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Subcellular Localization, Oligomerization, and ATP-Binding of *Caenorhabditis elegans* CED-4

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Caspase family cell death proteases are activated during apoptosis through the oligomerization of caspase-binding "adapter" proteins. In the nematode *Caenorhabditis elegans* one adapter protein, CED-4, exists. Here we report an analysis of CED-4 protein expressed in insect *Sf9* cells by infection with recombinant baculovirus. During expression, CED-4 assumed a perinuclear spherical or reticular localization where it was partly resistant to extraction with nonionic detergents. Both purified FLAG-CED-4 and GST-FLAG-CED-4 proteins were present in solution as large complexes. FLAG-CED-4 complexes were estimated by gel filtration to have a molecular weight of approximately 500 kDa to >1.2 MDa, while GST-FLAG-CED-4 complexes appeared somewhat smaller. Unlike its mammalian homologue Apaf-1, CED-4 exhibited a marked preference for ATP over dATP in filter binding studies and in competition experiments. ATP hydrolysis was required neither for complex stability nor for binding of CED-3. These features are likely to be relevant for CED-4's function as a caspase adapter.

Key Words: **CED-4; complex; ATP; localization.**

Apoptosis is the result of the activation of an intracellular signal transduction pathway. This apoptotic apparatus is the basis of an evolutionary conserved mechanism which is found in organisms as distinct as the nematode *Caenorhabditis elegans,* insects and humans. Although the applications are more extensive to which apoptotic cell death has been put in humans, the principles appear to be basically the same in all organisms (1). A host of information indicates that in all organisms apoptosis is implemented by the activity of cysteine proteases of the caspase family.

Caspases are present in probably all nucleated cells in an inactive form and are activated upon an apoptotic signal (for review see 2). In mammals, two levels of caspases appear to fulfill different roles. "Initiator caspases" are activated by a principle referred to as 'induced proximity', and their role is to activate "effector caspases." Effector caspases cause cell death by the cleavage of cellular substrates. An apoptotic signal induces the oligomerization of "adapter" molecules which in turn cause oligomerization of initiator caspases. Once thus brought into close proximity of each other, the intrinsic activity of the caspase molecules is thought to be sufficient to activate each other and start the chain of events of apoptosis (3).

C. elegans has probably one adapter, CED-4, and one cell death-relevant caspase, CED-3. In humans, caspase activation upon cytochrome *c* release requires either ATP or dATP to pass on the apoptotic signal (4). This requirement has its structural correlate in a nucleotide-binding site in the protein sequence of the caspase 9-adapter Apaf-1. The overall sequence similarity between Apaf-1 and CED-4 is relatively weak. Apaf-1 contains an additional C-terminal domain with a number of WD40 repeats, but both have clear resemblance in the N-terminal "Caspase Recruitment Domain" and their nucleotide binding sites (5). The biochemical and functional properties of Apaf-1 have been worked out in detail. Apaf-1 first binds cytochrome *c* with its WD40 domain. In the presence of ATP or dATP, this complex then oligomerizes and forms aggregates of about 700 and 1400 kDa also containing caspases (4, 6, 7). In the course of this complex formation the caspases become activated. The nucleotide binding site in Apaf-1 is somewhat unusual in that it shows a significant preference for dATP over ATP (8).

While the structural homology suggests that the principle of activation is the same for CED-4/CED-3, the lack of a direct biochemical approach has prevented the investigation of their function in the worm *in vivo.* Studies utilizing systems for expression of these proteins in yeast and mammalian cells have shown that they can interact with each other (9), that CED-4 can form at least homodimers and that dimerization of

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CED-3 leads to its activation (10). *In vivo* analyses of *C. elegans* have further demonstrated that CED-4 is normally sequestered by the anti-apoptotic protein CED-9 (a homologue of Bcl-2) on the mitochondria and released by expression of the 'BH3-only' protein EGL-1 (11, 12). In *C. elegans* embryos, CED-4 upon cell death induction assumes a localization around the nucleus, perhaps on the nuclear envelope (13); the significance of this process is uncertain.

In this study we expressed two CED-4-fusion proteins in insect cells by infection with recombinant baculoviruses. Purified recombinant proteins were used to investigate the properties of CED-4 with regard to subcellular localization, oligomerization, and nucleotide binding.

