Structural Basis for Stable DNA Complex Formation by the Caspase-activated DNase*

We describe a structural model for DNA binding by the caspase-activated DNase (CAD). Results of a mutational analysis and computational modeling suggest that DNA is bound via a positively charged surface with two functionally distinct regions, one being the active site facing the DNA minor groove and the other comprising distal residues close to or directly from helix α4, which binds DNA in the major groove. This bipartite protein-DNA interaction is present once in the CAD/inhibitor of CAD heterodimer and repeated twice in the active CAD dimer.

The caspase-activated DNase (CAD)² (DFF40) is the nucleosome subunit of the DNA fragmentation factor DFF, a heterodimeric complex that triggers DNA degradation in apoptotic cells (1–5). Within the complex, CAD is kept inactive by the inhibitory subunit ICAD-L (DFF45) that triggers DNA degradation in apoptotic cells (1–5). Within the core structure of the DNA fragmentation factor DFF, a heterodimeric complex repeated twice in the active CAD dimer.

As shown recently by biochemical and crystallographic studies, CAD belongs to the H-N-H or ββα-Me-finger superfamily of nucleases with a common active site motif (10–13). This motif, consisting of two anti-parallel β-strands and an α-helix, can be found in a large number of enzymes, ranging from nonspecific to highly sequence- or structure-specific nucleases with diverse structures and biological functions (14–16). CAD cleaves naked DNA as a nonspecific nuclease, yet in contrast to other nonspecific nucleases both free CAD and, notably, the inhibited enzyme are proficient in forming stable DNA complexes (17, 18). For this reason, CAD is often used for labeling of specific nucleic acid sequences by specific nucleases with diverse structures and biological functions (14–16).

Forte et al. (21) have shown that the active site facing the DNA minor groove and the other comprising distal residues close to or directly from helix α4, which binds DNA in the major groove. This bipartite protein-DNA interaction is present once in the CAD/inhibitor of CAD heterodimer and repeated twice in the active CAD dimer.

bacterial enzymes related to CAD, the Vibrio vulnificus nuclease (Vvn) and the Escherichia coli nuclease colicins E7 (ColE7) and E9 (ColE9) (14, 21–23). Vvn nuclease and ColE7/E9 are a periplasmic nuclease and bacterial toxins engaged in host defense and cell killing, respectively. These nonspecific nucleases mainly bind DNA in the minor groove, with their ββα-Me-finger motifs forming contacts to the phosphodiester backbone of one strand of the substrate and establishing only minor contacts to the DNA major groove (21–23). In contrast, the sequence-specific members of the H-N-H or ββα-Me-finger nucleases, such as the homing endonucleases I-PpoI or I-HmuI, also use their ββα-Me-finger motifs to contact the cleavage site via the DNA minor groove. In addition, they use more structural elements to form specific contacts to the phosphodiester backbone and the bases via the minor and major grooves of their DNA substrates (24–26).

With the aim of identifying the structural basis for the unusual capacity of CAD to form stable DNA complexes as a nonspecific nuclease, we performed a mutational analysis of putative DNA binding residues in combination with computational modeling of CAD/DNA complexes on the basis of DNA co-crystal structures of the above mentioned related nonspecific nucleases (21–23). Our results suggest that CAD forms stable DNA complexes via a DNA binding region that comprises residues close to or directly from helix α4 distal to the active site.

We describe a structural model for DNA binding by the caspase-activated DNase (CAD). Results of a mutational analysis and computational modeling suggest that DNA is bound via a positively charged surface with two functionally distinct regions, one being the active site facing the DNA minor groove and the other comprising distal residues close to or directly from helix α4, which binds DNA in the major groove. This bipartite protein-DNA interaction is present once in the CAD/inhibitor of CAD heterodimer and repeated twice in the active CAD dimer.

MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins—Recombinant DFF was produced by co-expressing GST-tagged CAD variants together with human ICAD-L (DFF45) in E. coli BL21Gold(DE3) cells harboring the two compatible expression vectors pGEX-2T-CAD and pACET-DFF45. For each DFF variant, recombinant protein from 2 liters of culture was purified via glutathione affinity chromatography. GST-tagged DFF was bound to glutathione-Sepharose 4-B beads in buffer A containing 750 mM NaCl. Purified complex was eluted from the glutathione-Sepharose 4-B beads using buffer A (20 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.01% Triton X-100) supplemented with 2.5 mM MgCl₂ treated with DNNase I (20 units) to digest contaminating DNA, and subsequently washed intensively with buffer A containing 750 mM NaCl. Purified complex was eluted from the glutathione-Sepharose 4-B beads using buffer A supplemented with reduced glutathione to 20 mM. Initially, protein was purified via GST affinity chromatography without DNNase I and high salt treatment; it was further purified by anion-exchange chromatography over a Mono-Q HR5/5 column by applying a linear gradient in 15 ml of 100–400 mM NaCl in buffer A at a flow rate of 1 ml/min. Eluted DFF was concentrated using Centriplus-30 ultrafiltration units. Caspase-3 was expressed and purified as described previously (27).

Generation of Arginine and Phenyllalanine Mutants—Arginine mutants of CAD were prepared as described previously (28). The following primers were used to introduce the desired mutations: R151A (AGGTTTGGAGTGCGCATTTCCGAGATAAGTCGGGCTA), R166A (ACAGCTTGGAGAGTCACCCAGTTGTCCTAAGAGA), R168A (TGAGAGTCTTTCCGAGATAAGTCGGGCTA).

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This paper is dedicated to Alfred Pingoud on the occasion of his 60th birthday.

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2 The abbreviations used are: CAD, caspase-activated DNase (synonym for DFF40); DFF40, DNA fragmentation factor 40-kDa subunit (synonym for CAD); DFF45, DNA fragmentation factor 45-kDa subunit (synonym for ICAD-L); EMSA, electrophoretic mobility shift assay; ICAD, inhibitor of CAD; ICAD-L, ICAD large isoform (synonym for DFF45); PAR, poly(ADP-ribose); CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
Conserved arginine residues contribute to DNA binding and cleavage by CAD.

A, alignment of the C-terminal region of CAD proteins from Mus musculus (mCAD, GenBank™ accession number AB009377), Rattus norvegicus (rCAD, GenBank™ accession number AF136598), Homo sapiens (hCAD, GenBank™ accession numbers AF064019, AF038910, AB019318), Gallus Gallus (chCAD, GenBank™ accession number AF406761), Danio rerio (fCAD, GenBank™ accession number AF286179), and Drosophila melanogaster (dCAD, GenBank™ accession number AF149797, AB036733). The black and gray vertical bars highlight conserved arginine, phenylalanine, and lysine residues investigated in this study. The black horizontal bar represents helix H9251. The two β-strands of the βαβα-Me-finger active site motif are shown as black arrows. Important active site residues (Asp262, His263, and His308) are highlighted by asterisks.

B, preparation of arginine variants of the caspase-activated DNase. Recombinant proteins (GST-CAD/DFF45) were expressed in E. coli and purified by glutathione affinity chromatography. Analysis by SDS-PAGE of the free nuclease variants released from the DFF complex through activation by caspase-3.

C, DNase activity of the arginine variants of CAD as determined in plasmid DNA cleavage assays.

**FIGURE 1.** Conserved arginine residues contribute to DNA binding and cleavage by CAD. A, alignment of the C-terminal region of CAD proteins from Mus musculus (mCAD, GenBank™ accession number AB009377), Rattus norvegicus (rCAD, GenBank™ accession number AF136598), Homo sapiens (hCAD, GenBank™ accession numbers AF064019, AF038910, AB019318), Gallus Gallus (chCAD, GenBank™ accession number AF406761), Danio rerio (fCAD, GenBank™ accession number AF286179), and Drosophila melanogaster (dCAD, GenBank™ accession number AF149797, AB036733). The black and gray vertical bars highlight conserved arginine, phenylalanine, and lysine residues investigated in this study. The black horizontal bar represents helix H9251. The two β-strands of the βαβα-Me-finger active site motif are shown as black arrows. Important active site residues (Asp262, His263, and His308) are highlighted by asterisks. B, preparation of arginine variants of the caspase-activated DNase. Recombinant proteins (GST-CAD/DFF45) were expressed in E. coli and purified by glutathione affinity chromatography. Analysis by SDS-PAGE of the free nuclease variants released from the DFF complex through activation by caspase-3. C, DNase activity of the arginine variants of CAD as determined in plasmid DNA cleavage assays.
DNA Binding by CAD

