Structural requirements of human DNase IIα for formation of the active enzyme: the role of the signal peptide, N-glycosylation, and disulphide bridging

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DNase IIα (EC 3.1.22.1) is an endonuclease, which is active at low pH, that cleaves double-stranded DNA to short 3′-phosphoryl oligonucleotides. Although its biochemistry is well understood, its structure–activity relationship has been largely unexamined. Recently, we demonstrated that active DNase IIα consists of one contiguous polypeptide, heavily glycosylated, and containing at least one intrachain disulphide linkage [MacLea, Krieser and Eastman (2002) Biochem. Biophys. Res. Commun. **292**, 415–421]. The present paper describes further work to examine the elements of DNase IIα protein required for activity. Truncated forms and site-specific mutants were expressed in DNase IIα-null mouse cells. Results indicate that the signal-peptide leader sequence is required for correct glycosylation and that N-glycosylation is important for formation of the active enzyme. Despite this, enzymic deglycosylation of wild-type protein with peptide N-glycosidase F reveals that glycosylation is not intrinsically required for DNase activity. DNase IIα contains six evolutionarily conserved cysteine residues, and mutations in any one of these cysteines completely ablated enzymic activity, consistent with the importance of disulphide bridging in maintaining correct protein structure. We also demonstrate that a mutant form of DNase IIα that lacks the purported active-site His^{295} can still bind DNA, indicating that this histidine residue is not simply involved in DNA binding, but may have a direct role in catalysis. These results provide a more complete model of the DNase IIα protein structure, which is important for three-dimensional structural analysis and for production of DNase IIα as a potential protein therapeutic for cystic fibrosis or other disorders.

Key words: deoxyribonuclease II, DNase IIβ, *DNASE2* gene, DNase II-like acid DNase (DLAD), endonuclease, NUC-1, peptide N-glycosidase (PNGase F).

INTRODUCTION

DNase II (EC 3.1.22.1), first identified in 1947, is the best characterized acid endonuclease identified in human tissues [1–3]. DNase II has been reported in lysosomes, nuclei and various secretions [4–8], and is critical to the process of mammalian development [9,10]. The activity of DNase II is distinguished from other endonucleases by its acidic pH optimum, the absence of a requirement for bivalent cations and its ability to hydrolyse double-stranded DNA to yield short oligonucleotides bearing 3′-phosphate groups, rather than 3′-hydroxyl groups. Acid endonucleases were originally reported at a variety of sizes [2,11–14], and subsequent identification of distinct enzymes has suggested that several unrelated enzymes appear to contribute to the acid-endonuclease activity found in mammalian tissues. These include DNase II, L-DNase II, Xib, DNase α and DNase β [1,15–19]. In addition to these unrelated enzymes, a new homologue of human DNase II has been recently cloned, named DNase IIβ (hence the original enzyme is now termed DNase IIα) [20].

We originally cloned both of the human DNase II family members, DNase IIα [21] and DNase IIβ [20]. These enzymes share extensive identity and similarity. Indeed, the human enzymes and those from other species share considerable homology at the amino acid level as well as at the genetic level. The signal peptide, conserved cysteine residues, purported active-site histidine residue and intron–exon structure all appear to be highly similar or identical and important in a variety of organisms [20,22]. DNase II homologues have been reported in human, mouse, rat, cow, pig, *Drosophila melanogaster* and *Caenorhabditis elegans* [20]. Recent database searches have yielded 29 additional homologues in 21 species, allowing clearer demarcation of the DNase IIα and DNase IIβ subfamilies as well as analysis of regions of shared similarity likely to be very important in enzyme activity [23].

Despite the understanding of its biochemical properties and role in development, only recently has any effort been undertaken to characterize the protein structure of DNase IIα. Because no member of the DNase II protein family has yet had its three-dimensional structure solved and no homology has been detected between any family members with other proteins for which the structure is known, there is currently no known tertiary structure data for DNase IIα. Indeed, the only work undertaken until recently had examined the domain structure of protein purified from whole organs [11,24,25]. Full-length porcine DNase IIα

Abbreviations used: CF, cystic fibrosis; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; MEF, mouse embryonic fibroblast; NEB, New England Biolabs; PNGase F, peptide N-glycosidase F; SRED, single radial enzyme diffusion; TM, tunicamycin.

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The nucleotide sequence data for human DNase IIα and human DNase IIβ have been submitted to DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AF047016 and AF274571 respectively.

was reported to undergo a series of proteolytic cleavage events: the removal of a hydrophobic N-terminal signal peptide and further cleavage to produce a three-chain ($\alpha1/\beta/\alpha2$) structure [11,24]. However, recent work in this laboratory showed that active protein consists of one contiguous polypeptide, heavily glycosylated and containing intrachain disulphide cross-links [26]. Our results suggest that the previously proposed structure for the porcine protein is the result of degradation during protein purification from whole organs.

One of our goals in studying DNase IIα has been to assess its potential as an improved mucolytic agent for improving pulmonary clearance in the genetic disorder cystic fibrosis (CF) [20,27]. Analysis of the elements of DNase IIα protein that are necessary for generating an active enzyme has great relevance to the choice of an expression system for the production of a testable therapeutic agent for CF. Our original attempts to produce the protein in bacteria were never successful at generating active protein (results not shown). In light of subsequent results suggesting the importance of glycosylation and disulphide bridging in activity [26], we investigated the use of a mammalian expression system for production, like that used in the production of recombinant human DNase I protein [28]. However, our previous results from transfection experiments in Chinese-hamster ovary cells found that DNase IIα overexpression resulted in apoptosis over short time courses [21]. Likewise, we were unable to produce stable transfectants, despite several attempts (results not shown).

