Experimental Evidence for a ββα-Me-Finger Nuclease Motif To Represent the Active Site of the Caspase-Activated DNase†

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ABSTRACT: The caspase-activated DNase (CAD) is an important nuclease involved in apoptotic DNA degradation. Results of a sequence comparison of CAD proteins with ββα-Me-finger nucleases in conjunction with a mutational and chemical modification analysis suggest that CAD proteins constitute a new family of ββα-Me-finger nucleases. Nucleases of this family have widely different functions but are characterized by a common active-site fold and similar catalytic mechanisms. According to our results and comparisons with related nucleases, the active site of CAD displays features that partly resemble those of the colicin E9 and partly those of the T4 endonuclease VII active sites. We suggest that the catalytic mechanism of CAD involves a conserved histidine residue, acting as a general base, and another histidine as well as an aspartic acid residue required for cofactor binding. Our findings provide a first insight into the likely active-site structure and catalytic mechanism of a nuclease involved in the degradation of chromosomal DNA during programmed cell death.

The degradation of chromosomal DNA is a hallmark of apoptosis (*1*). It includes the high molecular weight chromatin cleavage at the onset of apoptosis, resulting in 50−300 kb chromatin fragments and the subsequent nucleosomal cleavage of chromatin in the later stages of apoptosis detectable as the chromatin ladder (*2*−*5*). The caspase-activated DNase (CAD)[1] is an important nuclease involved in these processes (*6*−*8*). CAD resides in the nuclei of nonapoptotic cells as an inactive complex with its inhibitor ICAD (*9*, *10*), being released from the complex after the onset of apoptosis by caspase-3, which cleaves the inhibitor at two distinct positions (*11*). Nuclease activation results in the presence of active enzyme in the nuclei of apoptotic cells, leading to the destruction of the cell's genetic material. Despite its important role in apoptotic DNA degradation, little is known about the structural and the catalytic properties of CAD.

CAD is a 40 kDa protein consisting of two domains, the small regulatory N-terminal CAD- or CIDE-N domain (*12*−*15*) and the larger C-terminal catalytic domain (*16*). Previous mutagenesis studies identified several critical amino acid residues of murine CAD as essential for DNA cleavage by this enzyme (*17*−*19*). However, the particular roles of these amino acid residues in the catalytic mechanism remained uncertain since the interpretation of mutagenesis data in terms of a comprehensive model for the mechanism of catalysis of CAD requires structural information about the active site of the enzyme, and vice versa. Recently, Walker et al. (*20*) have presented a manual alignment of colicin E9 with CAD proteins, suggesting that the essential H-N-H motif residues His103, Asn118, and His127 of colicin E9 align with His263, Asn299, and His308 of murine CAD. This alignment was in part based on previous results identifying His263 and His308 as essential for phosphodiester bond cleavage by CAD (*17*−*19*). This sequence similarity could mean that CAD proteins and colicin DNases share a common active-site fold and a similar catalytic mechanism, since colicin DNases together with the DNA/RNA nonspecific nucleases and T4 endonuclease VII-family enzymes as well as certain homing endonucleases, are all members of the superfamily of ββα-Me-finger nucleases. The members of this superfamily are characterized by a common active-site structural fold, the ββα-Me-finger (Figure 1 B), and similar catalytic mechanisms (*21*−*24*). To verify the hypothesis that CAD belongs to the ββα-Me-finger nucleases, we carried out a mutational analysis. For this purpose, we have extended the manual alignment by including other protein families belonging to the ββα-Me-finger nuclease superfamily. Amino acid residues of murine CAD identified in this bioinformatic analysis as probably being important for catalysis were exchanged by site-directed mutagenesis, and the variants were characterized in DNA cleavage experiments. Our results demonstrate that CAD proteins indeed