MATERIALS AND METHODS

Production and purification of recombinant CED-4. The *C. elegans ced-4*-coding region and construct for bacterial expression was kindly provided by Dr. David Vaux, Melbourne, the plasmid pBluescript carrying the *ced-3* gene by Dr. Barbara Conradt, Munich. For expression in *E. coli,* plasmids were transformed into the bacterial strain BL21(DE3) and expressed with IPTG following standard protocols. For baculovirus-driven production, full-length *ced-4* was modified to contain an N-terminal FLAG-epitope. The PCR-generated 5′ part was cloned either into the vector pMelBacB to yield FLAG-CED-4 or into the GST vector pAcG1 (glutathione-*S*-transferase [GST]-FLAG-CED-4). The 3′ part was complemented by subcloning. The constructs were introduced into the baculovirus genome by cotransfection. *Sf9* cells were grown in tissue culture plates in TNM-FH medium supplemented with Gentamicin and Ciprobay. For protein production, cells from *Sf9* cell monolayers were infected at an MOI between 1 and 10 and lysed at 66 h after infection by extraction with 1% Triton X-100 in Tris-buffered saline (50 mM Tris–HCl, pH 7.5, 150 mM NaCl). Lysates were purified over either anti-FLAG M2 affinity gel (FLAG-CED-4) or glutathione Sepharose 4B, Amersham (GST-CED-4). Although a cleavage site was present in the protein, thrombin was unable to cleave the GST tag off the protein. For FLAG-CED-4: positive fractions were pooled, frozen and lyophilized. The resulting pellets were re-dissolved and dialyzed against 20 mM Hepes-KOH, pH 7.5. For GST-FLAG-CED-4: positive fractions were pooled and dialyzed. The amount of protein was estimated by absorbance at 280 nm and by protein staining upon SDS–PAGE and comparison to an albumin standard.

Intracellular staining and microscopy. At various times after infection, cells were fixed with either 100% methanol or acetone at −20°C. Cells were then stained with mouse monoclonal antibodies specific for the FLAG-epitope (M2-antibody, Sigma) or GST (generously provided by Dr. Flaswinkel), followed by a Cy3-coupled goat anti-mouse antiserum (Dianova). Cells were analyzed either by flow cytometry (Becton Dickinson) or by laser-scanning microscopy.

Gel filtration. 1–2.5 μg baculovirus-expressed FLAG-CED-4 or GST-FLAG-CED-4 protein was run over a Superose 6 HR 10/30 gel filtration column (Pharmacia) calibrated with S-100 buffer (20 mM Hepes–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA). Size profiles were calculated by linear extrapolation from the elution characteristics of standard proteins. 1 ml fractions were collected, in some experiments precipitated with trichloroacetic acid and analyzed by Western blotting (M2 anti-FLAG, followed by goat anti-mouse-HRP). In some experiments, CED-4 proteins were preincubated with cytosolic extract from Jurkat human T cells (2 mg protein in 50 or 100 μl) prior to gel filtration. Alternatively, cytosolic extract from infected *Sf9* cells prepared as above was directly run on the column and fractions were analyzed the same way.

Nucleotide-binding and competition. This assay was a modification of a filter binding assay reported recently (8). Reactions were set up to contain aliquots of various CED-4 proteins in binding buffer (20 mM Hepes–KOH, pH 7.5, 10 mM KCl, 3.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 100 μg/ml bovine serum albumin) to a total volume of 20 or 40 μl. 7400–14800 Bq of [α-^{32}P]ATP or [α-^{32}P]dATP were added, and reactions were incubated as indicated. Proteins were transferred onto a nitrocellulose membrane by suction through a 96-well dot blot apparatus (Schleicher and Schuell), followed by 4 washes of 500 μl wash buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 40 mM $MgCl_2$). Retained radioactivity was measured by exposure of the membrane to a PhosphoImager (Molecular Systems) for 1–16 h and quantitated using the software ImageQuant 4.2a (Molecular Systems).

Cross-linking. 100–250 ng FLAG-CED-4 or GST-FLAG-CED-4 was incubated at room temperature for 30 min in the presence or absence of 200 μg cytosolic cell extract to allow complex formation. In reactions containing no extract the reaction buffer S-100 was supplemented with 2 mM ATP and 0.1 mg/ml BSA. Cross-linkers dithiobis-(sulfosuccinimidyl propionate) (DTSSP; thiol-cleavable) or bis-(sulfosuccinimidyl)suberate (BS^3; both from Pierce) were added to a final concentration of 2 mM. After 15 min the cross-linking reaction was stopped with 50 mM Tris, pH 7.5. Reactions were boiled in Laemmli buffer with or without DTT and analyzed by Western blotting.

