ca$^{2+}$. In both cases, maximal excitation and emission occurred at 283 and 348 nm, respectively. The presence of calcium ions resulted in a rapid increase in the fluorescence emission intensity of MBP-ALG-2a. This could be reversed by addition of an excess of EGTA (Fig. 3A). Identical data (not shown) were obtained with MBP-ALG-2b. These results reveal for both proteins conformational changes sensed by the sensor chip was unable to interact with MBP-ALG-2a (see above) to induce a conformational change and to expose hydrophobic regions.

The lipophilic fluorescent probe, TNS, was used to evaluate the exposure of ALG-2 hydrophobic regions upon Ca$^{2+}$-induced change of conformation (37). In the absence of calcium ions, the fluorescence of TNS alone was very weak. Addition of MBP-ALG-2a led to only a small increase. Addition of calcium ions resulted in a large increase in TNS fluorescence and indicates that TNS is bound to newly exposed hydrophobic region(s) of MBP-ALG-2a (Fig. 3B). Addition of EGTA fully reversed the fluorescence induced by the calcium ions. Similar results were obtained for MBP-ALG-2b (data not shown).

The affinity of MBP-ALG-2a and -2b for calcium was estimated from intrinsic and TNS fluorescence experiments, where the variations of fluorescence intensity were recorded as a function of calcium ion concentration. Intrinsic and TNS fluorescence responses exhibited a similar trend and gave an apparent $K_D$ for Ca$^{2+}$ in the 15–40 $\mu$M range for MBP-ALG-2a and in the 300–600 $\mu$M range for MBP-ALG-2b (Fig. 3C). These data indicate that the observed conformational changes and exposure of hydrophobic stretches are likely to be related to binding of calcium ions at the same sites. The affinity of MBP-ALG-2b for calcium was roughly 10–20 times lower than that of Dd-ALG-2a. These values are noticeably higher than the affinity (6 $\mu$M) for Ca$^{2+}$ reported for mouse ALG-2 (37). Magnesium ions did not replace calcium ions in inducing conformational changes. Adding 2 mM Mg$^{2+}$ to the assay buffer did not affect the titration curves. These observations indicate that the binding sites are specific to calcium ions.

**MBP-ALG-2a and -2b Associate in the Presence of Calcium Ions**—The ability of Dd-ALG-2a and -2b to form dimers was studied using surface plasmon resonance spectroscopy. The results (Fig. 4A) indicate that MBP-ALG-2a in the analyte binds efficiently to immobilized MBP-ALG-2a in the presence of Ca$^{2+}$. This does not occur in the presence of EGTA. Furthermore, the value of $k_{off}$ measured during the dissociation phase increased by a factor of ~5 when the calcium ions were chelated with EGTA. Sensograms recorded at different calcium ion concentrations were used to determine the calcium ion concentration for oligomerization of MBP-ALG-2a. A plateau in the values of the resonance units at equilibrium ($R_{eq}$) was observed for calcium ion concentrations higher than 100 $\mu$M. Half-maximum binding was obtained at a concentration of 13 ± 4 $\mu$M ($n = 7$) (Fig. 4B). Such Ca$^{2+}$ concentrations agree with those needed in MBP-ALG-2a (see above) to induce a conformational change and to expose hydrophobic regions.

The kinetic parameters of the MBP-ALG-2a dimerization were determined by recording sensograms at different protein concentrations with a fixed saturating Ca$^{2+}$ concentration (100 $\mu$M). The association phases from a representative series of experiments performed with six MBP-ALG-2a concentrations (0.07–1.53 $\mu$M) were determined from a nonlinear least squares fit to give a value for $k_a$ at each concentration. A plot of $k_a$ as a function of MBP-ALG-2a concentration gave the association rate, $k_a = 1.9 \times 10^{-3}$ s$^{-1}$ (Fig. 4C). Analysis of the dissociation phases gave a value of $k_{off}$ = 0.2 ± 0.1 $\mu$M $n = 7$). As the binding of MBP-ALG-2a is close to equilibrium, the affinity of the interaction could be determined from a $R_{eq}/C$ versus $R_{eq}$ plot. A representative plot gave a $K_D$ value of 0.86 ± 0.05 $\mu$M ($n = 7$). This is consistent with the $K_D$ value derived from the kinetic analysis of the data (Fig. 4D) described above.