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[1] Abbreviations: CAD, caspase-activated DNase; CIDE, cell death inducing DFF-like effector; ICAD, inhibitor of CAD; GST, glutathione-*S*-transferase; EDAC, (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide); Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; kb, kilo base pairs; kDa, kilo Dalton; Mes, 2-morpholinoethanesulfonic acid monohydrate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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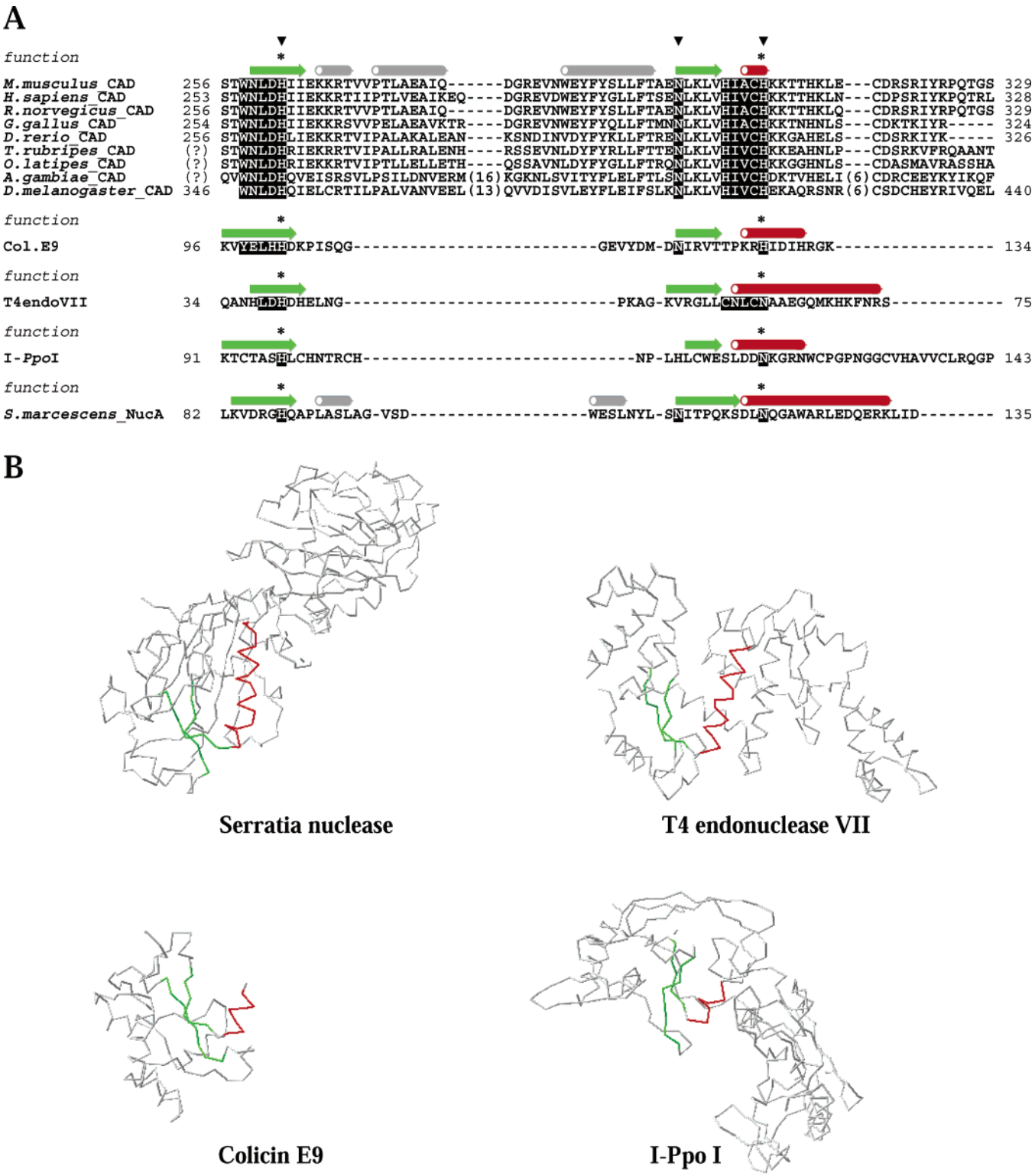


FIGURE 1: Sequence alignment and active-site structures of $\beta\beta\alpha$-Me-finger nucleases. (A) Sequence comparison of selected CAD proteins with structurally analyzed members of the $\beta\beta\alpha$-Me-finger nuclease superfamily. Confined sequence similarities can be seen at the site of the general base and cofactor binding residues, indicated by asterisks. The two β-strands and the α-helix of the $\beta\beta\alpha$-Me-finger motif are indicated by green arrows and red tubes, respectively; predicted α-helices in the catalytic core of CAD and those found in the *Serratia* nuclease structure are indicated in gray. The amino acid residues representing the H-N-H motif are indicated by arrowheads. This motif is only strictly conserved in the CAD proteins and the colicin DNases, as well as some homing endonucleases of the H-N-H family (not shown). Black shading denotes residues conserved in each protein family. Each of the nucleases listed is a representative of a larger family of nucleases belonging to the $\beta\beta\alpha$-Me finger nuclease superfamily. (B) Structures of the $\beta\beta\alpha$-Me-finger nucleases colicin E9 (PDB 1emv), the structure-specific T4 endonuclease VII (PDB 1e7d), the His-Cys box containing homing endonuclease I-*Ppo*I (PDB 1a74), and the *Serratia* nuclease (PDB 1g8t). The $\beta\beta\alpha$-Me-finger motifs of these nucleases are highlighted.

constitute a novel $\beta\beta\alpha$-Me-finger nuclease family. On the basis of these novel results, we present models for the active-site structure and the mechanism of catalysis of this key apoptotic nuclease.