CED-3 pull-down assays. [^{35}S]Methionine-labeled CED-3 was produced by *in vitro* rabbit reticulocyte transcription/translation (Promega). Pull-down reactions were set up in binding buffer containing 600–900 ng GST-FLAG-CED-4, various concentrations of ATP or ATP-γ-S and 1–3 μl of [^{35}S]-CED-3. After incubation at 30°C, 20 μl glutathione Sepharose 4B (Amersham) and 500 μl HNTG buffer (20 mM Hepes–KOH, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) were added. Samples were rotated at 4°C for 2–4 h on an overhead wheel. Sepharose beads were washed, resuspended in Laemmli buffer and subjected to SDS–PAGE. After drying, the gel was exposed to a PhosphoImager screen.

RESULTS

Intracellular Localization of CED-4 during Expression in Insect Cells

Full-length *C. elegans* CED-4 was expressed as two different fusion proteins in insect *Sf9* cells. During the expression we noticed that both FLAG-CED-4 and GST-FLAG-CED-4 were, although expressed to high levels, difficult to extract from infected cells. FLAG-CED-4 had been designed for release from the infected cells by virtue of an N-terminal secretion signal from the honey bee melittin but no secretion was detected (not shown). Very little FLAG-CED-4 protein was contained in cytosolic extracts prepared by hypotonic lysis. By treatment with nonionic detergents (*n*-octyl-β-D-glucopyranoside, Igepal CA-630, Triton X-100) approximately 20–40% of the protein could be extracted while the remainder was consistently nonextractable by this treatment (Fig. 1A). GST-FLAG-CED-4 behaved principally the same although a greater part could be extracted (Fig. 1A). Intriguingly, a similar pattern was seen when FLAG-CED-4 was expressed in mammalian cells: extracts from transfected COS7 cells prepared by

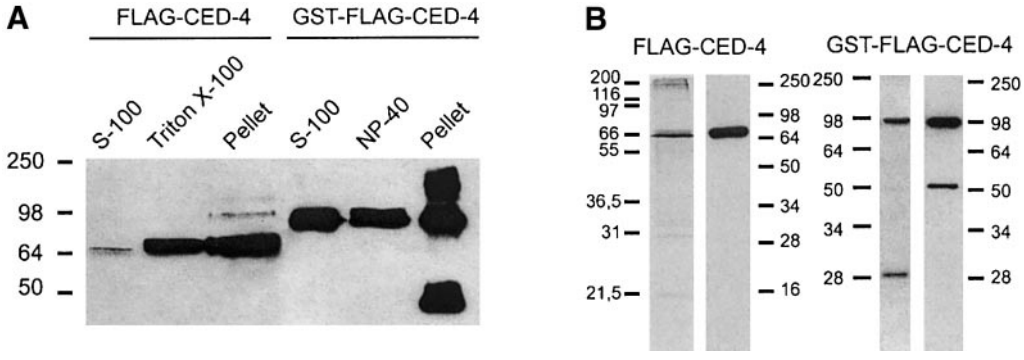

FIG. 1. Extraction and purification of CED-4 proteins. (A) *Sf9* cells were infected with a baculovirus driving the expression of either FLAG-CED-4 or GST-FLAG-CED-4. 3 days postinfection, cells were lysed by incubation in hypotonic buffer and passage through a 26-gauge needle until >90% of the cells were lysed (supernatants from this step are referred to as S-100 extracts). Lysates were centrifuged, and pellets were further extracted with buffer containing 1% of either Triton X-100 or Igepal CA-630 (NP-40) and centrifuged again. Pellets from this step were resuspended in Laemmli buffer, and equivalent amounts from each step were subjected to anti-FLAG Western blotting. (B) 1 μg of purified CED-4 proteins was analyzed by SDS–PAGE and silver staining (left panel for each protein) and anti-FLAG Western blotting (right panel). The FLAG-negative contaminant (about 30 kDa) in the case of GST-CED-4 was seen in most preparations and was in some experiments further removed by gel filtration (not shown).

either hypotonic lysis or by detergent containing buffers contained very little CED-4 protein, while the vast majority was nonextractable (not shown).

In order to assess the subcellular localization of CED-4 when expressed in insect cells, proteins in infected cells were analyzed by confocal microscopy. Strong expression of CED-4 protein was seen by flow cytometry (not shown). When analyzed by confocal microscopy, both CED-4 fusion proteins were found to be localized in a thin sphere around the nucleus in infected *Sf9* cells. In some cells, the sphere was incomplete, in others it appeared continuous and complete; expression in a reticular pattern around the nucleus was also seen, although the protein appeared not to be attached to the nucleus (Fig. 2). No morphological signs of apoptosis were observed (Fig. 2 and data not shown). These data indicate that it is an intrinsic property of CED-4 to assume a perinuclear pattern, reminiscent of the distribution.