To determine best the choice of production and purification systems for DNase IIα requires knowledge of its structure–activity relationship, in particular which elements of the protein are necessary for the generation of an active protein. In the present study, we report a thorough analysis of elements required for the activity of human DNase IIα protein. In particular, we report the requirement of a signal peptide for both N-glycosylation and subsequent activity. In addition, the role of the individual N-glycosylation sites and the effect of enzymic deglycosylation are described. We report the effect of ablating each of the conserved cysteine residues, the role of the purported active site residue His^{295} in DNA binding and catalysis, and develop further a model of DNase IIα protein structure. These results are critical for understanding the DNase IIα protein structure–activity relationship and for producing the protein in large quantities for therapeutic testing and three-dimensional structural analysis.

EXPERIMENTAL

Vector construction of DNase IIα site mutants

A mammalian expression vector generated in this laboratory containing the cloned cDNA of DNase IIα in the pcDNA 3.1(−) vector (Clontech, Palo Alto, CA, U.S.A.), designated pD2 [26], was used as the template for mutagenesis. Primers identical with the region surrounding each mutation site, incorporating the desired mutation, were designed and synthesized (Dartmouth Molecular Biology Core Facility). For each mutant created, a forward and reverse primer were employed for use with the QuikChange® Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The primers are listed in Table 1. Transformants were screened by DNA sequencing using the ABI Prism® Big-Dye™ Terminator Cycle Sequencing Ready Reaction kit and analysed on an Applied Biosystems 370 automated sequencer. Each mutant construct was designated according to its wild-type and mutant residue and its appropriate residue number.

Table 1 Primer sequences used in the construction of DNase IIα site-specific mutants

One of the two complementary primers for each mutant is listed. Primers are named according to the mutation they introduce, e.g. N69Q is an $Asn^{69} \rightarrow Gln$ mutation. Underlined residues represent the introduced mutations.

Primer	Sequence
N69Q1	5′-GGCAGGGCACTCATCCAAAGCCCGGAGGGGGCC-3′
N86Q1	5′-CGCTGTACCGGAGCCAAACCAGCCAGCTCGC-3′
N212Q1	5′-GCCAAGAACCCTGGCAAAGCAGCATCACAC-3′
N266Q1	5′-GCATCCTGCCCTCTCAATGCTCGGATATCTG-3′
N290Q1	5′-GCCGGCCCAAGCTTCCAAAGCACAGAGGACCAC-3′
C11A1	5′-CTGGCAGCGCTGCTGGCCGTCCCCGCCGGG-3′
C19A1	5′-GCCGGGGCCCTGACCGCCTACGGGGACTCC-3′
C151A1	5′-CCTCATAGCGCCGCTACCTACGGGCAGACC-3′
C159A1	5′-GGGCAGACCCTGCTCGCTGTGTCTTTTCCC-3′
C267A1	5′-CTGCCCTCTAACGCCTCGGATATCTGGCAG-3′
C299AF	5′-GAGGACCACTCCAAATGGGCCGTGTCCCCAAAAGGGC-3′
C308A1	5′-GGGCCCTGGACCGCCGTGGGTGACATGAATC-3′
C327A1	5′-GGGGGCACACTGGCTGCCCAGCTGCCAGCC-3′
C347A1	5′-GAAGAACTACCAGCCCGCTAATGGCATGGCC-3′
D107N1	5′-CACGCATGGAAGAGTTCTGAGCCTTGCTGGG-3′

Multiple N-glycosylation site or cysteine mutants were generated by sequential mutagenesis with different primers using the same method. The N × 2 mutant construct converted the asparagine residues at both positions 86 and 266 into glutamine residues. N × 3 has mutations at positions 86, 212 and 266, and N × 4 has mutations at all four positions 86, 212, 266 and 290. The double cysteine mutant C × 2 is mutated to alanine at both position 19 and position 159.

Vector construction of truncated DNase IIα mutants

The pD2 vector containing the cloned cDNA of DNase IIα [26] was again used as the template for PCR amplification. Two truncation mutants were created: (i) Δ15, which deleted the nucleotides coding for the first 15 amino acids (the signal-peptide leader sequence) and replaced them with a new starting methionine residue, and (ii) Δ107, which deleted the region corresponding to the first 107 amino acids and replaced it with a methionine start site. Both truncation mutants used the same reverse primer to introduce a *Bam*HI site 3′ of the TAA termination codon, 5′-TGCGGATCC*TTAGATCTTATAAGCTCTGCT*-3′. Forward primers homologous with the appropriate 5′ coding region were used to introduce a *Xho*I site and ATG for the Δ15 mutant (5′-GACTCGAG*ATGGCCCTGACCTGCTAC*-3′) and for the Δ107 mutant (5′-GACTCGAG*ATGTCTTCCATGCGTGGG*-3′). Underlined regions correspond to the introduced mutations and italicized regions indicate the coding sequences of the genes following the restriction sites. The amplified gene products were purified using the Strataprep PCR Purification Kit (Stratagene), digested with *Xho*I and *Bam*HI restriction endonucleases (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.), electrophoresed on a 1% (w/v) agarose gel, and purified using Geneclean II (Bio 101, Inc., Vista, CA, U.S.A.). This fragment was ligated into the multiple cloning site of the pcDNA 3.1(−) vector (Clontech) that was first digested with the same enzymes. Transformants were screened by DNA sequencing and the resulting plasmids were designated pD2 Δ15 and pD2 Δ107.

Isolation of primary mouse embryonic fibroblasts (MEFs) deleted for DNase IIα

In our previous work, we described the development of a knockout mouse model for DNase IIα [9]. To produce primary MEFs, we harvested day-12.5 mouse embryos from a breeding of mice heterozygous for the null allele. Embryos had their head and liver removed prior to homogenization and were then plated in Dulbecco's modified Eagle's medium (DMEM; Cellgro, Herndon, VA, U.S.A.) supplemented with 10% (v/v) foetal bovine serum, antibiotics and L-glutamine. During this process, tissue from the starting embryo was genotyped as previously described in [9]. Cells from mice homozygous for the null allele were repeatedly passaged at a 1:4 dilution and replated over subsequent weeks. Spontaneously immortalized cells were derived, and the resulting cell line was denoted MEF6$^{-/-}$.