EXPERIMENTAL PROCEDURES

Expression and Purification of CAD. GST-mCAD/hICAD-L complex was produced in *Escherichia coli* using a two plasmid system as described previously (*18*). Free GST-mCAD was obtained by treating the purified complex with recombinant caspase-3 (*18*).

Site-Directed Mutagenesis. Site-directed mutagenesis of mCAD was performed as described previously (*25*). In brief, a first PCR was performed using a mutagenic primer and an appropriate reverse primer with pGEX-2T-mCAD as template and Pfu DNA polymerase. Then, a second PCR was performed using purified product from the first reaction as megaprimers for an inverse PCR following the instructions of the QuikChange protocol (Stratagene).

In Vitro Nuclease Activity Assays. Aliquots of GST-mCAD (400 nM final concentration) were incubated in 20 mM Tris-HCl pH 7.0, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol, 0.01% Triton X-100, 5 mM $MgCl_2$ for defined time intervals at 37 °C, using 25 ng of plasmid DNA (pBSK-VDEX) per microliter assay solution (10 nM final concentration) or an aliquot of HeLa-cell nuclei, isolated as described by Scaffidi et al. (*26*). The disappearance of the sc-plasmid DNA substrate upon incubation with the nuclease variants and the wild-type enzyme was used to determine relative activities. Cleavage products were analyzed on a 0.8% TBE-agarose gel (100 mM Tris-HCl pH 8.3, 100 mM borate, 2.5 mM EDTA) containing 0.05 μg/mL ethidium bromide.

DNA−Cellulose Binding Assay. DNA binding by GST-mCAD was measured by the DNA−cellulose binding assay since DNA binding by CAD could not be detected by a gel electrophoretic mobility shift assay (C. Korn, S. R. Scholz, and G. Meiss, unpublished work). GST-mCAD variants were investigated by incubating caspase-3-treated GST-CAD/hICAD-L complex with 50 μL of DNA−cellulose suspension in a buffer consisting of 20 mM Hepes pH 7, 100 mM NaCl, and 1 mM EDTA for 25 min at 4 °C. Bound protein was washed twice with 500 μL of the same buffer as before supplemented with 5 mM DTT, 10% glycerol, 0.01% Triton-X-100, and subsequently eluted by incubating the sample for 5 min at 95 °C in 10 μL of SDS gel loading buffer (160 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 40% glycerol, and 0.1% bromophenol blue) and analyzed by SDS−PAGE.

Chemical Modification of GST-mCAD with EDAC. Modification of free GST-mCAD and the GST-mCAD/hICAD-L complex was performed using different concentrations of EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) in 10 mM Mes pH 6.0, 100 mM NaCl, 1 mM EDTA, 4 mM DTT, 0.01% BSA, and 0.01% Chaps. To investigate protection against modification, free GST-mCAD was modified in the presence of plasmid DNA (pBSK-VDEX) at a concentration of 100 ng/μL assay solution. Residual activity of the modified nuclease was analyzed by transferring aliquots of the modification reaction mixtures into a buffer consisting of 20 mM Tris-HCl pH 7.0, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol, 0.01% Chaps and incubated with caspase-3 (for reasons of standardization always added) for 10 min at 37 °C. Where necessary, the aliquots were supplemented with plasmid pBSK-VDEX as substrate (20 ng/μL assay solution), and the DNA cleavage reaction (10 min at 37 °C) was started by adding $MgCl_2$ (5 mM final concentration). Cleavage products were analyzed as described above.

Structural Models. Modeling of the tertiary structure of the core region (aa 248−315) of the catalytic domain of murine CAD (mCAD) nuclease domain was performed by satisfaction of spatial restraints using Modeler v6.1 (*27*). The coordinates of superimposed template structures 1g8t, 1ipp, and 1emv provided the constraints to model the conserved regions, while the insertion unique to CAD was modeled using the secondary structure restraints according to the predictions generated by PHD (*28*), PSIPRED (*29*), and JPRED (*30*, *31*) to identify a model with the best energy profile. VERIFY3D (*31*) was used to identify models with the best energy profile. Metal-binding residues, which usually exhibit atypical scores, were disregarded during evaluation of the modeled structure of CAD.