For further studies FLAG-CED-4 and GST-FLAG-CED-4 were purified by affinity chromatography. Silver staining and anti-FLAG Western blotting showed proteins of the expected sizes. Typical examples of preparations are shown in Fig. 1B. FLAG-CED-4 ran as one FLAG-positive band (in some preparations, a slightly higher band was also seen; this could be FLAG-CED-4 which had retained the melittin tag). GST-FLAG-CED-4 appeared as one single band at the expected size and varying amounts of a FLAG-positive smaller product; one apparently cellular contaminant was also seen in these preparations. In some cases this contaminant was removed by gel filtration (not shown).

Complex Formation by Recombinant CED-4

One notable difference between Apaf-1 and CED-4 is that CED-4 does not contain a WD40 domain. The WD40 domain in Apaf-1 appears to inhibit oligomerization, and this inhibition is relieved upon cytochrome *c*-binding (8, 14). Structural considerations would therefore suggest that isolated CED-4 can spontane-

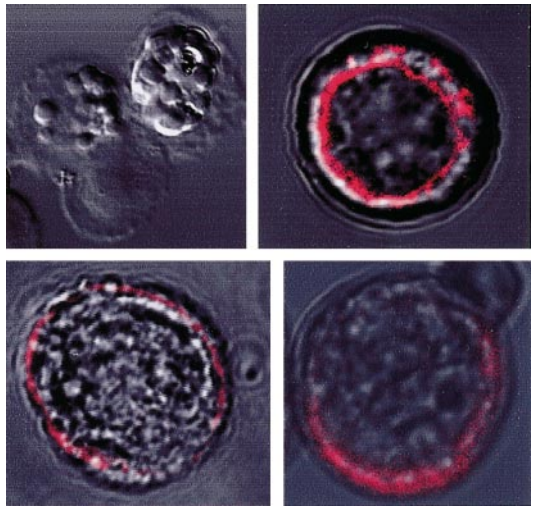

FIG. 2. Subcellular localization of CED-4 proteins in *Sf9* cells. *Sf9* cells were infected with the indicated baculovirus. On days 2 and 3 postinfection, cells were labeled with anti-FLAG or anti-GST antibodies, followed by Cy3-conjugated anti-mouse secondary antibody. Cells were analyzed by laser scanning microscopy. Confocal fluorescence images of immunolabeled cells are overlayed onto a differential interference contrast image. (Top left) Cells infected with the wild-type virus were stained with anti-FLAG, day 2 postinfection. Note the formation of polyhedrin occlusion bodies in infected cells. (Top right) FLAG-CED-4 detected with anti-FLAG, day 2 postinfection. (Bottom left) GST-FLAG-CED-4, detected with anti-FLAG, day 2 postinfection. (Bottom right) GST-FLAG-CED-4, detected with anti-GST, day 3 postinfection. Three cells infected with wild type virus (top left) and one cell each of the other conditions are shown. Specificity of FLAG/GST-staining was controlled by flow cytometry (not shown).

