Human lysosomal DNase IIα contains two requisite PLD-signature (HxK) motifs: Evidence for a pseudodimeric structure of the active enzyme species

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Abstract

Lysosomal DNase IIα is essential for DNA waste removal and auxiliary apoptotic DNA fragmentation in higher eukaryotes. Despite the key role of this enzyme, little is known about its structure–function relationships. Here, mutational and biochemical analyses were used to characterize human DNase IIα variants expressed in mammalian cells. The resulting data strongly support the hypothesis that the enzyme is a monomeric phospholipase D–family member with a pseudodimeric protein fold. According to our results, DNase IIα contains two requisite PLD-signature motifs (^{113}HTK^{115} and ^{295}HSK^{297}) in the N- and C-terminal subdomains, respectively, that together form a single active site. Based on these data, we present an experimentally validated structural model of DNase IIα.

Keywords: apoptosis; DNA fragmentation; phospholipase D; DNase; structure; active site

Lysosomal DNase IIα, also known as spleen acid DNase or acid DNase, is a physiologically important enzyme found in a wide variety of organisms and animal tissues (Evans and Aguilera 2003). The enzyme is active at acidic pH, cleaving DNA to produce 3′-phosphate termini. Unlike the majority of nucleases, DNase IIα functions in the absence of divalent metal ion cofactors (Yang et al. 2006). Discovered >50 years ago, numerous studies have focused on the enzymatic properties and biological roles of the enzyme and its homologs from various animal sources (Laskowski et al. 1954; Bernardi and Griffe 1964; Harosh et al. 1991). Several of these studies suggest that mammalian DNase IIα is involved in apoptosis by digesting chromosomal DNA of apoptotic cells after their engulfment by macrophages (Barry and Eastman 1993; Barry et al. 1993; McIlroy et al. 2000; Krieser et al. 2002). This process has been termed auxiliary DNA fragmentation to distinguish it from cell autonomous DNA fragmentation by apoptotic DNases such as the caspase-activated DNase or endonuclease G (McIlroy et al. 2000; Krieser et al. 2002; Samejima and Earnshaw 2005). It was also shown that the mammalian enzyme is essential for the digestion of expelled nuclei during erythropoiesis in mice, and it was demonstrated that a knockout of murine lysosomal DNase IIα leads to lethal anemia due to interferone-β production in mice embryos carrying cells with undigested DNA (Kawane et al. 2001; Yoshida et al. 2005). Investigations of the homologous Nuc1 in *Caenorhabditis elegans* confirmed the concept of auxiliary DNA fragmentation for lower eukaryotes by showing that phagocytic cells of nuc-1 mutants accumulate undigested nuclei of apoptotic cells (Wu et al. 2000). Thus, DNase IIα substantially contributes

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Abbreviations: CTD, C-terminal domain; NTD, N-terminal domain; PLD, phospholipase D.

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to apoptotic DNA fragmentation and the clearance of DNA during animal development (Samejima and Earnshaw 2005). These novel findings firmly demonstrate the biological importance of DNase IIα and have provoked new studies focusing on the role of DNase IIα in animal development and apoptosis.

Notwithstanding the numerous studies on the enzymatic properties of DNase IIα, the functional organization of the enzyme remains largely unknown. In particular, the nature of the active DNase IIα species is undetermined. Earlier studies on porcine and human DNase IIα suggested the processing of a single precursor polypeptide into three peptides, two of them cross-linked by disulfide bridges (Takeshita et al. 1998; Wang et al. 1998). Whereas the proteolytic processing of the porcine enzyme was confirmed in a very recent study on the involvement of histidine residues in the catalytic mechanism of the enzyme, studies on human DNase IIα nicely illustrate that this particular nuclease is active as a contiguous glycosylated polypeptide chain (MacLea et al. 2002, 2003; Cheng et al. 2006). Recently, we put forward a hypothesis based on a bioinformatics analysis that DNase IIα belongs to the phospholipase D (PLD) superfamily of enzymes, and we presented two alternative models of the human DNase IIα three-dimensional (3D) structure (Fig. 1; Cymerman et al. 2005). Known PLD family members either form homodimers with each subunit carrying a single HxK-motif (e.g., Nuc from *Salmonella typhimurium* or the restriction endonuclease R.BfiI from *Bacillus firmus* S8120) or pseudodimers with two HxK-motifs in one polypeptide chain (e.g., PLD from *Streptomyces* sp.) (Stuckey and Dixon 1999; Leiros et al. 2000; Grazulis et al. 2005). Given that DNase IIα exhibits two predicted PLD motifs, one in the N-terminal (^{113}HTK^{115}) and a second in the C-terminal (^{295}HSK^{297}) part of its sequence, the sequence analysis suggests that DNase IIα is likely to adopt a pseudodimeric protein architecture (Fig. 1A). However, since the similarity between the N-terminal HxK-motif of DNase IIα and those of other PLD family enzymes could not be statistically confirmed using bioinformatics, we were unable to exclude an alternative structural model in which two C-terminal domains dimerize to form a functional active site (Fig. 1B).

To discriminate between these two alternative hypotheses, we have performed a mutational and biochemical characterization of recombinant human DNase IIα expressed in mammalian cells. We show that the N-terminal PLD motif comprises catalytically relevant amino acid residues and that active, differently tagged DNase IIα variants behave as monomers in coimmunoprecipitation experiments. Summarizing, our data strongly support the model of human DNase IIα as a pseudodimeric PLD family member with a single active site formed by two subdomains.