RESULTS

Sequence Similarity between CAD Proteins and $\beta\beta\alpha$-Me-Finger Nucleases. Sequence comparisons of CAD proteins with members of the $\beta\beta\alpha$-Me-finger nucleases reveal significant local active-site sequence similarities not only between the CAD proteins and the colicins but also the T4 endonuclease VII family (Figure 1A). A confined active-site sequence similarity between colicin DNases and T4 endonuclease VII family members has already been described by Aravind et al. (*23*). CAD proteins display local sequence similarities to both protein families: colicins and CAD proteins share high local sequence similarity at the site of the predicted general base (CAD: His263; colicin E9: His103) and the structurally important asparagine residue, characteristic for the so-called H-N-H motif containing nucleases (CAD: Asn299; colicin E9: Asn118). The T4 endonuclease VII family members share high sequence similarity with CAD proteins at the site of the general base (CAD: His263; T4 endonuclease VII: His41) as well as the site of the metal-ion binding residue (CAD: His308; T4 endonuclease VII: Asn62). Notably, T4 endonuclease VII family members, Colicin DNases, and CAD proteins on one hand as well as DNA/RNA-nonspecific nucleases, such as *Serratia* nuclease or Endo G, and homing endonucleases, such as I-*Ppo*I on the other hand, do not show significant sequence similarity. In fact, these two groups of distinct nuclease families within the $\beta\beta\alpha$-Me-finger nuclease superfamily are related to each other based on their structurally conserved active-site fold rather than because of a pronounced similarity on the sequence level (Figure 1).

Alignment-Guided Site-Directed Mutagenesis. The alignment of CAD proteins with members of the $\beta\beta\alpha$-Me-finger nucleases provides a rationale for site-directed mutagenesis of corresponding amino acid residues in CAD. The catalytically relevant residues His263 and His308 that form the basis for the active-site comparison of CAD proteins with $\beta\beta\alpha$-Me-finger nucleases, along with additional conserved amino acid residues, have been characterized in previous studies (*17*−*19*, see also Table 1). To get further support for the hypothesis that CAD proteins belong to the $\beta\beta\alpha$-Me-finger nucleases, we have now replaced Asn260, Asp262, and Asn299 of murine CAD by alanine. Asn260 aligns with the important active-site residue Glu100 of colicin E9, which is involved in positioning the residues His102 and His127,

Table 1: Properties of Variants with Substitution of Conserved Amino Acid Residues in the C-Terminal Catalytic Domain of Murine CAD and Comparison with Active-Site Residues of the Different $\beta\beta\alpha$-Me-Finger Nucleases Colicin E9, the Structure Specific T4 Endonuclease VII, the DNA/RNA-Nonspecific *Serratia* Nuclease, and the Homing Endonuclease I-*Ppo*I

CAD	variant	rel. act. (%)[a]	Col. E9	T4endoVII	*Serratia* nuc.	I-*Ppo*I	putative function
Asn260	Ala	7.2	Glu100				active-site conformation
Asp262	Ala	1.3	His102	Asp40			general acid cofactor binding
His263	Asn[b]	<1.0	His103	His41	His89	His98	general base
	Asp[c]	<1.0					
	Arg[c]	<1.0					
Asn299	Ala	ndc	Asn118		Asn110		active-site conformation
His308	Asn[b]	9.3	His127	Asn62	Asn119	Asn119	cofactor binding
	Asp[c]	~1−2					
	Arg[c]	<1.0					

[a] % of wt activity. [b] Meiss et al. (*18*). [c] Korn et al. (*19*); ndc, no detectable cleavage.

suggested to be required for leaving group protonation and/or for cofactor binding in colicin E9 (*32*). Asp262 of CAD aligns with His102 of colicin E9 and Asp40 of T4 endonuclease VII. His102 of colicin E9 is supposed to play the role of the general acid in the presence of Mg^{2+} as cofactor, and to bind the divalent metal ion in the case of Ni^{2+} as cofactor, similarly as does Asp40 in the Ca^{2+}-containing structure of T4 endonuclease VII (*24*, *33*). Finally, Asn299 corresponds to the structurally important residue Asn118 of colicin E9, which is part of the H-N-H motif of this nuclease (*32*).