Cell culture and transient transfection

The DNase IIα-null MEF cell line (MEF6$^{-/-}$) was maintained in DMEM supplemented with 10% (v/v) foetal bovine serum, antibiotics and L-glutamine. MEF6$^{-/-}$ cells were co-transfected with 4 μg of pD2 plasmid, a mutant plasmid construct (see above) or no plasmid, together with 1 μg of pEGFP-C3 (Clontech) encoding the enhanced green fluorescent protein (EGFP) using DOSPER Liposomal Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.). After 36 h, cells were examined for transfection efficiency by fluorescence microscopy. For tunicamycin (TM) treatment, cells were treated 24 h before harvest with 2.5 μg/ml TM (Sigma, St. Louis, MO, U.S.A.). All cells were harvested using a cell scraper and pelleted. Pellets were washed once with cold PBS and resuspended in cold 0.1 M sodium acetate buffer, pH 5.0, containing 20 mM EDTA, 2 mM *N*-ethylmaleimide, 1 mM PMSF and 1 μg/ml pepstatin A (Sigma). Cells were lysed by snap-freezing in liquid nitrogen and were needle-sheared using ten passes through a 21-gauge needle. The protein content was determined using the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.) and was standardized by serial dilution of BSA.

Enzymic deglycosylation of endogenous DNase IIα

T47D human breast carcinoma cells (American Type Culture Collection, Manassas, VA, U.S.A.), grown in DMEM/F12 supplemented with 10% (v/v) foetal bovine serum, antibiotics and L-glutamine, were in the exponential phase of cell growth at the time of harvest (performed as above). Deglycosylation of both T47D lysates and MEF6$^{-/-}$ lysates was performed identically using peptide N-glycosidase F (PNGase F) enzyme [New England Biolabs (NEB), Beverly, MA, U.S.A.]. Briefly, cell lysates were incubated for 2 h or 6 h at 37 °C in 1 × G7 buffer (NEB) in the presence or absence of 500 or 1500 NEB units of PNGase F (where 1 NEB unit is defined as the amount of enzyme required to remove > 95% of the carbohydrate from 10 μg of denatured RNase B in 1 h at 37 °C in a total reaction volume of 10 μl). Treated cell lysates were examined by Western blot (see below) or single radial enzyme diffusion (SRED) activity assay (see below).

Western blot analysis

Cell lysates were boiled in Laemmli buffer in the presence of 2-mercaptoethanol and separated by SDS/PAGE (10% gels) using the Mini-PROTEAN II gel system (Bio-Rad Inc., Hercules, CA, U.S.A.). Proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.) and blocked in TBSTM [25 mM Tris, pH 8, 125 mM NaCl, 0.1% (v/v) Tween 20, 5% (w/v) non-fat dried milk] for 1 h at room temperature (22 °C). The membrane was then probed with the DNase IIα-specific rabbit polyclonal antibody (ProSci, Inc., Poway, CA, U.S.A.), at a 1:500 dilution, and/or the mouse anti-EGFP monoclonal antibody (A-6455; Molecular Probes, Eugene, OR, U.S.A.), at a 1:3000 dilution in TBSTM, for 1 h, or the new anti-DNase IIα polyclonal antibody, anti-hnD2 (a gift of Dr S.-C. Lu, National Taiwan University, Taipei, Taiwan) [29], diluted 1:55 in TBSTM for 2 h, followed by goat anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, U.S.A.), at a dilution of 1:3000, for 30 min. Chemiluminescent detection of DNase IIα or EGFP was performed with the ECL® (enhanced chemiluminescence) kit (Amersham Biosciences, Piscataway, NJ, U.S.A.). Protein molecular masses were estimated by comparison with a standard curve of sizes using the Precision Protein Standards (Bio-Rad).

Analysis of DNase II activity in cell lysates

Lysates in sodium acetate buffer were assayed for endonuclease activity using the SRED method [7,30]. In brief, digestion of ethidium bromide-stained salmon sperm DNA in the agarose gel assay plate was determined for each lysate sample by measurement of its diffusion diameter under UV illumination. Radial diffusion diameters were compared with serial dilution of bovine DNase II (Sigma). Lysate with equivalent total protein content was applied in 4 μl aliquots to each well of the SRED assay plate, with five replicate wells for each experiment.

DNA binding of wild-type and mutant DNase IIα in cell lysates

Lysates from wild-type or mutant plasmid transfection (see above) were assayed for the ability to bind DNA using a modification of the method in [31]. In brief, lysates were incubated with native DNA–cellulose beads (Amersham Biosciences) in Bind A buffer [0.1 M sodium acetate, pH 5.0, containing 10 mM dithiothreitol, 20% (v/v) glycerol, 0.02% (v/v) Tween 20, 1 mM EDTA, 1 mM PMSF and 1 mg/ml BSA] for 1 h at 4 °C with end-over-end continuous mixing. After incubation, the beads were pelleted for 5 min at 10000 ***g*** and the supernatant was separated from the bead fraction. The bead fraction was washed for 5 min at 4 °C, with mixing, in cold Bind A buffer, pelleted, and wash buffer was removed. Input lysates and bead fractions were boiled for 5 min at 95 °C in Laemmli buffer in the presence of 2-mercaptoethanol and separated by SDS/PAGE (10% gel). One half of each input lysate or bead fraction was analysed by Western blot analysis (as above) and the other half of each sample was stained for total protein using Coomassie Blue stain.