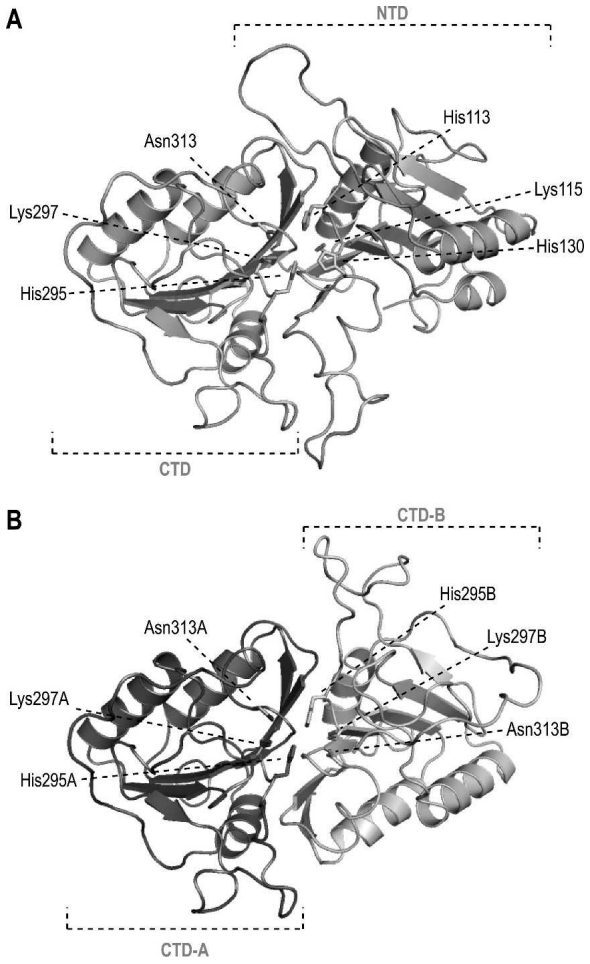

Figure 1. Alternative structural models for human lysosomal DNase IIα. (*A*) Ribbon representation of the putative pseudodimeric structure of DNase IIα with the C-terminal domain (CTD) and the N-terminal domain (NTD) forming one catalytic center at the CTD–NTD interface; amino acid residues from the C-terminal (His295, Lys297, and Asn313) and from the N-terminal (His113, Lys115, and His130) PLD-signature motifs are indicated. (*B*) Ribbon representation of a putative dimeric structure of DNase IIα with two C-terminal domains (CTD-A, dark gray; CTD-B, light gray) forming one catalytic center at the CTD–CTD interface (note that NTD is not shown); amino acid residues from both C-terminal PLD-signature motifs (His295, Lys297, and Asn313) denoted as "A" and "B" forming a symmetrical active site of a dimeric DNase IIα are indicated.

Results

C-terminally tagged recombinant DNase IIα localizes to lysosomes and is catalytically active

Native DNase IIα mainly localizes to lysosomes with some fraction of the enzyme also secreted into the extracellular space (Liao et al. 1989; Chang and Liao 1990; Baker et al. 1998). To track the cellular localization and to verify the catalytic proficiency of tagged DNase IIα variants used in this study, we overexpressed DNase

IIα fusion proteins in transiently transfected mammalian cells. To this end, constructs were generated leading to the production of enzyme variants C-terminally tagged with either enhanced green fluorescent protein (EGFP) or with a Flag-His_6 tandem tag. Confocal laser scanning microscopy was used to monitor enzyme distribution in a variety of mammalian cell lines, including CV1, HeLa, COS-1, HEK-293-T, NIH-3T3, and LNCaP cells. In all cell types tested, the transiently expressed DNase IIα–EGFP fusion protein was found distributed between the endoplasmic reticulum (ER), colocalizing with a DsRed-Express variant used as an ER marker (data not shown) and the lysosomal compartment, colocalizing with the lysosomal marker LysoTracker as shown for LNCaP cells (Fig. 2A), indicating that the lysosomal localization and hence the proper processing of recombinant DNase IIα is not affected by the C-terminal EGFP tag. Furthermore, using an anti-Flag affinity gel, we were able to immunoprecipitate a C-terminally Flag-His_6-tandem-tagged version of recombinant DNase IIα that was catalytically active, as shown by immunoblotting as well as a plasmid DNA cleavage assay, respectively (Fig. 2B). A Flag-tagged version of YFP (yellow fluorescent protein) was used as a control.

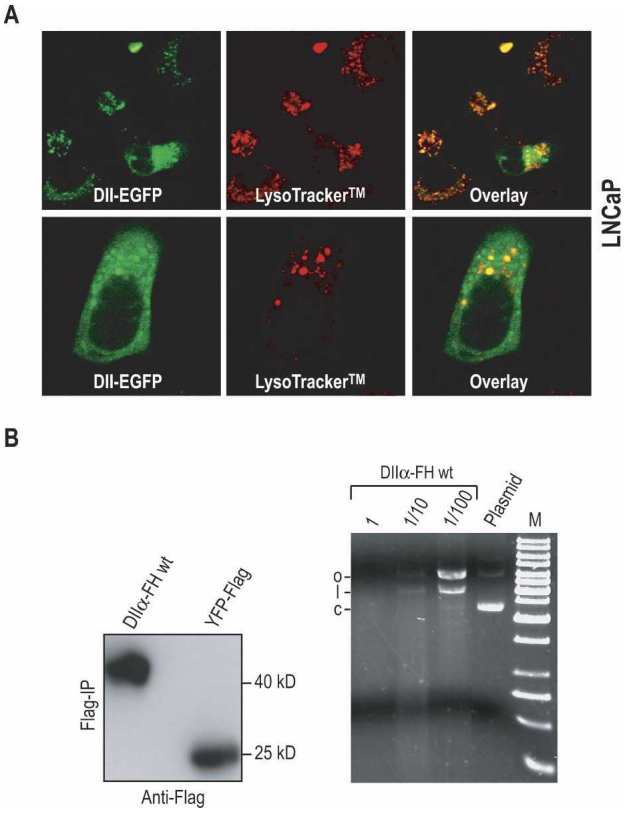

Figure 2. Intracellular localization and catalytic activity of transiently expressed DNase IIα fusion proteins. (*A*) Transiently expressed DNase IIα–EGFP localizes to the lysosomal compartment as exemplified using LNCaP cells. In these cells, a minor fraction of the enzyme is also found in the endoplasmic reticulum (ER). ER-staining was achieved by coexpression of a red fluorescent protein variant targeted to the ER (DsRed-Express-ER) (data not shown). Lysosomal staining was achieved using LysoTracker. (*B*) Transiently expressed Flag-His_6-tandem-tagged recombinant DNase IIα purified by immunoprecipitation using ANTI-FLAG M2 Affinity Gel (Sigma) (*left* panel) is catalytically active as shown by a plasmid DNA cleavage assay (*right* panel). Flag-tagged YFP was used as a control. In addition to the undiluted enzyme preparation, 10-fold and 100-fold dilutions of wild-type DNase IIα were used to monitor nuclease activity.