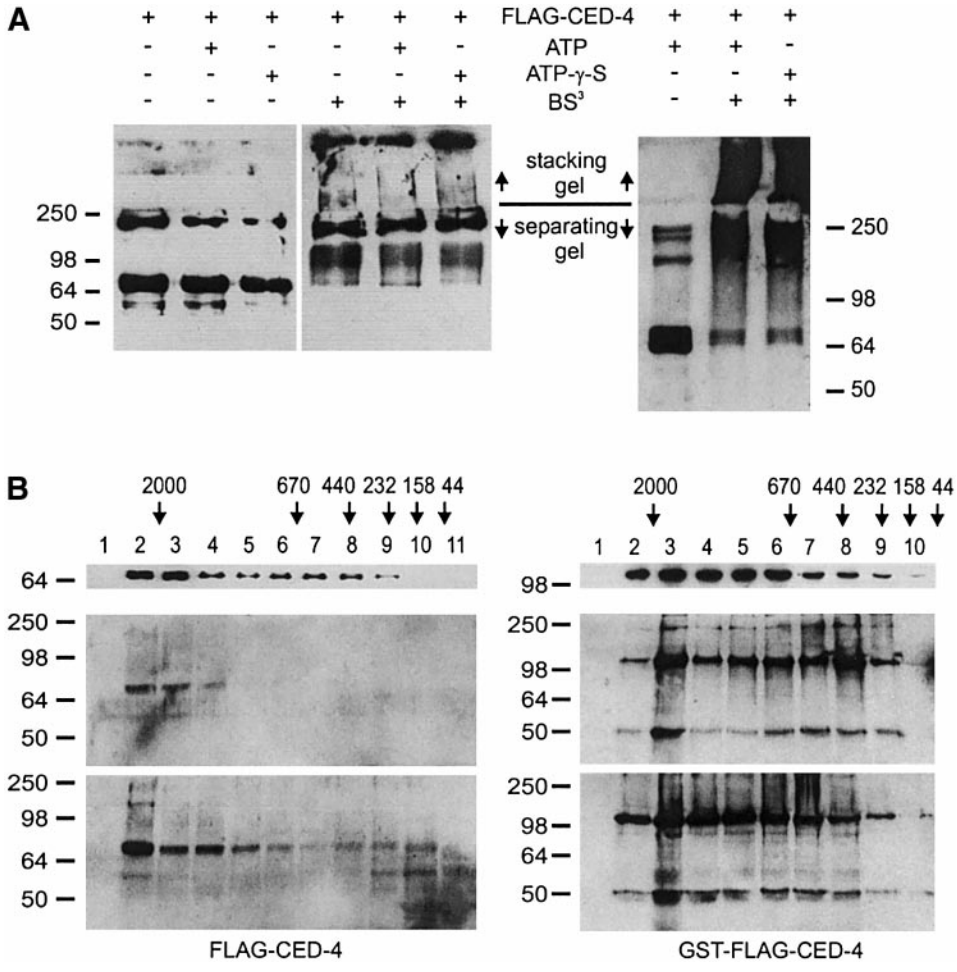

FIG. 3. Complex formation of CED-4. (A) 100–200 ng of either FLAG-CED-4 (left) or GST-FLAG-CED-4 (right) was incubated in the absence or the presence of the cross-linking agent BS^3. ATP (2 mM) or ATP-γ-S (1 mM) were included in the reaction as indicated. Concentrations up to 10 mM of ATP-γ-S were used but no effect was seen (not shown). To show the range of variations observed, two separate experiments are shown. (B) CED-4 proteins were run over a gel filtration column as described. Fractions were collected, precipitated, and CED-4 proteins were detected by anti-FLAG Western blotting. Elution times for marker proteins are indicated on top; the 2000 marker indicates the position of the void volume. Experiments were performed with FLAG-CED-4 (left) and GST-FLAG-CED-4 (right). The following preparations were separated: cytosolic extract from infected *Sf9* cells prepared by hypotonic lysis (top), purified protein (middle) and purified protein preincubated with cytosolic extract from Jurkat human T cells (bottom). The results are representative of at least two independent experiments with each protein and condition. In the case of GST-FLAG-CED-4, a band corresponding to the intact protein (about 90 kDa) and a breakdown product (about 45 kDa) is seen.

ously oligomerize, perhaps with the requirement for nucleotides. We next investigated whether purified CED-4 did auto-oligomerize and whether factors from the cytosol of human cells affected this organization. Two techniques were used for these experiments: chemical cross-linking and gel filtration.

Recombinant CED-4-protein was exposed to chemical cross-linking, and complex formation was assessed by Western blotting. As shown in Fig. 3A, cross-linking led to the appearance of high molecular weight complexes of both FLAG-CED-4 and GST-FLAG-CED-4. The complexes could be dissolved and ran mostly as monomers when reduction-sensitive cross-linking agents were used and the samples were boiled in the presence of DTT (not shown). Preincubation of the purified CED-4 proteins with cytosolic extracts from Jurkat human T cells did not affect complex formation (not shown).

Apaf-1 requires binding of dATP for the formation of large complexes; CED-4, however, appeared to form such complexes in the absence of added nucleotide. Including the noncleavable ATP derivative ATP-γ-S which can compete away ATP (see below) did not disrupt the complexes even at high concentrations (Fig. 3A); although this still leaves the possibility that ATP-binding is required for the initial complex formation, it shows that the complex is stable when a noncleavable ATP-analogue has bound.