In contrast to these results, similar homotypic experiments with MBP-ALG-2b at Ca$^{2+}$ concentrations (and up to 5 mM) for which Ca$^{2+}$ binding to MBP-ALG-2b occurred, showed no association between immobilized and soluble proteins. Nevertheless, when immobilized on the sensor chip, MBP-ALG-2b in the presence of Ca$^{2+}$ was able to form heterodimers with MBP-ALG-2a. From the plot of $k_a$ versus MBP-ALG-2a concentration, an apparent equilibrium dissociation constant, $K_D$, was calculated from the ratio $k_{off}/k_{on}$, and was 2.0 $\mu$M for the MBP-ALG-2a/MBP-ALG-2b interaction (Fig. 4C). The dissociation constant derived from a $R_{eq}/C$ versus $R_{eq}$ plot was 1.7 ± 0.2 $\mu$M ($n = 6$) for that interaction (data not shown). These values are similar to those found for the homotypic MBP-ALG-2a interaction. Surprisingly, in a mirror experiment, MBP-ALG-2b in the analyte was unable to interact with immobilized MBP-ALG-2a.

**ALG-2 Proteins and Calcium Signaling in Dictyostelium**—The primary sequence data and homology modeling obtained for Dd-ALG-2a and -2b clearly showed that they belong to the penta EF-hand family. The strong homology with murine ALG-2 suggests that the functional interaction could well be conserved in a cross-species assay. To examine whether...
the amebal proteins represent genuine orthologues of mammalian ALG-2 proteins, we explored their capacity to bind mouse Alix, a protein previously shown to directly interact with the ALG-2 protein (10, 12). Mouse Alix was immobilized on the sensor chip, and MBP-ALG-2a or -2b were added in the analyte fluid. MBP-ALG-2a was found to bind with increasing concentration, and protein-protein interaction was dependent on the calcium ion concentration (Fig. 5). From the plot of $k_\text{i}$ versus $C$ (Fig. 5, inset), we calculated that mouse Alix was able to bind MBP-ALG-2a with an apparent affinity constant of 0.6 mM. No interaction was detected when MBP-ALG-2a was immobilized on the sensor chip, and with Alix present in the analyte. Mouse Alix bound MBP-ALG-2aΔEF5 with an apparent affinity constant of 1.2 mM (Fig. 5, inset), but no interaction with Alix was observed for MBP-ALG-2aΔNter. For MBP-ALG-2b, or its truncated form MBP-ALG-2bΔEF5 in the analyte fluid, there was no interaction with immobilized Alix. This was independent of the presence or absence of calcium ions.

Intracellular Localization of Dd-ALG-2a and -2b—Protein localization was determined by subcellular fractionation and immunocytochemistry. Immunofluorescent staining of overexpressed Dd-ALG-2a was found to be associated with both the cytosol and membrane/cytoskeleton (Fig. 6A). Dd-ALG-2b showed a similar localization together with some nuclear enrichment (Fig. 6A). Subcellular fractionation of a post-nuclear extract on a Percoll gradient (Fig. 6B), Dd-ALG-2a/2b were mainly recovered in the soluble cytosolic fraction (tubes 9–12). It was also found broadly distributed in deeper fractions (tubes 4–7), which did not coincide with the endolysosomal compartment as determined by the acid phosphatase distribution (Fig. 6B, dashed line).