Mutational analysis of DNase IIα reveals two requisite HxK-motifs in the polypeptide chain

Our previous fold recognition analysis allowed us to confidently assign the PLD fold to the C-terminal domain of human DNase IIα (Fig. 1). Nevertheless, we were not able to confirm the significance of low, but intriguing, similarity between the N-terminal domain (NTD) of DNase IIα and its own C-terminal domain (CTD) or other PLD family members. To ascertain whether DNase IIα acts as a monomer (pseudodimer of N- and C-terminal subdomains) or as a dimer of subunits interacting via their CTDs, we generated mutants of the Flag-His_6-tandem-tagged enzyme with amino acid residues changes in the C-terminal (His295, Lys297, Asp311, and Asn313) as well as the putative N-terminal (His113, Lys115, and His130) PLD-signature motifs (Fig. 3A). Mutant proteins were expressed in transiently transfected HEK 293-T cells and purified either by immunoprecipitation directed against the C-terminal Flag-epitope tag (data not shown) or by Ni^{2+}-NTA affinity chromatography using the C-terminal His_6 tag (Fig. 3B). Our mutational analyses identified several residues from the C-terminal PLD motif of DNase IIα as indispensable for the activity of the enzyme. In agreement with previous data identifying homologous histidine residues in the catalytic mechanism of human and porcine spleen DNase IIα (Liao 1985; MacLea et al. 2003; Cheng et al. 2006), H295A in human DNase IIα leads to an inactive enzyme (Fig. 3C). Mutations K297A and N313A also generated enzyme variants with little but detectable nuclease activity, as deduced by their ability to cleave the fraction of supercoiled plasmid DNA, demonstrating that the putative C-terminal PLD motif of human DNase IIα, indeed, contains catalytically relevant amino acid residues that are characteristic for the active site of PLD enzymes. Of the C-terminal PLD motif mutants, the D311A variant retains the highest residual activity, indicating that Asp311 of human DNase IIα very likely is not essential for catalysis.

Significantly and importantly, our mutational analysis clearly illustrates that the putative N-terminal PLD motif also harbors residues that prove to be critical for the catalytic activity of DNase IIα. When alanine substitutions were made for His113, Lys115, or His130, all of

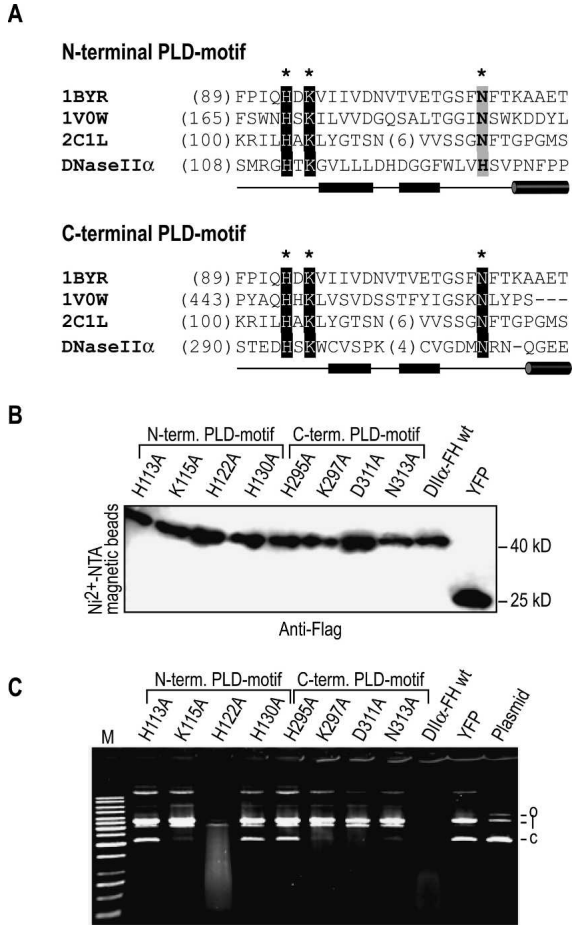

Figure 3. Alanine scanning mutagenesis of amino acid residues belonging to the putative C- and N-terminal PLD-signature motifs of human DNase IIα. (*A*) Sequence alignments showing conservation among structurally characterized PLD family members (1BYR, Nuc from *Salmonella typhimurium*; 1V0W, phospholipase D from *Streptomyces* sp.; and 2C1L, R.BfiI from *Bacillus firmus*) of putative active site residues (marked by asterisks) in the N- and C-terminal domains of DNase IIα. Secondary structure elements for DNase IIα are given *below* (rectangles, β-sheets; cylinders, α-helices). (*B*) All DNase IIα variants were overexpressed as Flag-His_6-fusion proteins in HEK 293-T cells and purified by IMAC using MagneHis magnetic Ni^{2+}-particles. In addition to the amino acid residues belonging to the PLD-signature motif, we also exchanged His122 to alanine and used the variant H122A of DNase IIα as a positive control in the cleavage assay shown in *C*. Flag-His_6-tandem-tagged YFP was purified in parallel and used as a negative control. Expression and protein preparation were monitored by immunoblotting using an anti-Flag antibody (Sigma). (*C*) Nucleolytic activity of DNase IIα variants was measured by cleavage of plasmid DNA in the presence of EDTA and at pH 4.5. (Plasmid) DNA incubated with reaction buffer only; (M) DNA marker; (YFP) yellow fluorescent protein; (c) supercoiled plasmid DNA; (l) linear plasmid DNA; (o) open circular plasmid DNA.

which are predicted to be part of the N-terminal PLD motif, residual activity was only seen in the case of the K115A variant, whereas nuclease activity was abolished in the case of variants H113A and H130A (Fig. 3A,C). As an internal positive control representing a catalytically active mutant enzyme, we also generated the H122A variant of DNase IIα whose high catalytic activity emphasizes the significance of our findings. Histidine residue 122 of human DNase IIα is predicted to be neither part of a conserved sequence motif nor part of the putative active site of DNase IIα, and thus, as observed, should not lead to an inactivated enzyme when exchanged to alanine. These results strongly argue that the enzyme contains two functional PLD motifs from which conserved amino acid residues contribute to the formation of a single functional catalytic center (Fig. 3A).