Conserved Arginine Residues Contribute to DNA Cleavage by CAD—Arginine residues are frequently involved in substrate binding by proteins interacting with nucleic acids (29–31). Primary sequence analysis of CAD proteins from diverse species suggests the presence of six conserved residues (Arg$^{151}$, Arg$^{166}$, Arg$^{168}$, Arg$^{212}$, Arg$^{250}$, and Arg$^{269}$) in the C-terminal catalytic domain of CAD (Fig. 1A). To investigate the contribution of these residues to DNA binding and cleavage by CAD, we produced CAD variants with substitution for alanine of these conserved residues, expanding previously performed mutational analyses of amino acid residues critical for activity of the enzyme (11, 27, 32).

Activity assays with a plasmid DNA substrate and recombinant CAD purified from $E$. coli by glutathione affinity chromatography revealed that all arginine variants of CAD tested here exhibit reduced cleavage activities, albeit to different extents, and thus contribute directly or indirectly to DNA binding and/or cleavage by this enzyme (Fig. 1, B and C). One of these conserved arginine residues (Arg$^{269}$) is located at the $\beta\beta\alpha$-Me-finger active site motif of CAD, two residues (Arg$^{250}$ and Arg$^{212}$) are involved in homodimerization of CAD or located in spatial proximity to the N-terminal end of helix $\alpha_4$ (residues Ser$^{156}$–Ser$^{178}$), respectively, and three of these residues (Arg$^{151}$, Arg$^{166}$, and Arg$^{168}$) belong to either a loop region preceding helix $\alpha_4$ or helix $\alpha_4$ itself (Fig. 1A), suggesting that this structural element plays an important role in DNA binding and/or cleavage by CAD (10, 17).

PAR Interferes with DNA Binding by CAD and the CAD-ICAD Complex—We have shown previously that DFF (CAD-ICAD) binds to DNA, though in the heterodimeric DFF complex the nuclease is inactivated by the inhibitory subunit (17). Given that a single amino acid substitution in the DNA subunit (Lys$^{555}$ → Gin) abolishes DNA binding by DFF and the free nuclease itself, we proposed that the nuclease subunit alone is responsible for DNA binding by DFF (17). In addition to the catalytic center of CAD, which consists of a $\beta\beta\alpha$-Me-finger motif and very likely contacts DNA via the minor groove, a structural analysis of the active CAD homodimer suggested that helix $\alpha_4$ is in a favored position to bind DNA via the major groove (10). As shown recently, this helix $\alpha_4$ overlaps with a conserved poly(ADP-ribose) binding motif (residues Arg$^{151}$–Arg$^{172}$ in CAD) that allows modulation of CAD activity by non-covalent interaction with PAR (Fig. 2A) (33). PAR is known to bind to specific domains in DNA damage checkpoint proteins (34–36). We have used this interaction of CAD with PAR to determine whether the DNA binding ability of not only the free nuclease, as shown earlier, but also DFF (CAD-ICAD) can be modulated by PAR. As a result, competition assays in which DFF and caspase-3-treated DFF were incubated with PAR prior to DNA binding revealed that PAR interferes with the ability of free CAD and CAD in the DFF complex to induce a mobility shift in the DNA (Fig. 2B). To interfere with DNA binding by DFF as compared with the free nuclease a higher concentration of PAR was needed, indicating that CAD in complex with the inhibitory subunit ICAD-L (DFF45) probably binds PAR more weakly than free CAD. These results suggest that helix $\alpha_4$ is indeed part of a DNA binding site accessible in free CAD as well as CAD bound to its inhibitor in the DFF complex.