RESULTS

Requirement of the signal-peptide leader sequence for N-glycosylation and activity

Analyses of DNase II family members have noted the presence of a conserved hydrophobic N-terminal signal-peptide leader sequence [20]. The presence of a signal peptide is usually required for N-glycosylation as well as for targeting of a protein to lysosomes or to the constitutive secretion pathway. An expression construct was developed in which the signal peptide was removed (pD2 Δ15). In addition, because our original purification of the bovine DNase IIα protein showed that the N-terminus of the commercially available bovine protein began at residue 108 of the human sequence [21], we re-created this form by generating a

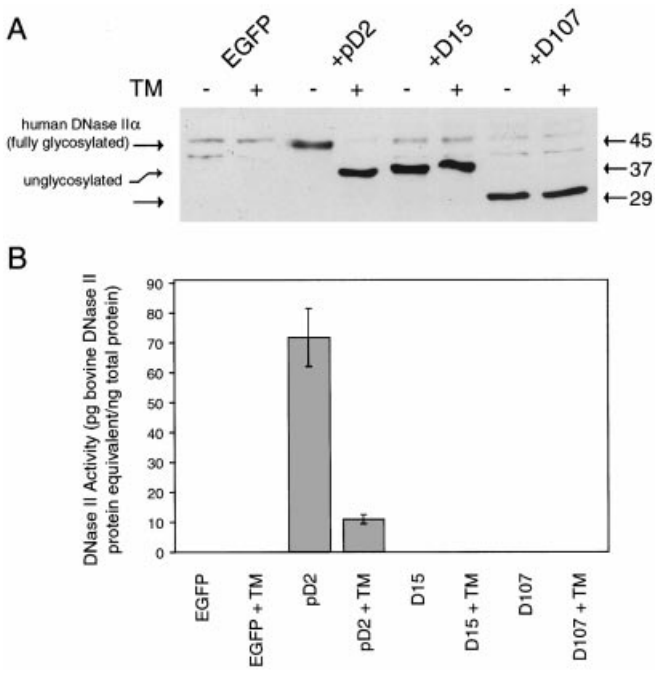

Figure 1 Analysis of MEF6$^{-/-}$ DNase IIα-null MEFs transiently transfected with truncated forms of DNase IIα

(**A**) Anti-DNase IIα immunoblot of mock-, pD2-, Δ15- and Δ107-transfected cell lysates. Cells were transfected with wild-type construct (pD2) or truncated mutant plasmid (all cells were co-transfected with EGFP and exhibited equal transfection efficiency) and grown for 24 h in the absence or presence of TM. Each lane represents identical total protein. (**B**) Acid endonuclease activity at pH 5 of the lysates from mock-, pD2-, Δ15- and Δ107-transfected cells. Results are means $\pm$ S.D.; $n = 5$.

construct in which the first 107 amino acids were removed (pD2 Δ107).

MEF6$^{-/-}$ cells null for DNase IIα expression and activity were transiently transfected with plasmid-expression constructs encoding wild-type human DNase IIα (pD2) or these mutant forms (Figure 1). We concurrently examined the effect of the N-glycosylation inhibitor TM on both the size and activity of expressed DNase IIα. Cells that were transfected with pD2 exhibited a predominant band at 45 kDa, which was reduced in size to 37 kDa after treatment with TM (Figure 1A), consistent with previous observations of DNase IIα as a glycoprotein [11,26]. As in our previous work [26], TM treatment greatly diminished, but did not completely ablate, acid endonuclease activity of this protein (Figure 1B). The slight residual activity observed may be due to an undetectable level of glycosylated protein in these samples, although it is possible that completely unglycosylated protein may retain a slight intrinsic activity. The truncated DNase IIα constructs were also expressed at similar levels (Figure 1A), but appeared to lack any activity above the mock-transfected baseline (Figure 1B). The Δ15 mutant protein exhibited an apparent molecular mass of 37 kDa, consistent with the peptide length of this protein fragment, and this was not altered by TM treatment. The Δ107 mutant showed an apparent mass of 29 kDa, and this also remained unchanged in the presence of TM. Hence the signal-peptide leader sequence is required for N-glycosylation and the formation of active enzyme.

Effect of site-specific mutations on N-glycosylation and activity of DNase IIα

DNase II proteins across species contain consensus sequences for N-linked glycosylation, Asn-Xaa-(Ser/Thr)-Xaa, where the sugar moiety is attached at the asparagine residue (and where a cysteine residue is also found, but rarely, in the third position and a proline residue is disfavoured in the second or fourth position) [32–34]. Indeed, both human DNase IIα and DNase IIβ contain four predicted N-glycosylation sites, including one site that is found in both proteins and one site found in DNase IIβ for which the corresponding site in DNase IIα does not fit the consensus (Table 2).

To examine the role of N-glycosylation in formation of the active endonuclease, the wild-type pD2 expression construct was altered by site-directed mutagenesis. Expression constructs were generated with mutations at each of the four glycosylation sites in DNase IIα as well as constructs with two (N $\times$ 2), three (N $\times$ 3) or four (N $\times$ 4) sites ablated. Constructs were also created in which the non-consensus Asn^{69} was mutated to glutamine or an additional site was created by mutation of Asp^{107} to asparagine. The original context for the latter mutant was to inhibit proteolytic cleavage of the predicted Asp–Ser scissile bond [21], before it was realized that the protein existed as a single, uncleaved polypeptide [26].

DNase IIα-null MEFs were transiently transfected with these constructs in the presence or absence of TM, and the corresponding immunoblot, loaded with equal total protein, is shown in Figure 2(A). It is worth noting that these proteins have different sizes when glycosylated, but each of the proteins was decreased to the approx. 37 kDa unglycosylated form upon TM treatment. In the glycosylated form, both the wild-type and N69Q mutants exhibit the normal full-length size of 45 kDa (labelled on the right as +4 glycosylation sites). Each of the single mutants, N86Q, N212Q, N266Q and N290Q, display a

Table 2 Consensus N-glycosylation sites among DNase II family members

Human DNase IIα and DNase IIβ are compared, and conservation is noted with ten homologues from other mammalian species, as well as *D. melanogaster* and *C. elegans* [20].

Human protein	Potential consensus N-glycosylation site						
DNase IIα	Asn^{69*}	Asn^{86}	–	–	Asn^{212}	Asn^{266}	Asn^{290}
DNase IIβ	Asn^{77}	–	Asn^{99}	Asn^{115}	–	Asn^{274}	–
Property							
Conservation in species	Mammal, except human and rat α	All mammal α	All known β	All known β	All mammal α	All mammal	Mammal α, except mouse
Glycosylation in DNase IIα	No	Yes	–	–	Yes	Yes	Yes
Importance for activity in DNase IIα	Yes	Yes	–	–	Yes	Yes	Yes

* Asn^{69} is not a consensus N-glycosylation site in human DNase IIα, though it is in all other mammalian species except rat.