Expression of Dd-ALG-2a and -2b mRNAs and Proteins during Differentiation—The levels of Dd-ALG-2a and -2b mRNA transcripts were developmentally regulated. The mRNAs were present in vegetative and early developing cells (Fig. 7A), in agreement with the transcriptional profile published by Van Driessche et al. (42). To assess whether protein levels were similarly regulated during the course of differentiation, Dd-ALG-2a and -2b were revealed on Western blots using polyclonal antibodies raised against the recombinant His$_6$-ALG-2b protein. Three bands in the 20-kDa region were detected in the parent strain (Fig. 7B), which were absent in alg-2а$^{-}$/alg-2b$^{-}$. From the patterns obtained on Western blots of Dd-alg-2а$^{-}$ (where only Dd-ALG-2b was expressed) and Dd-alg-2b$^{-}$ (where only Dd-ALG-2a was expressed), the upper band could be attributed to Dd-ALG-2a and the two lower bands to Dd-ALG-2b. The size and relative intensity of these two bands were unchanged no matter what protease inhibitor mixture was used during sample preparation. Translation of the gene sequences gave theoretical molecular masses of 22.3 and 23.2 kDa for Dd-ALG-2a and -2b, respectively. The reasons why Dd-ALG-2b migrated slightly faster than Dd-ALG-2a, despite a higher molecular mass, and exhibited two protein bands are not clear, but are probably not the result of a calcium-dependent migration artifact as for calmodulin or grancalcin (43).

Antibodies raised against Dd-ALG-2b were used to follow the expression of Dd-alg-2а$^{-}$ and -2b during development in the single null-mutants (alg-2b$^{-}$ and alg-2а$^{-}$). The amount of both proteins remained fairly constant throughout development (Fig. 7C), with only a slight decrease after 12 h of development. Development of Dd-alg-2 Simple and Double Knock-outs—Disruption of Dd-ALG-2 genes either individually in Dd-alg-2а$^{-}$ or Dd-alg-2b$^{-}$ mutants or in combination in Dd-alg-2а$^{-}$/2b$^{-}$ mutant resulted in strains that grew normally in axenic medium or on bacterial lawns. Although Dd-alg-2b-null mutants have a stalk base thicker than normal (data not shown), there was no major differentiation defect in the single knockouts or the double knockout Dd-alg-2а$^{-}$/2b$^{-}$ that produced mature fructifications and spores with identical kinetics and proportioning than the parent strain.

**DISCUSSION**

The experiments reported here were aimed at obtaining a better understanding of the role of ALG-2 calciproteins and to establish whether the apoptotic function described in mammals had a functional counterpart in Dictyostelium PCD. We have biochemically characterized two ALG-2 isoforms from *D. discoideum* and described their interaction both with calcium ions...
and the mouse protein Alix. The latter is one of the physiological binding partners of ALG-2 protein(s) in mammals.

Besides the calcioproteins with 4 EF-hands identified in D. discoideum (21–27), we describe two new Ca$^{2+}$-binding proteins with 5 putative EF-hands, Dd-ALG-2a and -2b. During this work, these proteins have been independently identified by others (28). On the basis of their high sequence identity/homology, both proteins clearly belong to the family that includes grancalcin, calpain, sorcin, and peflin in other organisms (16). The structural homology of the above amebal proteins with mouse ALG-2 is very striking, and both molecules superimpose remarkably well on the mouse ALG-2 structure (41).

Dd-ALG-2a and -2b exhibited the general biochemical properties shared by all members of the family, i.e. Ca$^{2+}$-induced conformational changes and exposure of hydrophobic domains. Their 10-fold difference in affinity toward calcium ions is unlikely to be related only to differences at the level of the EF-hand, because these regions of the proteins are similar in sequence alignments and homology modeling. The amino acid sequences of Dd-ALG-2a and -2b are noticeably different in their N terminus, with the insertion of a 10-amino acid hydrophobic sequence in Dd-ALG-2b. The N-terminal domain might be able to regulate the affinity for Ca$^{2+}$ through hydrophobic interactions with the cleft in the molecule.
Blue to assess equivalent protein loads along the time course (data not shown). After immunodetection, the membranes were stained with Coomassie blue, contained every 4 h during development were analyzed by Western blot. Indeed, we know from gel filtration experiments that MBP-ALG-2a is in solution as a monomer-multimer mixture, whereas MBP-ALG-2b exists mainly as a dimer (data not shown).