Models of CAD-DNA Complexes—to gain insight into the structural basis for stable DNA complex formation by CAD, we modeled CAD-DNA complexes by superimposing the active site motifs of ColE7 and $Vvn$ nuclease from DNA co-crystal structures with the active site of CAD (Fig. 3). The structural analysis yielded two models of CAD binding to DNA, with differences arising from dissimilar conformational changes induced in the DNA by the two bacterial nucleases. Although ColE7 does not produce major distortions in the DNA, $Vvn$ nuclease widens the minor groove and bends the DNA toward the major groove at an angle of $\sim$20° (21, 22). We also modeled the CAD-DNA complex with DNA from the ColE9-DNA-Mg$^{2+}$ ternary complex (23). Given the high similarity between ColE7 and E9 this yielded a model that, as expected, is highly similar to the one obtained with DNA from the ColE7-DNA complex (data not shown). The electrostatic potential distribution on the DNA binding surface of CAD suggests that DNA might indeed be bent toward the major groove, as seen in the $Vvn$-DNA complex (data not shown). As expected, in both models the active site motif of CAD faces the DNA minor groove, and helix- $\alpha_4$ nicely aligns with the DNA major groove.

Selection, Purification, and Stability of CAD Variants—To support our predicted model for CAD-DNA complexes and to analyze the CAD-DNA interaction in more detail we purified from $E$. coli an assortment of CAD variants with substitution of the conserved arginine residues described above and of selected variants (K155Q, K310Q, and H313N) described previously (11, 32). In addition we exchanged Phe$^{152}$ of CAD to alanine, because we suspected this highly conserved amino acid residue forms contacts to the DNA together with its neighboring residue Arg$^{151}$ from a loop region preceding helix $\alpha_4$ (Fig. 4A). Together, these variants carry amino acid substitutions at conserved residues that are either part of the $\beta\beta\alpha$-Me-finger active site motif of CAD or located...
FIGURE 2. Interference of DNA binding by DFF and free CAD with PAR. A, caspase-activated DNases contain a PAR binding consensus sequence (for sequence descriptions refer to Fig. 1). The residues comprising the PAR binding consensus sequence belong to the N-terminal part of helix α4. They overlap with a surface area that is involved in DNA complex formation by CAD. B, DFF and free CAD were incubated with indicated amounts of poly(ADP-ribose) (PAR) prior to binding to a 44-bp radiolabeled double-stranded oligodeoxyribonucleotide. Binding reactions were separated on 4% polyacrylamide gels and subsequently analyzed by autoradiography. PAR interferes with DNA complex formation by free CAD and the DFF (CAD-ICAD) complex. This suggests that helix α4, which overlaps with the PAR binding motif of CAD (33, 36), is crucial for stable DNA complex formation.
distal from the active site in the region around helix α4 that largely overlaps with the PAR binding region (compare Figs. 2A and 4A).

Because some of the DFF complexes with certain CAD variants when purified by ion-exchange chromatography over a Mono-Q column as described previously turned out to be unstable, we established a different protocol for DFF purification: DFF bound to glutathione-4B-beads was treated with DNase I and then extensively washed with high salt buffer to get rid of contaminating DNA that would otherwise interfere with the gel retardation analysis (17). Using this protocol the majority of desired DFF complexes could be produced, with the exception of the DFF_{N-F32A} variant (carrying the nuclease subunit with Phe^{152} exchanged to Ala), which could not be obtained as a DNA-free complex irrespective of the purification protocol applied (Fig. 4B). Intriguingly, most CAD variants that turned out to be proficient in forming stable DNA complexes (see below) could be readily purified by ion-exchange chromatography over a Mono-Q column, whereas those variants that turned out to be deficient in stable DNA complex formation caused dissociation of the DFF complex on the Mono-Q column, suggesting a correlation between the stability of the variant DFF complexes during the purification procedure and their ability to form stable DNA complexes. All of the nuclease variants produced showed little if any plasmid cleavage activity, with the notable exception of the R250A variant (Fig. 4C).