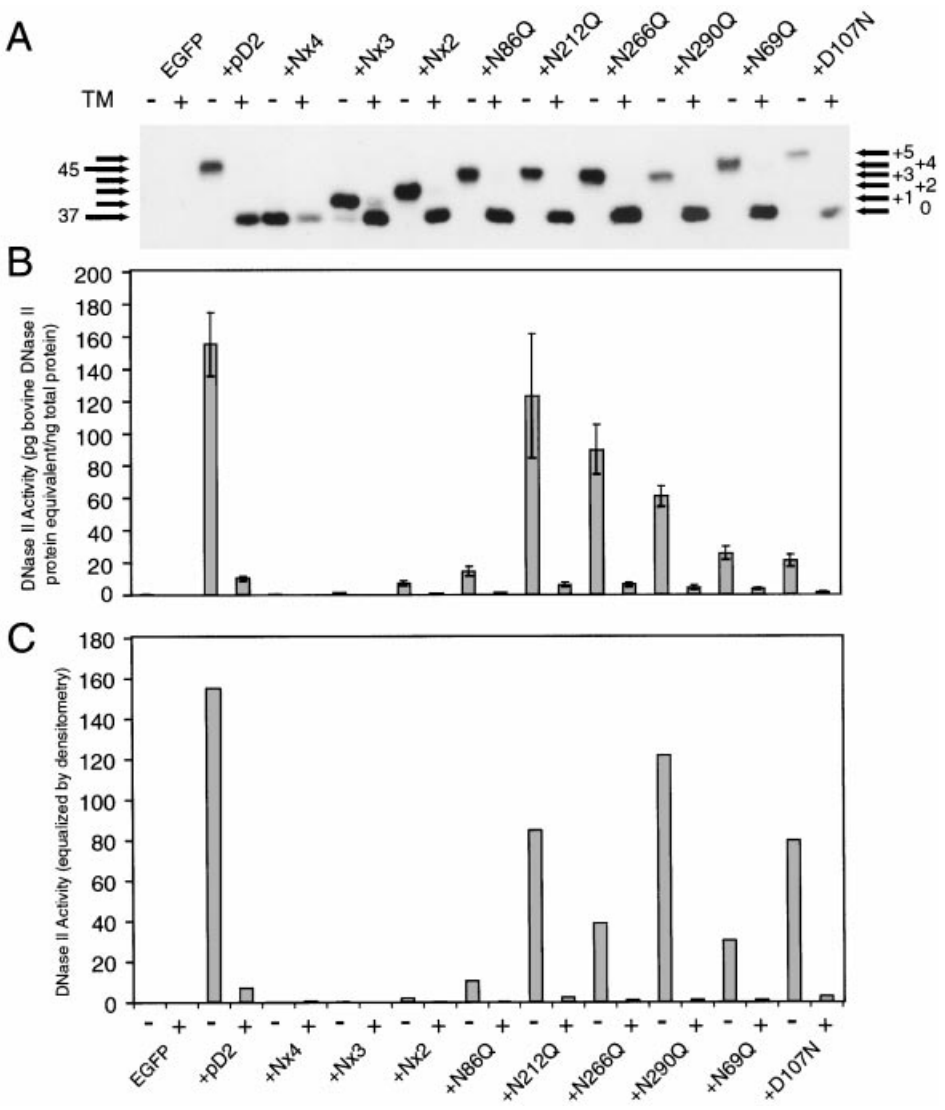

Figure 2 Analysis of cells transfected with mutant forms of DNase IIα which alter the observed N-glycosylation pattern

(**A**) Anti-DNase IIα immunoblot of mock-, pD2-, or N-glycosylation mutant-transfected cell lysates. MEF6$^{-/-}$ cells were co-transfected with EGFP and wild-type (pD2) or mutant plasmid and grown for 24 h in the absence or presence of TM. For details of the mutations, see the Experimental section. Each lane represents identical total protein. (**B**) Acid endonuclease activity at pH 5 of the lysates from mock-, pD2- and mutant-plasmid-transfected cells. Results are means ± S.D.; $n = 5$. (**C**) Acid endonuclease activity of the lysates, equalized by densitometry. Results are expressed in pg of bovine protein equivalents/ng of total protein adjusted relative to the ratio of the density of the pD2-transfectant without TM treatment [third lane from the left in (**A**)] to the density of the corresponding sample.

+3 size of approx. 43 kDa. N × 2 exhibits a +2 size of approx. 41 kDa, N × 3 has a +1 size of 39 kDa and N × 4 is never glycosylated and remains at 37 kDa. The addition of another N-glycosylation site at position 107 via the D107N mutation results in an apparent +5 size of 47 kDa. Hence it appears that the four sites (86, 212, 266 and 290) each contribute to glycosylation and no additional glycosylation sites are responsible for the observed size.

When lysates were examined in the SRED acid endonuclease activity assay (Figure 2B), substantial effects on activity were observed. To correct for the differences in expression level observed in Figure 2(A), the results were replotted after correction by densitometry (Figure 2C). The results of the SRED assay suggest that each of the N-glycosylation sites is important for activity of the DNase IIα protein. Double, triple or quadruple mutants effectively destroy most or all of the residual activity that is detectable in any of the single mutants. Though the N69Q protein is not normally glycosylated, the Asn^{69} residue appears to have a functional role in activity, since conversion of this residue into glutamine results in decreased activity. Also of interest, although the D107N mutant is capable of even greater glycosylation, this mutant also results in some loss of activity.