DNA Binding and Cleavage by CAD Variants. As can be seen from Figure 2A, all CAD variants were expressed in *E. coli* at more or less the same level and were able to bind to DFF45 (human ICAD-L) as deduced from coexpression and copurification of the nontagged chaperone and inhibitor with the GST-tagged nuclease. The variants, activated by recombinant caspase-3 in vitro, are markedly affected in their DNA cleavage activity (Figure 2B) as deduced from cleavage experiments with plasmid DNA and nuclear DNA, the natural substrate of CAD. In particular, Asn299, which aligns with the structurally important H-N-H motif residue Asn118 of colicin E9, when exchanged to alanine leads to an enzyme with no detectable cleavage activity, whereas the variants N260A and D262A show a strongly reduced but still measurable residual activity (Table 1). Despite their reduced activities, all mutant proteins were still able to bind to DNA (Figure 2C), ruling out that the drop in activity of the variants is due to impaired DNA binding. Cleavage of nuclear DNA by the mutant N260A is less affected than cleavage of naked plasmid DNA. Since DNA packed into chromatin is the natural substrate for CAD, these differences may reflect the different accessibilities of these substrates for a given variant.

Chemical Modification of CAD with EDAC. Supporting evidence for the importance of Asp262 of murine CAD for catalysis is provided by the results of chemical modification studies of murine CAD with the carboxylate-specific reagent EDAC. EDAC inhibits the nucleolytic activity of free CAD in a time and dose-dependent manner (Figure 3A). The presence of the inhibitor ICAD/DFF45 does not protect the nuclease from inactivation (Figure 3B), indicating that a putative catalytically relevant aspartic or glutamic acid residue is still accessible for the small inactivating compound in the nuclease/inhibitor complex, in accordance with an exosite mechanism of inhibition, which is defined by a binding of a protein inhibitor close to but not directly at the active site (*34*). In contrast, the presence of DNA efficiently protects the DNase from inactivation, indicating that an aspartic (or glutamic) acid residue is indeed involved in interaction with the DNA (Figure 3B). In general, several amino acid residues contribute to a DNA binding interface of a nuclease. In most cases, substitution of one of these residues alone does not lead to a loss of DNA binding activity. For example, three DNA binding residues of the colicin E9 DNase have to be exchanged to abrogate DNA binding. Nevertheless, every single DNA binding residue is in close contact to the DNA and thus can be protected from

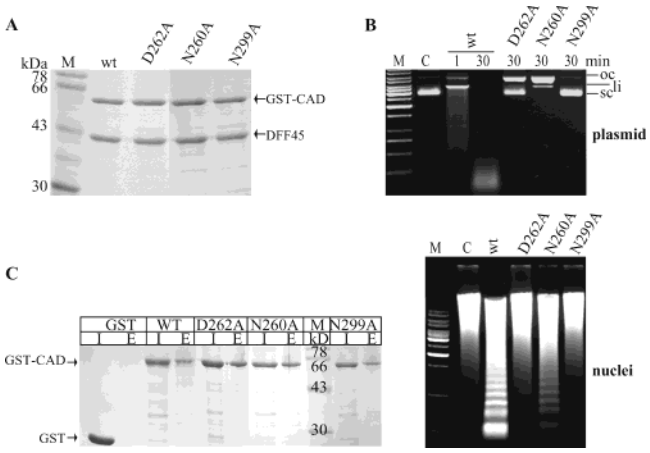
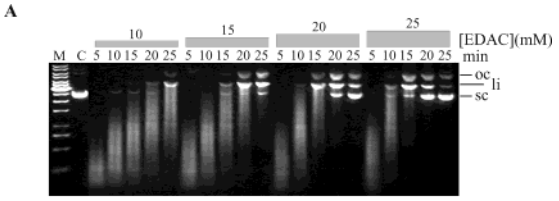
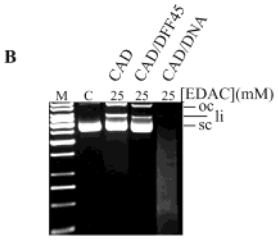