Variants of CAD in Complex with ICAD (DFF) and as the Free Enzyme Behave Similarly in the EMSA—To test the DNA binding ability of the selected CAD variants in complex with ICAD and as the free enzyme, we first carried out EMSA with DFF (CAD-ICAD) and with the free nuclease released from DFF by caspase-3 treatment. To this end, DFF with mutated nuclease subunits at a final concentration of 5 μM was incubated with plasmid DNA and the binding reactions analyzed by agarose gel electrophoresis (Fig. 5A, upper panel). In parallel, DFF complexes with mutated nuclease subunits were treated with recombinant caspase-3 to release the nuclease, and DNA binding reactions were analyzed as above (Fig. 5A, lower panel). Generally, in the assay DFF complex and the free nuclease behave similarly with only slight differences in the appearance of the protein-DNA complexes, suggesting that ICAD does not interfere with the function of residues involved in stable DNA complex formation by CAD.

As seen from Fig. 5A, only one of the selected variants (H313N) displays wild type-like DNA binding activity in the EMSA with plasmid DNA, whereas all other mutants exhibit a reduced ability to induce a mobility shift in the DNA, albeit to different extents. Although the variants R166A, K310Q, and R269A are still able to shift plasmid DNA in the assay, the variants R151A, K155Q, R168A, and R212A do not induce a detectable mobility shift in the DNA under the conditions applied, with the exception of R250A, which leads to a slight mobility shift in the DNA. As shown above (Figs. 1C and 4C), most of the nuclease variants, as expected, show little if any plasmid cleavage activity, with the exception of the R250A variant. This variant cleaves plasmid DNA with reasonable activity, even at nanomolar enzyme concentration. Because the R250A variant is the only variant tested so far that readily cleaves DNA but fails to induce a strong mobility shift at the concentrations used above, we prepared larger amounts of this variant and tested its DNA binding activity at concentrations exceeding 5 μM. In these experiments the variant R250A induces a moderate mobility shift in plasmid DNA at relatively high concentrations compared with the wild type enzyme (Fig. 5B).

Relative DNA Binding Activities of CAD Variants in Complex with ICAD—All the nuclease variants analyzed so far show diminished DNA cleavage activity, and some also lost the ability to induce a mobility shift in a plasmid DNA substrate. To gain information on the relative binding activities of the CAD variants, we used an assay that allowed us to obtain more quantitative data on DNA complex formation by CAD. We carried out EMSA with DFF variants and a 273-bp PCR-derived double-stranded DNA substrate (Fig. 6A). Analysis of the DNA binding data in conjunction with the localization of the amino acid residues exchanged revealed that the variants fall into three groups. The first group comprises variants that are deficient in DNA cleavage yet proficient in inducing a mobility shift in the DNA (variants with amino acid substitution of Arg^{166}, Arg^{296}, Lys^{310}, and His^{313}). This behavior can be con-
considered typical for variants mainly affected in catalysis. The second group consists of variants that are deficient in DNA binding as well as DNA cleavage (variants with amino acid substitution of Arg151 and Arg168), which can be considered typical for mutants mainly affected in substrate binding. Phe152 most likely also contacts DNA directly; however, exchanging this residue in CAD leads to a highly unstable variant (Fig. 4A). Variants with substitution of residues close to or directly from helix α4 (Arg166 and Arg168) span important interactions with the DNA minor groove (Figs. 3 and 7). In contrast to other ββα-Me-finger nucleases, CAD aligns a loop rather than an α-helix with the DNA minor groove. This loop contains the active site residues His308, His313, and Lys310 involved in cofactor binding and substrate positioning (10, 11, 32). Also included in this part of the bipartite DNA binding surface is Arg269, conserved in all CAD proteins (Fig. 1A).

DISCUSSION

In the present study we have reported the modeling of CAD-DNA complexes accompanied by a mutational and biochemical analysis of the DNA binding activities of selected CAD variants. On the basis of their DNA binding properties and available structural data, the variants investigated here can be sorted into three groups, those with substitution of active site residues having no influence on stable DNA binding and those with substitution of residues close to or directly from helix α4, being directly or indirectly important for stable DNA complex formation, respectively. Indeed, these different categories of amino acid residues reflect distinct functional regions of a bipartite DNA binding surface important for stable DNA complex formation and DNA cleavage by CAD as well as a structurally important region that contributes indirectly to stable DNA complex formation and DNA cleavage by the enzyme.