Effect of enzymic deglycosylation of active DNase IIα on structure and activity

Although we have observed that N-glycosylation is important for the formation of the active enzyme, the question of whether

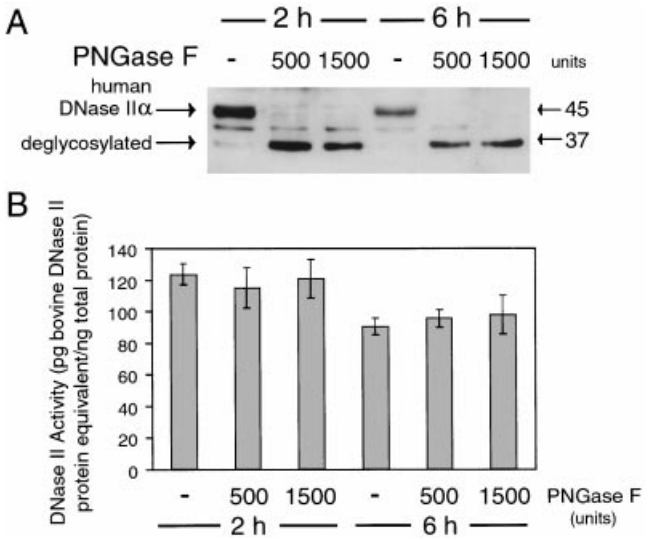

Figure 3 Analysis of enzymically deglycosylated DNase IIα from T47D cell lysates

(**A**) Anti-DNase IIα immunoblot of T47D human breast carcinoma cell lysates incubated in the presence or absence of 500 or 1500 units of PNGase F enzyme for 2 h or 6 h at 37 °C. (**B**) Acid endonuclease activity of the lysates in (**A**) at pH 5. Results are means ± S.D.; $n = 5$.

glycosylation is intrinsically required for activity has not been resolved. To address this question, DNase IIα protein produced under normal glycosylating conditions (in a high-activity cell line) was subjected to enzymic deglycosylation with PNGase F. After 2 h or 6 h of 37 °C incubation with or without PNGase F, the size of wild-type protein from T47D human breast carcinoma cells matched that seen with TM treatment (Figure 3A). However, there was no loss of activity (Figure 3B). This suggests that glycosylation is not intrinsically required for DNase IIα activity.

Analysis of site-specific mutations of conserved cysteines on DNase IIα activity

Human DNase IIα possesses six cysteine residues conserved in all of the DNase II family members examined at the present time, an additional site found in all mammalian homologues, and two non-conserved sites [20]. DNase IIβ contains the seven conserved sites from DNase IIα and two more that are found in all reported DNase IIβ species (Table 3), as well as one non-conserved site (Cys^{185}, unlisted). The presence of such highly conserved cysteine residues presumably reflects the importance of these residues in forming disulphide bridges that are critical for maintaining correct protein structure. Indeed, our previous work has confirmed that at least one intrachain disulphide linkage is present in DNase IIα [26]. To examine the role of disulphide bridge formation on DNase IIα activity, each of the nine cysteine residues was mutated to an alanine in an expression construct.

When these constructs were transiently transfected into $MEF6^{-/-}$ cells, similar expression levels were detected in each lysate (Figure 4A), except for the C151A mutant, which remained undetectable in repeat transfection experiments. Only proteins with mutations at the less conserved cysteine residues retained any activity (Cys^{11}, which is present in the signal peptide and presumably cleaved off; and Cys^{299}, which is only conserved in the reported mammalian species; Figure 4B). There was complete loss of activity in the other cysteine-to-alanine mutants. Because the C151A mutant remained unexpressed, it was not possible to determine its activity. A protein with both Cys^{19} and Cys^{159} mutated to alanine also exhibited no activity. The finding that this latter mutant, which clearly cannot form an 'interchain' bridge as originally proposed [11,24], is expressed and is of identical size to the wild-type protein, confirms that DNase IIα is a single-chain polypeptide.

One unexpected result of this experiment was the observed larger size of the C267A mutant protein. In Figure 4(C), PNGase F treatment demonstrates that this observed size difference is due to N-glycosylation. Because Asn^{266}, immediately adjacent to Cys^{267}, is the sugar attachment point of a consensus N-glycosylation site, this may indicate an interference with normal N-glycosylation at this site when disulphide linkages at Cys^{267} are allowed to form. In the absence of this linkage, the protein can then be N-glycosylated at this site. However, this suggests, in opposition to our glycosylation data (Figure 2A), that normal glycosylation at Asn^{266} may not occur. One possible reason for the observed reduction in protein size in the N266Q mutation could be that the configuration of the protein had been altered in a manner that resulted in inhibition of glycosylation at another site. Then, mutation of Cys^{267} could have the opposite effect, now allowing Asn^{266} to be available for glycosylation.

Examination of DNA binding of wild-type and mutant DNase IIα protein

Work with the porcine DNase IIα protein had suggested that the histidine residue at position 295 of the human sequence was required for activity [11]. In our previous work, site-directed mutagenesis was used to mutate this histidine residue to an alanine in our pD2 H295A expression construct [26]. Protein with this ablated His^{295} residue was expressed and showed identical sizing to the wild-type, but lacked activity. However, the question remained whether this protein was unable to cleave DNA because it was unable to bind it or because it was itself inactive. To test this question, we subjected lysates from wild-

Table 3 Distribution of cysteines among DNase II family members

Human DNase IIα and DNase IIβ are compared, and conservation is noted with ten homologues from other mammalian species, as well as *D. melanogaster* and *C. elegans* [20]. The activity of each cysteine-to-alanine mutant construct (Figure 4) is also noted. Note that, for the C151A mutant, no protein was expressed, hence no activity was detected.