Truncated forms of Dd-ALG-2a/2b proteins in their N-terminal (amino acids 1–27) and C-terminal (α8 helix) extremities were generated to determine the relative importance of these domains for dimerization. MBP-ALG-2aΔEF5 is able to form a complex with MBP-ALG-2a, but no homodimer was detected. This is a strong indication that one α7–α8 helix pair is necessary and sufficient to stabilize an MBP-ALG-2a/MBP-ALG-2a dimer. No MBP-ALG-2a/MBP-ALG-2aΔNter dimers were observed, despite the presence of the two pairs of α7–α8 helices. This indicates that the N terminus participates in the dimer formation. We cannot exclude that shortening the distance between the MBP-tag and EF-hand 1 in MBP-ALG-2aΔNter may grossly affect the overall structure of the protein and hence its capacity to interact.

In surface plasmon resonance experiments, Dd-ALG-2a in solution is able to bind to immobilized Alix from mouse and the interaction is strictly Ca\(^{2+}\)-dependent, two arguments that the amebal protein is indeed a genuine functional homologue of mammalian ALG-2. Dd-ALG-2b was unable to interact with Alix, whatever the calcium concentration. Both Alix and Dd-ALG-2b are thus binding partners of Dd-ALG-2a. In view of its longer hydrophobic N-terminal sequence, Dd-ALG-2b could be closer to a peffin, which, in mammals, regulates the ALG-2/Alix interaction by competing for ALG-2 (14, 15). However, the strictly calcium-dependent interaction between Dd-ALG-2a and Dd-ALG-2b is not the mirror image of mammalian ALG-2/peffin interaction that is observed even in the absence of Ca\(^{2+}\) (14). The moderate affinity observed for the Ca\(^{2+}\)-dependent complex between mouse Alix and Dd-ALG-2a is not surprising, considering that we are using recombinant proteins from heterologous sources. Furthermore, an additional level of regulation is likely to occur in vivo, such as interaction with partners, in particular the protein SETA (44, 45) or phosphorylation (46). Only a 2-fold reduction in the binding affinity between the Dd-ALG-2aΔEF5 and Alix was noticed. On the other hand, deletion of the N-terminal sequence of Dd-ALG-2a completely abolished its ability to interact with Alix. This points again to the importance of the N-terminal stretch of Dd-ALG-2a for interaction with partners. Whether the hydrophobic N terminus is directly used for the interaction with Alix or is necessary for the right structuring of ALG-2a to bind to Alix will be examined with truncated proteins with their MBP tag removed.

Immunofluorescence and subcellular fractionation experiments indicate that Dd-ALG-2a and -2b are essentially cytosolic, with some association with the plasma membrane/cytoskeleton and with the nucleus. The latter localization is more prominent for Dd-ALG-2b.

Western blot analysis indicate that the level of ALG-2a/2b proteins is stable during development. Ohkouchi et al. (28) established by in situ hybridization that Dd-ALG2a was enriched in the prespore region and that Dd-ALG-2b was enriched in the prestalk region. The reported analysis of the cell type-specific transcriptional profile provided an opposite trend. Dd-ALG-2a mRNA, followed by its representative clone SSG263, was maximal in stalk cells, whereas Dd-ALG-2b mRNA, represented by clone SSB886, was maximal in prespores (42). Simple and double knock-outs of Dd-ALG-2a/2b produced no obvious developmental phenotype, thus excluding an indispensable role for Dd-ALG-2a/2b in the calcium response during differentiation, as also reported for other calciproteins in D. discoideum (21–27). Nevertheless, an alix-null mutant has a strong defect in development7 and Dd-ALG-2a, by binding Alix, may participate in the overall regulation of that pathway.

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