One part of the bipartite DNA binding surface encompasses the active site of CAD with a ββα-Me-finger motif that faces the DNA minor groove (Figs. 3 and 7). In contrast to other ββα-Me-finger nucleases, CAD aligns a loop rather than an α-helix with the DNA minor groove. This loop contains the active site residues His308, His313, and Lys310 involved in cofactor binding and substrate positioning (10, 11, 32). Also included in this part of the bipartite DNA binding surface is Arg269, conserved in all CAD proteins (Fig. 1A). Variants with substitution of amino acid residues from this region, K310Q, H313N, and R250A (and also H263N characterized previously, Ref. 17), retain their ability to form stable DNA complexes yet show little DNA cleavage activity, suggesting that these surface-exposed residues from the active site are not required for stable DNA complex formation by CAD. However, different from ColE7 and Vvn nuclease, CAD has established pronounced interactions with the DNA major groove via a second DNA binding region. This region, which locates distal to the active site, represents the second part of the bipartite DNA binding surface and largely overlaps with the previously identified PAR binding site (Figs. 2A and 7B) (33). It includes residues nearby or directly from helix α4 of CAD that nicely align with the DNA major groove (Figs. 3 and 7). Conserved arginine residues from helix α4 (Arg166 and Arg168) span the major groove, with the side chain of Arg168 being very close to the

**FIGURE 4.** Selection, purification, and stability of CAD variants. A, distribution of residues selected for the mutational analysis of stable DNA complex formation by CAD.

The selected residues are either part of the ββα-Me-finger active site motif of CAD (Arg166, Lys160, and His163) or located distal from the active site in the region around helix α4 (Arg151, Phe152, Lys155, Arg166, Arg168, Arg212, and Arg250). Also shown are the catalytic residues Asp262, His263, and His308. B, preparations of selected DFF variants. Recombinant proteins (GST-CAD/DFF45) were expressed in *E. coli* and purified using different protocols described under “Materials and Methods.” Analysis by SDS-PAGE of the purified protein complexes illustrates that with certain variants complex stability is affected by the purification protocol (e.g., compare the purification profiles of R166A to R168A). C, DNA cleavage activities of the CAD variants released from the DFF complexes purified by the DNase I/high salt purification protocol. Concentration of CAD variants in the different DFF complexes was normalized to the concentration of wild type CAD, similar to the arginine variants shown in Fig. 18.
active site and contacting the phosphodiester backbone with the scissile bond and the side chain of Arg^{168} pointing away from the active site, contacting the phosphodiester backbone of the opposite strand (Fig. 7A). Upon amino acid substitution of Arg^{166} or Arg^{168}, CAD variants display drastically reduced DNA cleavage activities. However, variant R166A, in contrast to variant R168A, retains the ability to form stable DNA complexes, suggesting that Arg^{166} falls into the class of an active site residue, whereas Arg^{168} is indeed required for stable DNA complex formation. Two other conserved surface-exposed amino acid residues (Arg^{151} and Phe^{152}), located farthest away from the active site in a loop and a short β-strand directly preceding helix α4, are also part of this second DNA binding region and thus critical for stable DNA complex formation by CAD. Unfortunately, the likely role of Phe^{152} in DNA binding could not be analyzed because the F152A variant was unstable. Considering the correlation we observed between the stability of CAD variants during the purification procedure and their ability to form stable DNA complexes, it seems very likely that Phe^{152} together with its neighboring residue Arg^{151} directly contacts the DNA. Finally, the residues Lys^{155}, Arg^{212}, and Arg^{250} represent a group of residues that define a structurally important region in CAD. Whereas Lys^{155} is buried in the protein and located at the N-terminal end of helix α4, Arg^{212} and Arg^{250} are surface-exposed residues but no part of the DNA binding surface of CAD. These residues are most likely indirectly involved in DNA binding and seem to play an important structural role in stabilizing helix α4 (Lys^{155} and Arg^{212}) as well as supporting homodimerization of CAD (Arg^{250}) (Fig. 7) (10).