FIGURE 2: Expression and activity of murine CAD variants with amino acid substitutions at positions corresponding to catalytically important residues in Colicin E9 and T4 endonuclease VII. (A) SDS−PAGE analysis of CAD variants coexpressed and purified from *E. coli* in complex with DFF45 (human ICAD-L). M, protein standard. (B) DNA cleavage assays with wild-type CAD and the variants D262A, N260A, and N299A. All variants are markedly affected in their plasmid DNA cleavage activities (upper panel), as identified by an agarose gel electrophoretic analysis of the CAD-induced conversion of the supercoiled (sc) plasmid DNA to the open circular (oc) and linear (li) form, which subsequently is degraded to give a smear, as well as their capacity to induce chromatin laddering in isolated HeLa nuclei (lower panel). C, control without nuclease; M, DNA marker. Neither mock purified GST nor GST-ICAD or caspase-3 purified from *E. coli* are associated with a nuclease activity, demonstrating that the nuclease activity we observe is due to CAD (not shown). (C) All CAD variants are able to bind to DNA as shown by an SDS−PAGE analysis of the result of an affinity chromatography on DNA cellulose, whereas GST, used as a control, does not bind. I, input; E, eluate; and M, protein standard. The extra bands running below CAD are fragments of caspase-3 cleaved ICAD and recombinant caspase-3 itself.





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	CAD	CAD/DFF45	CAD/DNA
DEPC	+	+	-
NAI	+	-	-
TNBS	+	-	-
EDAC	+	+	-

FIGURE 3: Chemical modification of CAD with EDAC. (A) Chemical modification of free CAD reveals a time and dose-dependent inactivation of the enzyme through EDAC, indicating the presence of catalytically relevant aspartic and/or glutamic acid residues. Free CAD was treated with different concentrations of EDAC (10, 15, 20, 25 mM) for the indicated time (5, 10, 15, 20, and 25 min), and the residual activity of the chemically modified nuclease was determined by a plasmid DNA cleavage assay as described in the Experimental Procedures. (B) The presence of DNA, not however of the inhibitor ICAD-L/DFF45, protects the nuclease from modification through EDAC. (C) A summary of chemical modification studies of free CAD, the CAD/DNA complex, and the CAD/DFF45 complex with various amino acid specific compounds reveals similar results with DEPC and EDAC (see text for references), arguing for a close proximity of histidine and aspartic and/or glutamic acid residues at the enzyme's active site. + denotes inactivation of CAD by chemical modification through the respective compound; − denotes protection from modification through the presence of ICAD-L/DFF45 or DNA, respectively.

chemical modification. On the basis of the results of our mutational analysis, Asp262 of murine CAD is a likely candidate for such a residue. Interestingly, modification of histidine residues by DEPC (*17*, *18*) and aspartic and/or glutamic acid residues by EDAC is prevented by the presence of DNA in both cases, not however by the presence of ICAD (DFF45), indicating that catalytically relevant histidine and aspartic and/or glutamic acid residues are probably in close spatial proximity and most likely form the active site of CAD, which is not directly contacted by ICAD/DFF45. In contrast, modification of lysine and tyrosine residues is prevented by the presence of DNA and the presence of the inhibitor ICAD/DFF45 (*19*) (Figure 3C).

Active-Site Structural Model and Catalytic Mechanism of CAD. Our results provide a basis for homology modeling of the active-site region of CAD. Crystal structures are available for five different members of the $\beta\beta\alpha$-Me-finger nuclease superfamily, the *Serratia* nuclease (*35*−*37*), the H-N-H family members colicin E9 and E7 (*38*−*40*), the structure-specific T4 endonuclease VII (*24*, *33*), and the His-Cys box containing homing endonuclease I-*Ppo*I (*41*) (Figure 1B). The modeled structure of mCAD shows the common features of the $\beta\beta\alpha$-Me core of known $\beta\beta\alpha$-Me-finger nucleases, with