Gel filtration of recombinant human DNase IIα confirms that the enzyme is active as a contiguous polypeptide chain

In an attempt to determine the apparent molecular mass of the active enzyme species, we performed gel filtration experiments with cell extracts from HEK 293-T cells transiently transfected with constructs coding for Flag-His_6-tandem-tagged DNase IIα. Enzyme was immunoprecipitated from fractions eluting from the gel filtration column and subjected to plasmid cleavage assays, SDS-PAGE, and immunoblotting (Fig. 4). Both mock-transfected cells and cells transfected with an inactive DNase IIα variant (H295A) construct were used as controls. Upon gel filtration on a Superdex 75 column, DNase IIα protein and activity mainly appeared in fractions corresponding to an apparent molecular mass of 44–22 kDa, indicative of a monomeric enzyme as the active molecular species. The peak of DNase IIα activity eluted at the size of chymotrypsinogen α (22 kDa), which was used as a gel filtration marker (Fig. 4A). However, SDS-PAGE and immunoblotting show that the molecular species eluting at this particular apparent molecular mass also represents a contiguous polypeptide chain of ~45 kDa (Fig. 4B). The strong retention of DNase IIα on the column most likely is due to an unfavorable interaction of the glycosyl groups displayed by the mature enzyme with the gel filtration matrix. Unfortunately, due to this exceeding retention of DNase IIα, the gel filtration data do not allow us to draw any conclusions about the oligomerization state of DNase IIα; however, in combination with immunoblotting and plasmid cleavage assays, the data confirm that the recombinant human enzyme is active as a contiguous, proteolytically unprocessed polypeptide chain of ~45 kDa.

Coimmunoprecipitation assays support the idea of a DNase IIα monomer being the active species

In addition to the gel filtration analysis of DNase IIα, we used coimmunoprecipitation assays with Flag-His_6-tandem-tagged and HA-tagged DNase IIα to assay for dimerization of the

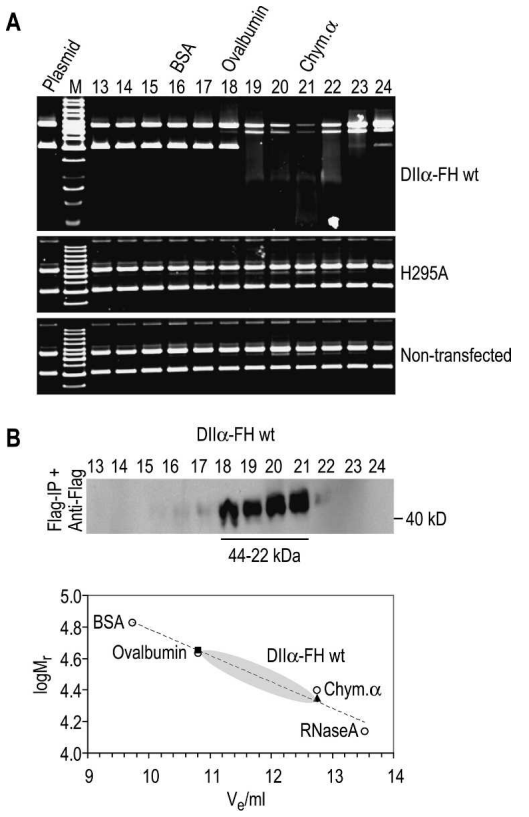

Figure 4. Gel filtration of extracts from HEK 293-T cells overexpressing DNase IIα. (*A*) Extracts from HEK 293-T cells transiently transfected with constructs coding for wild-type DNase IIα-FH, the inactive H295A-variant, and nontransfected cells were subjected to gel filtration over a Superdex-75 HR 10/30 column. Fractions (0.6 mL) were collected and supplemented with anti-Flag beads to allow immunoprecipitation of recombinant DNase IIα. Aliquots of the immunoprecipitated protein were assayed for DNase IIα activity using a plasmid DNA cleavage assay. In the eluted fractions of cellular extract from cells expressing the H295A variant and from nontransfected cells, virtually no DNase IIα activity was detectable, while from cells expressing wild-type DNase IIα, strong nucleolytic activity could be found in the fractions 19–24 with a peak activity in fraction 21, corresponding to an apparent molecular mass of 22 kDa. (*B*) Immunoprecipitated proteins were also blotted and probed with anti-Flag antibody. Flag-tagged DNase IIα protein was strongly detectable in fractions 18–21 with minor bands also in neighboring fractions, corresponding to an apparent molecular mass from 44 to 22 kDa.

enzyme. To this end, HEK-293-T cells were cotransfected with constructs coding for Flag-His_6-tandem-tagged wild-type DNase IIα and either the inactive H113A variant or HA-tagged wild-type DNase IIα. Constructs coding for Flag-tagged and HA-tagged YFP (yellow fluorescent protein) were used as controls. Immunoprecipitated proteins were assayed for dimer formation by Western blotting using anti-HA or anti-Flag antibodies and by activity assays using plasmid DNA as a substrate (Fig. 5). Dimerization was not detected in either coimmunoprecipitation experiments with anti-Flag agarose or in complementary assays using anti-HA agarose. These results were confirmed by activity assays with the immunoprecipitated proteins (Fig. 5B). Nucleolytic activity was only detected when active DNase IIα, either Flag-His_6-tandem-tagged or HA-tagged, respectively, was immunoprecipitated directly but not detected when active enzyme (e.g., HA-tagged wild-type DNase IIα) was cotransfected and coimmunoprecipitated with an inactive enzyme variant (e.g., Flag-His_6-tandem-tagged active-site mutant H113A).