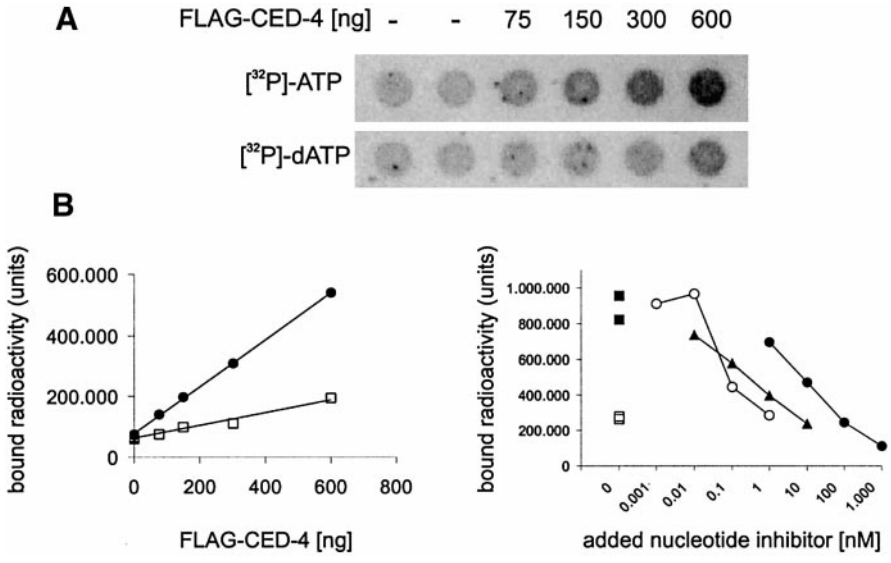

FIG. 4. Nucleotide binding characteristics of CED-4. (A) Various amounts of FLAG-CED-4 were incubated with the same activity (7.4 kBq) of [^{32}P]ATP (filled circles) or [^{32}P]dATP (open squares) for 30 min at 37°C. Proteins were then immobilized on nitrocellulose membrane, and the membrane was exposed to a PhosphoImager screen for 16 h. Binding of the nucleotides to CED-4 was visualized as PhosphoImager signals (top) and is quantified as detected radioactivity units (bottom). Similar results were obtained with two independent preparations of FLAG-CED-4 as well as preparations of GST-FLAG-CED-4 and preparations of *E. coli*-derived FLAG-CED-4. (B) 14.8 kBq of [^{32}P]ATP was incubated either without (open squares) or with 1 μg of FLAG-CED-4 protein (filled squares). To some samples, cold competitor nucleotide was added prior to the addition of the [^{32}P]ATP (open circles, ATP, triangles, ATP-γ-S, filled circles, dATP). After incubation for 30 min at 37°C, samples were blotted onto nitrocellulose and bound radioactivity was measured as above. Similar results were obtained with two independent preparations of FLAG-CED-4 and with two independent preparations of GST-FLAG-CED-4.

Next, preparations of CED-4 were run on a gel filtration column to assess the size of the complexes. Both GST-FLAG-CED-4 and FLAG-CED-4 mainly eluted in fractions with a high apparent molecular mass; FLAG-CED-4 ran as one major peak at a MW of at least 1–1.5 MDa and a smaller fraction around 500–700 kDa, while GST-FLAG-CED-4 was eluted mainly at sizes between 700 and at least 1200 kDa, trailing off down to monomeric sizes around 50–100 kDa (Fig. 3B). This indicates that the majority of CED-4 in solution is organized into complexes containing several CED-4 molecules; the observed sizes suggest that the complexes contained at least 10 CED-4 molecules. The pattern observed was very similar when purified CED-4, *Sf9* extracts containing CED-4 or purified CED-4 incubated in the presence of Jurkat extract was used (Fig. 3B). Including 150 mM NaCl did not change the observed characteristics (not shown). The difference between the two proteins could result from a hindering effect of the GST part of the fusion protein on the association. A FLAG-positive breakdown product of GST-FLAG-CED-4 ran with the same characteristics as the intact protein suggesting that the complex formation is mediated through the N-terminal part of the protein (Fig. 3B). Presence of cytosolic proteins from insect or human cells had no great impact on the sizes determined suggesting that these proteins did not affect the formation of complexes.