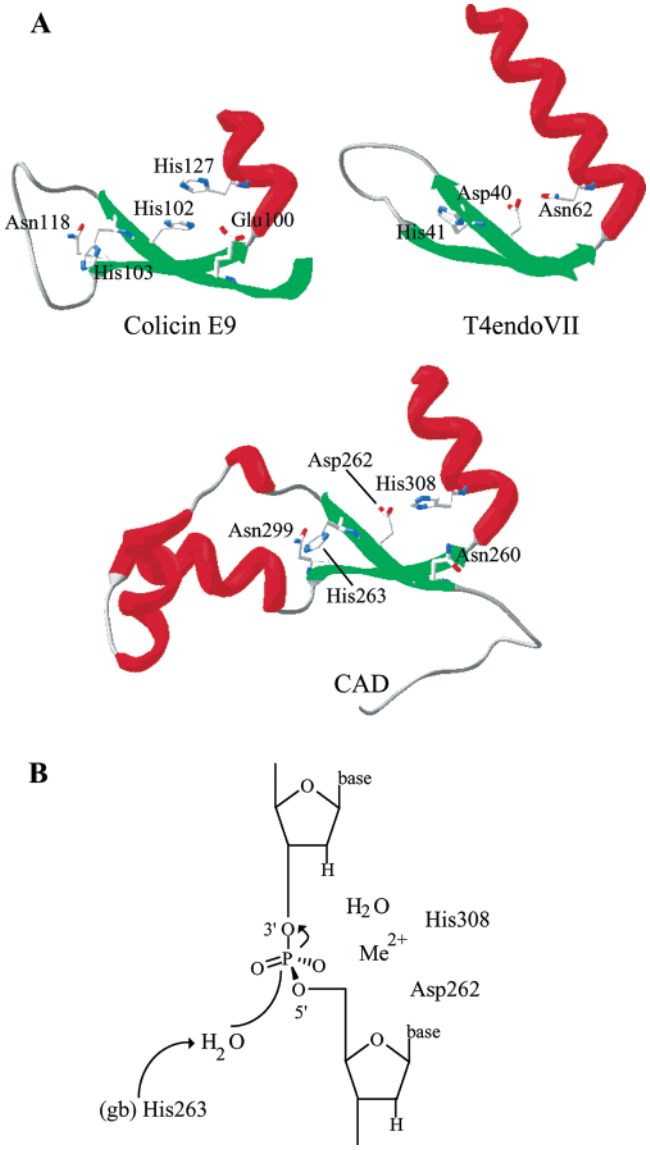

FIGURE 4: Structural model of the active-site region and catalytic mechanism of murine CAD. (A) Structural model of the $\beta\beta\alpha$-Me-finger motif of murine CAD in comparison with the motif of colicin E9 (PDB 1emv, aa 97−130) and T4 endonuclease VII (PDB 1e7d, aa 38−74). The amino acid residues indicated are important for the activity of each of these nucleases. Homologous catalytically relevant residues that are found in colicin E9 and T4 endonuclease VII are also found in CAD. (B) His263 of murine CAD, as the general base (gb), generates a hydroxyl ion for a nucleophilic attack on the scissile phosphodiester bond by activating a water molecule. Asp262 together with His308 could serve to bind the metal ion as seen for Asp40 and Asn62 in the T4 endonuclease VII structure or His102 and His127 in the Ni^{2+}-containing colicin E9 structure. The general acid in the mechanism of catalysis in murine CAD might be a water molecule from the hydration sphere of the divalent metal-ion cofactor but could in principle also be Asp262 or His308 themselves, respectively.

an insertion, that has been modeled as an extension of the short helical structures present in the template structures (Figure 4A); this segment exhibits a well-packed hydrophobic core and hydrophilic surface and has been evaluated as well-folded by VERIFY3D. In addition, there are several hydrophobic residues exposed on the surface of the common $\beta\beta\alpha$-Me segment in murine CAD, which suggests that in the native protein this region is part of a larger globular structure, as it has been observed in other $\beta\beta\alpha$-Me-finger nucleases

nucleases. However, in the known structures (with the exception of the two closely related colicins E7 and E9), the other parts of the proteins among themselves are nonhomologous and structurally dissimilar.

On the basis of the presumptive structural relationship between CAD and $\beta\beta\alpha$-Me-finger nucleases and based on our mutational analysis, we propose that His263 of murine CAD acts as the general base (gb), which activates a water molecule for the nucleophilic attack. His308 is likely to be the principal metal ion binding residue in the active center of the nuclease, like His127 in colicin E9 or Asn62 in T4 endonuclease VII. On the basis of the sequence comparison in which Asp262 of CAD aligns with His102 of colicin E9 and with Asp40 of T4 endonuclease VII (*24*, *32*) and the results of the mutational analysis, we suggest that this residue, like the corresponding residues in colicin E9 and T4 endonuclease VII, is involved in metal-ion binding at the active site. As such, as we have shown in our chemical modification experiments, it would not be accessible for modification when the active site is occupied by the DNA substrate (Figure 4).