Refined structural model of human DNase IIα

We recently proposed a preliminary model of DNase IIα (one of two alternatives) that implicated residues from

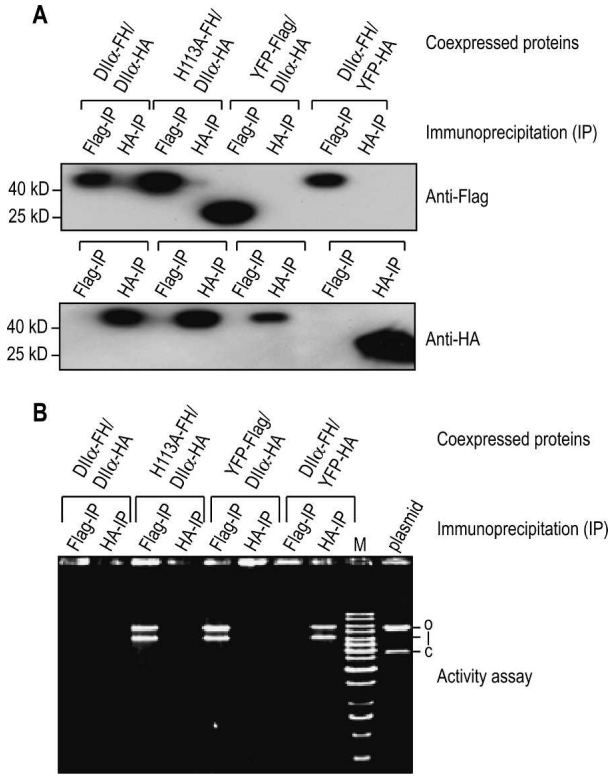

Figure 5. Immunoprecipitation of coexpressed DNase IIα variants with different epitope tags. Flag- and HA-tagged DNase IIα variants were coexpressed in HEK 293-T cells and (co-)immunoprecipitated using anti-Flag or anti-HA affinity beads, respectively, in order to detect possible dimer formation. The Flag-tagged inactive variant H113A as well as Flag- and HA-tagged YFP were used as controls. (*A*) Blots with anti-Flag and anti-HA antibodies, respectively, of the immunoprecipitated proteins show that apparently none of the coexpressed proteins displays significant interaction. (*B*) Beads used for the (co-)immunoprecipitation of differently epitope-tagged proteins were checked for DNase IIα activity with plasmid DNA serving as substrate. Only those beads with the wild type of either Flag- or HA-tagged DNase IIα directly bound yield a high nuclease activity, while those with the variants H113A-Flag, YFP-Flag, and YFP-HA directly bound do not show any detectable nuclease activity irrespective of the presence or absence of a differently tagged coexpressed wild-type DNase IIα.

the C-terminal (His295, Lys297, Asp311, and Asn313) and N-terminal (His113 and Lys115) PLD motifs but not the invariant and catalytically essential His130 in the putative active site. Using this model as a starting point for the refinement, we adjusted the sequence–structure alignment to match His130 of DNase IIα with the catalytic residue Asn111 of Nuc (1byr), Asn125 of PLD (2c1l), and Asn187 of R.BfiI (1v0w). The resulting 3D model of DNase IIα was evaluated as well-folded by VERIFY3D (0.34 for amino acids 30–329). It now accounts for all experimental data available, including the monomeric character of the whole protein, the 3D fold of the domain predicted by bioinformatics methods, identities of putative catalytic residues, proximal positions of disulfide bonds between cysteine residues, and the exposed character of *N*-glycosylated residues (Fig. 6). The new model better exemplifies intramolecular symmetry than the previous preliminary model, not only in the active site, but also in the length and conformation of the putative DNA-binding loops, for example, Asp97–Arg111 and Pro133–Tyr153. The model is available to the public at ftp://genesilico.pl/iamb/models/DNaseII/.

Discussion

DNase IIα is an enzyme essential for accessory apoptotic DNA fragmentation and DNA clearance during development and tissue regeneration in higher eukaryotes (Samejima and Earnshaw 2005; Yoshida et al. 2005). Despite its decisive role in those fundamental cellular processes, investigations into the structure–function relationship of DNase IIα have been hampered by the enzyme's requirement for *N*-glycosylation at several sites to acquire nucleolytic activity. This poses a serious problem for heterologous expression in bacterial systems and has made structural investigations hardly possible.

Based on the detection of the remote relationship between DNase IIα and structurally characterized PLD superfamily members, we previously predicted the 3D fold of DNase IIα and proposed two models. Whereas the dimer model predicts that the active site is composed of