Nucleotide-Binding of CED-4

One of the surprising features of Apaf-1 is its preference for the binding of dATP over ATP. In cell extracts, dATP is superior to ATP in the activation of caspases (15) and purified Apaf-1 displays a significantly higher binding affinity for dATP than for ATP (8). We tested the nucleotide binding characteristics of purified recombinant CED-4 protein using an assay similar to the one described for Apaf-1 (8). Binding studies were performed with FLAG-CED-4, GST-FLAG-CED-4 and *E. coli*-derived FLAG-CED-4. The data shown here were generated using FLAG-CED-4; the two other purified proteins showed very similar relative binding of dATP and ATP (not shown). FLAG-CED-4 bound [α-^{32}P]ATP significantly better that [α-^{32}P]dATP (Fig. 4A). Consistent with this finding, cold ATP was approximately 100 times more efficient than dATP in competing for binding of [α-^{32}P]ATP. The noncleavable ATP-analogue ATP-γ-S was able to compete for ATP-binding with a somewhat lower affinity than ATP itself (Fig. 4B). ADP was found to compete with ATP for binding to CED-4 with an affinity in a similar range as dATP (not shown), although it appeared to be higher than the affinity of dADP for Apaf-1 (8). These data show a distinct preference of CED-4 for the binding of ATP over dATP; CED-4 therefore appears to have features which differ significantly from Apaf-1.

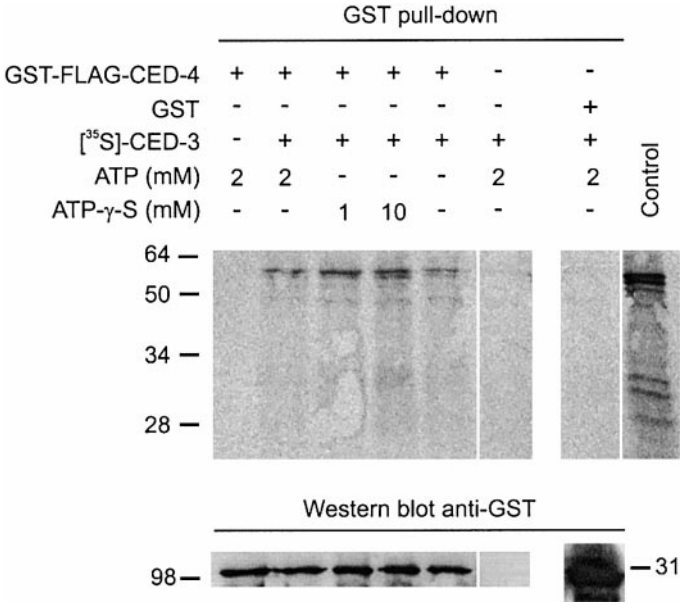

FIG. 5. Binding of CED-3 to GST-FLAG-CED-4. Reactions were set up to contain approximately 900 ng of GST-FLAG-CED-4 or GST alone, various concentrations of ATP or ATP-γ-S as indicated and 3 μl *in vitro* translated [^{35}S]-CED-3. Reactions were incubated for 60 min at 30°C, and GST-FLAG-CED-4 was precipitated with glutathione–Sepharose beads. Reactions were washed, boiled in Laemmli buffer and radiolabeled proteins were visualized by SDS–PAGE, followed by transfer onto nitrocellulose and autoradiography (top panel; control, 0.5 μl of *in vitro* translated CED-3 was loaded directly; the smaller bands around 20–32 kDa possibly result from the low self-cleaving activity of CED-3). Precipitation of the proteins was verified by anti-GST immunodetection on the same membrane (bottom panel).

Interaction between FLAG-CED-4 and CED-3

CED-4 has been shown directly to interact with the *C. elegans* caspase CED-3 (9). We tested whether ATP hydrolysis was necessary for CED-4 to bind CED-3. Interaction of recombinant CED-4 with *in vitro* translated CED-3 was tested by pull-down experiments; to analyze for the requirement for ATP-hydrolysis, ATP-γ-S was included during the incubation of the proteins. GST-FLAG-CED-4 was able to bind *in vitro* translated CED-3 (Fig. 5; similar results were obtained for FLAG-CED-4, not shown). When even higher concentrations of ATP-γ-S were included in the reaction, no decrease in the amount of CED-3 precipitated was observed (Fig. 5). Since ATP-γ-S, a nonhydrolyzable ATP analogue, can replace ATP from CED-4, and GST-FLAG-CED-4 still (in the presence of high concentrations of ATP-γ-S) binds CED-3 apparently equally well, these data suggest that hydrolysis of ATP is not required for binding to CED-3.

DISCUSSION

In this study we investigated molecular properties of *C. elegans* CED-4 using a baculovirus expression system and purified recombinant CED-4. CED-4 was found to form a distinctive pattern around the nucleus of *Sf9* cells during expression in the absence of apoptosis induction. Purified CED-4 spontaneously formed large homo-oligomeric complexes. Nucleotide binding studies indicate that, unlike Apaf-1, CED-4 binds ATP with significantly higher affinity than dATP; ATP hydrolysis was required neither for the stability of CED-4 complexes nor for CED-3-binding.