Interestingly, exchange of Arg^{250} that is located at the dimerization interface of CAD influences the DNA binding and cleavage activity of CAD. The effect seen upon substitution of this conserved arginine residue is likely to be indirect because it is too far away from the DNA substrate. It could arise from structural perturbation of the DNA binding surface and/or the dimerization interface of CAD. Provided that disturbing the homodimerization of the enzyme is responsible for the drop in DNA binding and cleavage by CAD, our finding would support the hypothesis that preventing homodimerization of CAD by ICAD is the principle mechanism of inhibition of this nuclease (10). It would also mean that dimerization of CAD already occurs at pico- to nanomolar concentrations.

FIGURE 6. Relative DNA binding activities of CAD variants in complex with ICAD-L/DFF45. A, EMSA with a 273-bp PCR-derived double-stranded DNA fragment incubated with the indicated amounts of CAD variants in complex with ICAD-L/DFF45 and analyzed by PAGE as described under “Materials and Methods.” B, plot of DNA binding data from panel A. Variants fall into three groups, those with active site residues having no influence on stable DNA binding and those with residues close to or directly from helix α4, being either directly involved in stable DNA binding or being structurally important and thus indirectly influencing DNA complex formation.
concentrations because recombinant CAD is reasonably active at these concentrations.

With its dimeric structure and its ability to form stable DNA complexes, CAD on the one hand significantly differs from other nonspecific nucleases and instead displays similarities to sequence-specific nucleases such as restriction enzymes or homing endonucleases (16, 17, 37). Whereas the latter mostly act as dimers or pseudo-dimers and exhibit complex protein-DNA interfaces, the nonspecific nucleases typically act as monomers or dimers with independent active sites. They usually form only a minimum of contacts sufficient to bring about DNA cleavage, at the same time avoiding specific DNA complex formation (14, 19, 38). On the other hand, different from restriction enzymes or homing endonucleases, the stable DNA complexes formed by CAD are nonspecific and thus enable the enzyme to degrade DNA, still without selectivity, a prerequisite for the physiological function of the enzyme, which is to cut any accessible linker DNA it engages. Because CAD forms stable DNA complexes in the presence of its inhibitor ICAD, it is tempting to speculate that the biological function of stable DNA complex formation by the CAD-ICAD complex is to bind the DNA in apoptotic cells prior to nuclease activation by caspase-3 in order to transform the proximity to its substrate into highly efficient DNA fragmentation (17). It could well be that in non-apoptotic cells the CAD-ICAD complex forms transient interactions with chromatin, employing direct contacts to the DNA as well as indirect contacts to its substrate mediated by chromatin-associated proteins such as histone H1 and HMGB1 and 2, which have been shown to interact with CAD and stimulate its activity (18, 39–41). Interaction of CAD with these proteins might be important for the enzyme to gain access to the linker DNA between nucleosomes.

In summary, our results demonstrate that CAD possesses, in addition to the active site, a second DNA binding region responsible for stable DNA complex formation by the free enzyme and the DFF (CAD+ICAD) complex. This binding site is formed by conserved surface-exposed amino acid residues close to and directly from helix α4, which is distal to the βαα-Me-active site motif of this nuclease and binds in the DNA major groove. In addition, we identified a region important for stabilization of helix α4 that comprises amino acid residues in spatial proximity to the N-terminal end of this helix. The bipartite protein DNA interaction is found once in the CAD-ICAD heterodimer and is repeated twice in the active CAD homodimer (Fig. 7B). Our model explains the unusual capacity of CAD to form stable DNA complexes as a nonspecific nuclease on the structural level.

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FIGURE 7. Bipartite DNA binding surface of CAD. A, view along the DNA major groove bound by CAD (red, helix α4; green, βαα-Me-active site motif). Space-filled representation of the DNA from the ColE7/DNA co-crystal structure (Protein Data Bank code 1pt3) with one strand orange and the opposite strand purple. B, surface representation of a CAD homodimer illustrating the bipartite DNA binding surface without (upper panel) and with (lower panel) DNA from the Vvn/DNA co-crystal structure (Protein Data Bank code 1oup). Residues close to or directly from helix α4, which overlaps largely with the PAR binding surface highlighted in red, form the DNA binding site responsible for stable DNA complex formation, whereas residues from the βαα-Me-active site motif are responsible for DNA cleavage.
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