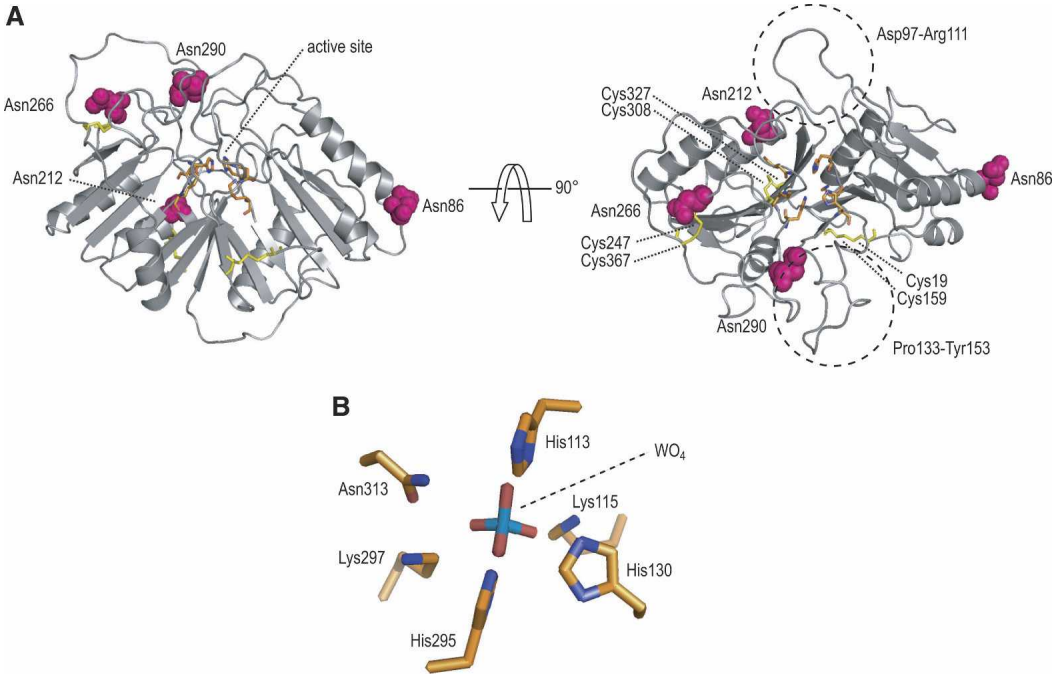

Figure 6. Refined model of the 3D structure of human DNase IIα. (*A*) According to our data, human DNase IIα adopts a monomeric structure with a pseudodimeric architecture. The two PLD motifs in the N- and in the C-terminal domains contain the catalytically relevant amino acid residues (orange), which collectively form a single active site (see below). In addition to the identities of putative catalytic residues, our structural model accounts for the monomeric character of the nuclease, the proximal positions of cysteine residue disulfide bonds (yellow, amino acid residues Cys19–Cys159, Cys267–Cys347, and Cys308–Cys327), and the exposed character of *N*-glycosylated residues (magenta, Asn86, Asn212, Asn260, and Asn290, represented as space-filled residues). Putative DNA-binding loops (Asp97–Arg111 and Pro133–Tyr153) are circled. (*B*) Active site of pseudodimeric DNase IIα including a bound tungstate ion modeled from the Nuc×tungstate cocrystal structure (PDB code 1byr). Amino acid residues His113, Lys115, and His130 from the N-terminal domain and His295, Lys297, and Asn313 from the C-terminal domain are in a similar arrangement as seen for homologous residues in structurally characterized PLD enzymes and form a single functional active site.

amino acids from two copies of the C-terminal domain, the monomer (pseudodimer) model implicates the N- and C-terminal domains folding into a single active site (Cymerman et al. 2005). The PLD superfamily of enzymes includes phosphodiesterases and phospholipases with a PLD signature motif HxK-x_n-N-x_n-(E/Q/D) (Ponting and Kerr 1996). A single active site of PLD enzymes is formed by two such motifs either as homodimers or monomers with a pseudodimeric structure. The first structures of PLD enzymes solved by X-ray crystallography were that of a nonspecific dimeric nuclease, Nuc from *S. typhimurium*, and of the monomeric phospholipase D from *Streptomyces* sp. (Stuckey and Dixon 1999; Leiros et al. 2000). More recently, the structure of the EDTA-resistant BfiI restriction endonuclease (R.Bfi) was solved. This REase represents a novel subtype of Type IIS restriction endonucleases with a DNA-recognition domain fused to a nonspecific nuclease domain from the PLD superfamily (Sapranauskas et al. 2000; Zaremba et al. 2004; Grazulis et al. 2005).

Our experimental validation of a previously proposed structural model of DNase IIα supports the hypothesis that human DNase IIα is monomer with a pseudodimeric fold. Whereas Nuc and R.Bfi are dimeric nucleases, human DNase IIα represents the first example of a monomeric nuclease of the PLD superfamily investigated so far. It should be noted here that the C-terminal tags fused to recombinant DNase IIα and the relatively low enzyme concentration obtained from preparations of overexpressing mammalian cell lines in principle could interfere with detection of protein dimers. However, our localization and activity studies suggest: (1) proper processing of the C-terminally tagged enzyme, as it is localized to the lysosomal compartment, and (2) formation of the enzymatically active molecular species, as the C-terminally tagged highly dilute DNase is catalytically active. The latter is even true for the enzyme immobilized via its C terminus to either Flag-, HA-, or Ni^{2+}-NTA-magnetic beads, suggesting that a freely accessible C-terminal end is no prerequisite for activity of this enzyme.

In our previous work, we were unable to identify all amino acid residues comprising the active site of the pseudodimeric structure and the common motif of the PLD superfamily, HxK-x_n-N-x_n-(E/Q/D). Using site-directed mutagenesis, gel filtration, and coimmunoprecipitation, we have filled this gap. Interestingly, according to our data, the N-terminal PLD motif of human DNase IIα contains another conserved histidine residue (His130) that in principle can functionally replace the asparagine residue found in the consensus PLD motif, HxK-x_n-N-x_n-(E/Q/D). This residue could be involved in binding the scissile phosphodiester bond of a DNA substrate. Homologous histidine residues are found in human DNase IIβ (DLAD) and in *C. elegans* Nuc1. Thus, His130 appears to play a similar role as the asparagine residue from the active site motif in the N-terminal domain of DNase IIα. In a recent study, the homologous residue of the porcine enzyme (His132) has been claimed to be important for the proper folding of the enzyme (Cheng et al. 2006).