CED-4 was assembled in a sphere-, sometimes net-like structure around the nucleus of the infected cells and was found to be hard to extract from the cells. Although the FLAG-CED-4 expressed was designed to be secreted into the culture medium and would therefore be targeted to the ER, this was not the case for GST-FLAG-CED-4, and this protein behaved the same in terms of localization; this suggests that it is an intrinsic property of CED-4 to assume this unusual localization at least in some cell types. The resistance to extraction might signify a solid association with some cellular component which itself is firmly attached for instance to the cytoskeleton. It would also be conceivable that CED-4 is extremely insoluble. We think that this is unlikely, however, since CED-4 upon extraction showed no propensity to form precipitates and stayed perfectly soluble during all procedures of handling, such as during lyophilization and resolubilization. In its natural environment in *C. elegans*, CED-4 has been observed to assume a similar ring-like shape around the cell nucleus; furthermore, when *C. elegans* embryos were then fractionated, CED-4 was found not in the cytosolic or membrane/organelle fraction but in the nonextractable fraction containing the nuclei (13). Although costaining with nuclear lamins in that study suggested a localization on the nuclear envelope, we believe that the available evidence points to a localization on a structure attached to the nucleus or in its proximity. CED-4 can simultaneously interact with both CED-3 and CED-9 (reviewed in 16). CED-3 might therefore already be bound to CED-4 while held inactive by CED-9 but clustering around the nucleus could be necessary for efficient activation of CED-3.

We show that purified CED-4 protein spontaneously forms large homomeric complexes in solution. Predictions based on structural similarities to other proteins have suggested that CED-4 upon ATP-binding forms homohexamers (17). The gel filtration profile of FLAG-CED-4 suggests that a large portion is organized into complexes containing even more CED-4 molecules, in the order of 20 or more while some protein eluted at around 500 kDa. GST-FLAG-CED-4 ran as a mixture of complexes of apparently different stoichiometry, ranging from monomers up to hexamers and large complexes. This difference might be the result of steric hindrance by the GST fusion partner; the results therefore suggest that more than 6 CED-4 molecules can form a complex in solution.

Since CED-4 may have bound cellular ATP during expression in *Sf9* cells we cannot make a statement about the nucleotide requirement for the initial oligomerization. However, if ATP was bound to CED-4, it could be replaced by externally added ATP and the nonhydrolyzable ATP-γ-S. Since ATP-γ-S was unable to disrupt the complexes, ATP hydrolysis is very likely not required for complex stability. Furthermore, ATP-cleavage was not required for the binding of CED-3. It has been suggested that ATP-hydrolysis might be used to trigger disassembly of the complex (17). Although GST-FLAG-CED-4 bound CED-3, we did not observe activation of CED-3 in the course of the precipitation experiments. If CED-4/CED-3 interaction was the same as the one between Apaf-1 and caspase-9, one would expect to see such activation. It is conceivable that this lack of activation is a reflection of the reaction conditions. CED-3-binding and CED-4 oligomerization might also require a certain orderly procedure which was not given under these conditions.

It is remarkable that the relative binding affinities are significantly different between Apaf-1 and CED-4: Apaf-1 binds dATP with a higher affinity than ATP while the opposite is the case for CED-4. Furthermore, dADP was unable to replace dATP bound to Apaf-1 even at high concentrations (8) but ADP was able to compete with ATP for binding to CED-4 (although with a lower affinity). It is therefore conceivable that ATP is hydrolyzed during the molecular function of CED-4 and that ADP remains bound to the complex. The different sensitivity of Apaf-1 toward nucleotides could reflect the broader task in the induction of apoptosis in response to a much larger number of stimuli (compared to the induction of programmed cell death by CED-4).

The data presented here show characteristics of CED-4, some of which put it in clear contrast with Apaf-1. It is uncertain why CED-4 assumes the peculiar localization pattern seen; it might reflect the different mode of regulation of CED-4 and Apaf-1, i.e., release from the CED-9-"chaperone" vs conformational change upon cytochrome *c*-binding. In a similar way, complex formation is regulated by cytochrome *c*/dATP-binding in Apaf-1 and probably occurs spontaneously in CED-4, presumably after release from CED-9.

However, a number of features, such as complex formation, nucleotide-binding and caspase-binding are clearly conserved between the species and can be assumed to represent a well-functioning system as it has been conserved over a long evolutionary distance.

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