DISCUSSION

CAD contains histidine residues that are essential for catalysis (*17*−*19*). This finding has led to different suggestions regarding the relationship of this enzyme with other nucleases. Nagata and co-workers recently proposed that the caspase-activated DNase belongs to the DNase I-like nuclease family (*17*, *42*), whereas Kleanthous and co-workers suggested that CAD is a member of the $\beta\beta\alpha$-Me-finger nucleases (*20*). The latter suggestion was supported by a manual alignment of the colicin E9-DNase active-site motif with CAD proteins. On the basis of this alignment, it was proposed that the H-N-H motif residues of colicin E9 including the general base His103, the structurally relevant Asn118, and the metal-ion binding residue His127 have their counterparts in His263, Asn299, and His308 of murine CAD, respectively. Although highly speculative without supporting experimental evidence other than the presence of essential histidines residues in CAD, a corollary of this conjecture is that the structure of the active site and the mechanism of catalysis for CAD proteins and the members of the of $\beta\beta\alpha$-Me-finger nuclease superfamily, to which colicin E9 belongs, are similar. The manual alignment that revealed the sequence similarities between CAD proteins and colicin E9 was based on the results of our previous studies, from which it was speculated, that His263 of murine CAD acts as the general base and His308 as the general acid in the mechanism of DNA cleavage by CAD (*18*, *19*).

In the present study, we have extended the alignment by including other families of the $\beta\beta\alpha$-Me-finger nuclease superfamily and gathered further biochemical evidence for the hypothesis that CAD proteins belong to the $\beta\beta\alpha$-Me-finger nucleases. Although the nucleases of that superfamily fulfill very different biological functions, and range in their specificity from highly nonspecific to sequence or structure specific enzymes, they share a common active-site fold and exhibit similar catalytic mechanisms with some differences in detail. Local sequence similarities between CAD proteins, colicins, and T4 endonuclease VII family members can be found at critical positions, such as the amino acid residues presumably functioning as the general base and the putative metal-ion binding residues, characterizing these distinct protein families as members of a common superfamily.

Sequence homologous residues that are important for the active-site conformation and the structure of colicin E9 (Glu100, Asn118), or those that are involved in cofactor binding in colicin E9 (His102) and T4 endonuclease VII (Asp40), have been exchanged in the present study by site-directed mutagenesis in murine CAD (Asn260, Asp262, and Asn299) and were shown to be essential for the DNA cleavage activity of this DNase, providing strong evidence for a similarity in active-site structure and mechanism of the CAD proteins with these members of the $\beta\beta\alpha$-Me-finger nucleases.

On the basis of our results, we have generated a model for the active-site structure and the mechanism of catalysis of CAD (Figure 4). In the model, the active-site region of CAD shows an α-helical insertion between the two β-strands of the $\beta\beta\alpha$-Me-finger motif but otherwise closely resembles the common features of the $\beta\beta\alpha$-Me core of known $\beta\beta\alpha$-Me-finger nucleases (Figure 4). Previous mutagenesis data in combination with the sequence similarities of CAD proteins with the $\beta\beta\alpha$-Me-finger nucleases clearly identify His263 as the general base in the catalytic mechanism of CAD. In contrast, it is still not clear which is the general acid. Leaving group protonation could in principle be carried out directly by His308 or Asp262, or as concluded for the *Serratia* nuclease and I-*Ppo*I, by a water molecule from the hydration sphere of the divalent metal ion bound to these residues (*41*, *43*, *44*). The divalent metal ion, which is an obligatory cofactor for the catalytic mechanism of CAD, could also serve to stabilize the transition state during catalysis of the hydrolysis reaction, as suggested recently for I-*Ppo*I and colicin E7 (*45*, *46*).

In colicin E9, the divalent metal-ion cofactor (in the case of Ni^{2+}) can be bound by two histidine residues, His102 and His127 (*32*), and in the Ca^{2+}-containing structure of T4 endonuclease VII, the cofactor is bound to Asp40 and Asn62 (Figure 4) (*24*). The pronounced sequence similarity between CAD proteins and T4 endonuclease VII as well as colicin E9 let it appear likely that in the case of CAD the metal-ion cofactor may also be bound by the two corresponding residues, Asn262, which aligns with Asp40 in T4 endonuclease VII and His102 in colicin E9 as well as His308, which aligns with Asn62 in T4 endonuclease VII and His127 in colicin E9 (Figure 4). This would mean that the active site of CAD displays features that in one part resemble the colicin E9 active site and in another part the T4 endonuclease VII active site. It is clear that structural investigations are necessary to clarify this issue.

Taken together, we have presented evidence that CAD proteins constitute a novel subfamily of $\beta\beta\alpha$-Me-finger nucleases. Local sequence similarities between CAD proteins, the colicin DNases, and T4 endonuclease VII family members and the results of previous and present studies strongly support the hypothesis that CAD proteins have an active-site fold and follow a catalytic mechanism very similar to that of colicin DNases and T4 endonuclease VII family members. Our results provide a first insight into the likely catalytic mechanisms of an important nuclease involved in the degradation of chromosomal DNA during programmed cell death.

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