As a consequence of its pseudodimeric structure and the distant relationship of the N-terminal domain to the PLD family of enzymes, the active site structure of DNase IIα is hypothesized to be slightly asymmetric. This differs from phospholipase D or dimeric nucleases, which have symmetric active sites made up of symmetry-related residues from two identical or highly conserved PLD-signature motifs. In addition, in the more conserved C-terminal PLD motif of human DNase IIα, a characteristic negatively charged residue found in other PLD family members (Glu122 in Nuc, Glu136 in R.Bfi, and Asp202 and Asp473 in the two subdomains of phospholipase D, respectively) is not conserved. A CTD–CTD dimer of human DNase IIα would thus be symmetric but lack an active-site residue characteristic of the PLD-signature motif found in other PLD enzymes. Despite its presence in the PLD motifs of the above mentioned PLD enzymes, the role of this acidic residue is not clear. It has been speculated that in Nuc, Glu122 lowers the effective pK_a of the active site histidine residue and helps in stabilizing different protonation states (Stuckey and Dixon 1999). In human DNase IIα, this role could be filled by other residues in spatial proximity to the active site histidine residues. To the best of our knowledge, no mutagenesis data about this residue are available in the literature.

More critical for the catalytic mechanism than the glutamic or aspartic acid residues found in some PLD family members are the histidine and the lysine residues that form the core of all PLD motifs and are fully conserved in human DNase IIα (His113 and Lys115, and His295 and Lys297, respectively). Whereas the lysine residues are thought to bind the scissile phosphate, the histidine residues are very likely required for the formation of the phosphoenzyme intermediate and subsequent activation of a water molecule that attacks the covalently bound phosphoryl moiety (Gottlin et al. 1998; Stuckey and Dixon 1999; Waite 1999). In principle, each of the histidine residues could serve as the primary nucleophile in attacking the scissile phosphate forming the phosphohistidine intermediate or serve as a general base by activating a water molecule generating a hydroxyl ion for the nucleophilic attack on the phosphoenzyme intermediate, respectively. From our present mutagenesis data we cannot yet assign a specific role for the histidine residues in human DNase IIα with respect to their particular role in the catalytic mechanism, and it cannot be excluded that each of the histidine residues has a dual

role and functions in both ways. It should be mentioned here that upon a scanning mutagenesis study, Cheng et al. (2006) recently identified three out of nine histidine residues from porcine DNase IIα (His115, His132, and His297) that turned out to be catalytically and/or structurally important. Based on biochemical analyses, a catalytic mechanism was proposed that involves histidine residues His115 and H297 (corresponding to His113 and His295 in the human enzyme) as a general acid/general base pair during catalysis. Intriguingly, it seems that porcine and human DNase IIα differ in that the porcine enzyme is processed into two noncovalently linked peptides of 35 and 10 kDa, respectively, whereas the human enzyme is active as a contiguous polypeptide chain (Liao 1985; Wang et al. 1998; MacLea et al. 2002; Cheng et al. 2006). This would in the case of the porcine enzyme lead to a different number of polypeptide chains in the active enzyme species; however, the catalytic center of the porcine DNase IIα would still be composed of the two requisite N- and a C-terminal HxK-motifs arranged in a pseudodimeric way, since the proteolytic processing of this enzyme leaves a C-terminal fragment comprising amino acid residues 110–364, including the two PLD motifs.

Summarizing, our structural model of DNase IIα in combination with an alignment- and structure-based mutational analysis identifies two active site motifs in the N- and C-terminal domains of DNase IIα, arguing for a monomer with a pseudodimeric structure being the active enzyme species. A putative catalytic mechanism could involve two histidine residues, His113 and His295, as well as two lysine residues, Lys115 and Lys295, that form the core of the catalytic center. In addition, His130 from the N-terminal domain and its counterpart Asn313 from the C-terminal domain are likely to be involved in the catalytic mechanism by binding to the scissile phosphate of the DNA substrate. Intriguingly, the structure-based results regarding the important catalytic role of conserved histidine residues in human DNase IIα presented herein are in perfect agreement with results of a recent mutational analysis of the porcine homolog (Cheng et al. 2006). It should be stressed that our structural analysis was only possible due to the integration of bioinformatics and experimental validation of the predictions. On the one hand, for 50 years the DNase II 3D structure and active center could not be defined, and because of the resistance of this enzyme to crystallization, the identification of catalytic residues required bioinformatics input. On the other hand, it would not be possible to discriminate between the two proposed structural modes of DNase IIα organization (dimer vs. monomer) without experimental validation. Thus, our data provide the first experimental evidence for human DNase IIα being a monomeric PLD family member with a pseudodimeric fold.

Materials and methods

Plasmid construction

To allow for expression of DNase IIα fusion proteins with different C-terminal affinity tags in transfected mammalian cells, a cDNA coding for a C-terminal Flag-His_6-tandem-tagged version of human DNase IIα was inserted into the NheI- and XhoI-digested vector pCI (Promega). The plasmid was named pCI-DII-FH. Similarly, a cDNA coding for a C-terminal HA-tagged version of human DNase IIα was inserted into the NheI- and NotI-digested vector pCI, resulting in the plasmid pCI-DII-HA. Constructs coding for a DNase IIα–EGFP fusion protein were generated by insertion of the cDNA of human DNase IIα in frame into the NheI- and XhoI-digested vector pEGFP-N1 (Clontech).

Cell culture and transfection

For the production of DNase IIα in mammalian cells, HEK-293T cells (1 maxi dish/transfection) cultured at 37°C in a humidified atmosphere of 5% CO_2 in DMEM with 10% fetal calf serum, 100 units of penicillin, and 100 μg/mL streptomycin were transfected with 15 μg (7.5 μg + 7.5 μg when cotransfecting cells with two plasmids) of an appropriate expression construct using Transfast transfection reagent (Promega) according to the supplier's recommendations.

To study the subcellular distribution of EGFP-tagged DNase IIα, cells cultured in six-well dishes, under the same conditions as described above for HEK-293T cells, were transfected with 1.5 μg of each expression construct (750 ng + 750 ng when cotransfecting cells with two plasmids) using Polyfect transfection reagent (QIAGEN) as described by the supplier's recommendations.