Protein	Cysteine residue										
DNase IIα	Cys^{11}	Cys^{19}	Cys^{151}	Cys^{159}	–	–	Cys^{267}	Cys^{299}	Cys^{308}	Cys^{327}	Cys^{347}
DNase IIβ	–	Cys^{28}	–	Cys^{167}	Cys^{192}	Cys^{209}	Cys^{275}	Cys^{305}	Cys^{317}	Cys^{336}	Cys^{356}
Property											
Conservation in species	–	All	–	All	All β	All β	All	All mammal	All	All	All
DNase IIα Cys → Ala mutant activity	Yes	No	? (No)	No	–	–	No	Yes	No	No	No

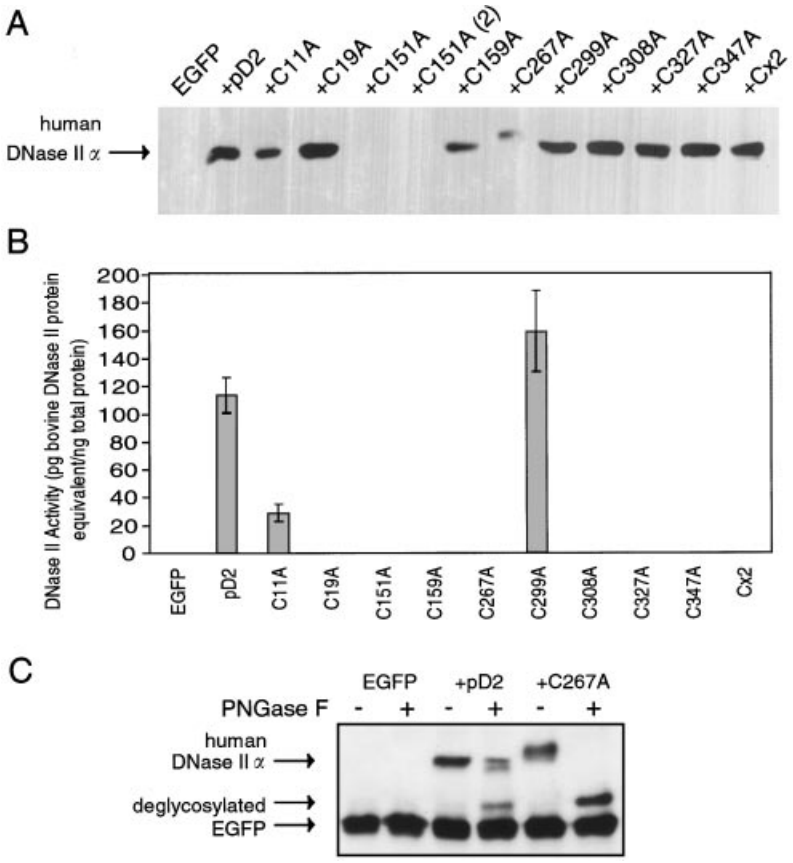

Figure 4 Analysis of cells transfected with mutant forms of DNase IIα which alter disulphide bridging

(**A**) Anti-DNase IIα immunoblot of mock-, pD2- or cysteine-mutant-transfected cell lysates. MEF6$^{-/-}$ cells were co-transfected with EGFP and wild-type (pD2) or mutant plasmid. For details of the mutations, see the Experimental section. Each lane represents identical total protein. (**B**) Endonuclease activity at pH 5 of the lysates from mock-, pD2- and mutant-plasmid-transfected cells. Because of the inter-experimental variability in transfection efficiency, results from different experiments could not be used to calculate a mean. Results are means ± S.D. calculated from five replicates within one representative experiment. (**C**) Anti-DNase IIα immunoblot of enzymically deglycosylated C267A-transfected cell lysate compared with mock- and pD2-transfected cell lysates. The same MEF6$^{-/-}$ lysate from (**A**) was subjected to deglycosylation with PNGase F enzyme for 2 h at 37 °C. Each lane represents identical total protein. The membrane was concurrently immunoblotted with antibody to detect transfected EGFP protein, which was co-transfected along with the DNase IIα-expression constructs.

type or H295A transfections to a native DNA pull-down assay using DNA–cellulose beads, as previously described for the caspase-activated DNase [31]. As observed in the Western blot of input compared with bead fractions in Figure 5(A), both wild-type and mutant DNase IIα are capable of binding DNA. As a control, the proteins binding to the DNA beads were stained with Coomassie Blue and compared with the starting protein (Figure 5B). It is clear that the majority of proteins failed to bind to the DNA–cellulose beads, demonstrating the selectivity of this purification procedure. The major band observed in the bead fractions is non-specific, as it is also found in beads in the absence of lysate (results not shown). These results suggest that His^{295} may indeed be involved in catalysis and not simply in DNA binding.

Comparison of protein processing using a new DNase IIα antibody

The antibody used throughout the present study recognizes an epitope at the extreme C-terminus of DNase IIα. It is therefore possible that other forms of DNase IIα exist that have cleaved off this non-conserved portion of the protein. During the course of the present study, a new DNase IIα antibody became available [29], which allowed us to verify our model of protein structure. Using this new antibody, protein bands were detected of identical size to DNase IIα in glycosylated (45 kDa) or unglycosylated (37 kDa) states (Figure 6). No specific smaller or larger forms were noted, though several non-specific bands were noted. Although the epitope that is recognized by this antibody is unknown, this provides preliminary evidence confirming the revised protein structure model.

DISCUSSION

Despite its extensive early biochemical characterization, the higher-order protein structure of DNase IIα protein has only recently begun to be examined. A common feature of all reported DNase II family members is the presence of an N-terminal signal-peptide leader sequence [20], which have been found to be important in other proteins for subcellular localization (e.g. to the lysosomes) and targeting of proteins to the secretion pathway. Signal-peptide sequences are highly degenerate [35] and eukaryotic signal peptides tend to feature hydrophobic amino acids,