Microscopy

Transfected cells were examined with a Leica TCS4D confocal laser scanning microscope using a Leica HCX APO L40X/0.80 W U-V-1 objective. Excitation of EGFP was achieved at 488 nm by a 75-mW Omnichrome argon/krypton laser. Emission was observed using a 580-nm beam splitter and a 510-nm-long pass filter. Individual images were pseudocolored.

Protein purification

Twelve to 20 h after transfection of HEK-293T cells with pCI-DII-FH or pCI-DII-HA, the medium was removed and the cells were harvested in 10 mL of phosphate-buffered saline (PBS) with a rubber-policeman. Subsequently, the cell suspension was centrifuged at 800 rpm for 2 min, and the supernatant was disposed of. Next, the cells were washed again with 1 mL of PBS. The pelleted cells were then lysed in 1 mL of lysis buffer A [10 mM Tris-HCl at pH 7.5, 200 mM NaCl, 1 mM EDTA, 2% (v/v) Triton X-100, and protease inhibitor cocktail (Roche)] for 1 h at 4°C, after which the samples were centrifuged at 14,000 rpm for 40 min at 4°C. From the lysate supernatant, the protein of interest was purified according to the affinity matrix used: DNase IIα with a Flag-His_6 tandem tag was purified by either Flag-affinity chromatography using EZview Red ANTI-FLAG M2 Affinity Gel (Sigma) or IMAC using magnetic Ni^{2+}-NTA particles (see below); whereas for DNase IIα fused to the

hemagglutinin-epitope tag, HA-affinity chromatography using EZview Red ANTI-HA Affinity Gel (Sigma) was performed. The beads (40 μL/construct) were first pretreated as recommended by the supplier, and subsequently the supernatants of the abovementioned cell lysates were added and left to incubate for 1 h at 4°C. The samples were then centrifuged at 9000 rpm for 30 sec, the supernatants containing unbound components were discarded, and the affinity resins were washed twice with lysis buffer and once with Tris-buffered saline (TBS). After the final washing step, a reaction buffer consisting of 200 mM NaAc (pH 4.5) and 5 mM EDTA was added to the beads.

DNase IIα with a Flag-His_6 tandem tag was also purified via Ni^{2+}-NTA-affinity chromatography using MagneHis magnetic Ni^{2+}-Particles (Promega). Transfected HEK 293T-cells were collected and lysed as described above, but using lysis buffer B [10 mM HEPES at pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 20 mM imidazole, 1% (v/v) Triton X-100, and protease inhibitor cocktail (Roche)]. The lysate supernatant was then added to Ni^{2+} beads (20 μL/construct) and equilibrated with lysis buffer, and the protein was left to bind for 0.5 h at 4°C, after which the bound protein was pulled down using magnetic force, and the supernatant including unbound components was discarded. Subsequently the beads were washed three times with washing buffer (10 mM HEPES at pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 20 mM imidazol, 1 mM PMSF), and, finally, reaction buffer was added to the beads.

Immunoblotting

Flag-His_6-tandem-tagged DNase IIα and HA-tagged DNase IIα were detected after SDS-PAGE and electroblotting onto Hybond-ECL nitrocellulose membranes or Hybond-PVDF membranes (Amersham Biosciences) using ANTI-FLAG M2 Monoclonal Antibody-Peroxidase Conjugate (Sigma) and ANTI-HA Monoclonal Antibody-Peroxidase (Roche), respectively, in combination with enhanced chemiluminescence detection reagents.

Site-directed mutagenesis

Site-directed mutagenesis of DNase IIα was performed as described by Kirsch and Joly (1998). Briefly, a first PCR was performed using a mutagenic primer and an appropriate reverse primer with pCI-DII-FH as template, respectively, 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 QuickChange Protocol (Stratagene).

Nuclease activity assay using plasmid

DNA aliquots of the suspensions of DNase IIα-Flag-His_6 bound to Flag beads were incubated for defined time intervals at 37°C in the presence of 20 ng/μL assay solution (10 nM final concentration) of plasmid DNA (pBSK-VDEX or pCI) in reaction buffer (150 mM NaAc-HCl at pH 4.5 and 5 mM EDTA). Cleavage products were analyzed by agarose gel electrophoresis.

Gel filtration

For an analysis of the quaternary structure of the active DNase IIα species, cell lysates (1 maxi dish per construct) of 293-T cells transiently transfected to express either wild-type DNase IIα or the H295A variant were loaded onto a Superdex-75 HR 10/30 gel filtration column (bed dimensions: 10 mm × 30 cm) equilibrated with 10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, 0.01% (v/v) Triton X-100, and 0.5 mM PMSF using a Beckman BioSys 2000 HPLC-System. Fractions of 0.6 mL were collected at a flow rate of 1 mL/min. In order to pull down DNase IIα-Flag-His_6, 10 μL of a suspension of ANTI-FLAG M2 Affinity Gel (Sigma) was added to each fraction, and the protein was purified as described above. For analysis of the fractions, aliquots of the beads were resuspended in SDS-gel loading buffer, and the eluted protein was subjected to SDS-PAGE and blotted. In addition, aliquots of the bead suspension were washed once with TBS, resuspended in 40 μL of reaction buffer (150 mM NaAc-HCl at pH 4.5 and 5 mM EDTA), and tested for activity using the plasmid cleavage assays.

Molecular modeling

The construction of two preliminary, alternative models of DNase IIα were described earlier (Cymerman et al. 2005). The refinement of the pseudodimeric variant, based on experimental data, was carried out by introducing local shifts into the sequence alignment between the N-terminal domain of DNase IIα and the structural templates used for modeling: nuclease Nuc (1byr), phospholipase D from *Streptomyces* sp. (1v0w), and the recently solved R.BfiI (2c1l), as to superimpose the residues demonstrated to be essential for the nuclease activity. The final model was built using the FRankenstein's monster approach (Kosinski et al. 2003) by iterating model generation using MODELLER (Fiser and Sali 2003), evaluation of sequence-structure fit by VERIFY3D (Luthy et al. 1992) via the COLORADO3D gateway (Sasin and Bujnicki 2004), and re-alignment of the poorly scoring regions.

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