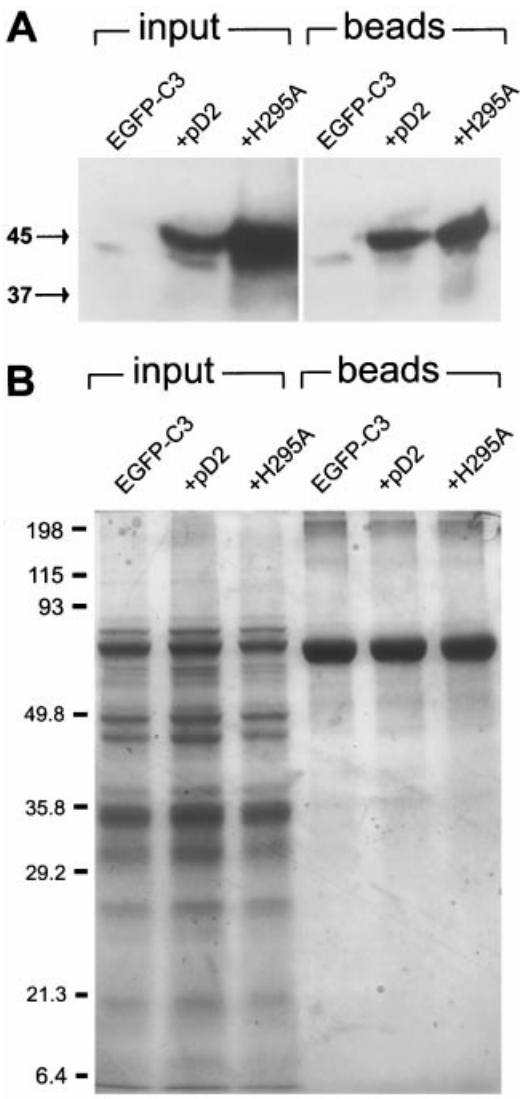

Figure 5 Analysis of the ability of wild-type and H295A mutant DNase IIα to bind DNA

(**A**) An anti-DNase IIα immunoblot of mock-, pD2- and H295A-transfected cell lysates purified on DNA–cellulose beads. Input (pre-binding) and bead fractions are compared. (**B**) An identical SDS/polyacrylamide gel stained with Coomassie Blue. Only a small subset of the proteins found in the input fractions are effectively bound by the DNA–cellulose beads. The predominant band in the Coomassie Blue-stained gel is a non-specific band found in the beads alone (results not shown).

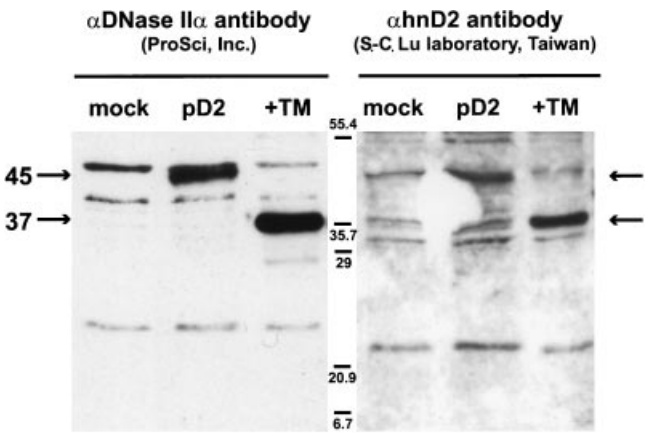

Figure 6 Immunoblot comparison of DNase IIα protein visualized using the commercial antibody and a new polyclonal antibody [29]

Left panel: anti-DNase IIα antibody immunoblot of lysates from mock- or pD2-transfected MEF6$^{-/-}$ cells grown in the absence or presence of TM to inhibit glycosylation. Each lane represents identical total protein. Right panel: anti-hnD2 antibody immunoblot of the same samples resolved on the same gel.

which are clearly observed in the human DNase IIα protein sequence. When this sequence was examined for predicted signal peptides using the SignalP v1.1 online server (http://www.cbs.dtu.dk/services/SignalP/), the protein was predicted to be cleaved between residues 18 and 19, Ala-Leu-Thr-cleavage-Cys-Tyr [36]. A plasmid encoding a truncated form of DNase IIα, Δ15, which lacks most of the signal-peptide sequence, remained both unglycosylated and inactive (Figure 1), consistent with the importance of this region of the protein in targeting the protein for correct processing in the endoplasmic reticulum and Golgi complex. The same result was observed in a truncated form that was designed to mimic the N-terminus of the commercially available bovine DNase IIα protein, Δ107. These results demonstrate that the signal peptide sequence is critical for N-glycosylation and activity of human DNase IIα.

Likewise, overexpression of mutant forms of DNase IIα that lack single or multiple N-glycosylation sites (Figure 2) have substantially reduced activity and predictable decreases in protein size. This verifies that each of the reported N-glycosylation sites (Table 2) possesses an attached sugar moiety and, although the protein can tolerate losses of single sites, multiple ablations result in severe loss of activity.

An earlier study examined the effect of a subset of the N-glycosylation mutants examined in the present paper [37]. However, this previous work detected substantially smaller proteins following immunoprecipitation and failed to detect expression of each mutant form. Additionally, no expression was detected in any of the multiple mutant constructs, in contrast with our results. The results of the present study of all four N-glycosylation sites, singly or in combination, in the presence or absence of the N-glycosylation inhibitor TM, are substantially more complete. Also, unlike the earlier study [37], these results are consistent with the revised domain structure of DNase IIα [26].

Despite the importance of N-glycosylation in forming the active enzyme, as seen by both site-directed mutagenesis and inhibitor treatment (Figures 1 and 2), it was unclear if sugar attachment was itself required for activity, or merely for correct acquisition of the active form. To evaluate this, wild-type DNase IIα protein produced under fully glycosylating conditions was subjected to PNGase F deglycosylation (Figure 3). Although the protein was diminished in size from 45 kDa to 37 kDa, the size of the unglycosylated protein produced during TM treatment, there was no effect on protein activity. This provides support for the hypothesis that N-glycosylation is only required for protein to become active and is not intrinsically required for DNase IIα activity.

Previously, the reported structure of DNase IIα purified from porcine spleen [11,24] suggested a disulphide bridge between dissociable α1 and α2 fragments at sites which correspond to Cys^{19} and Cys^{159} of the human DNase IIα sequence. However, our recent work [26] proposed a revised model of DNase IIα: a

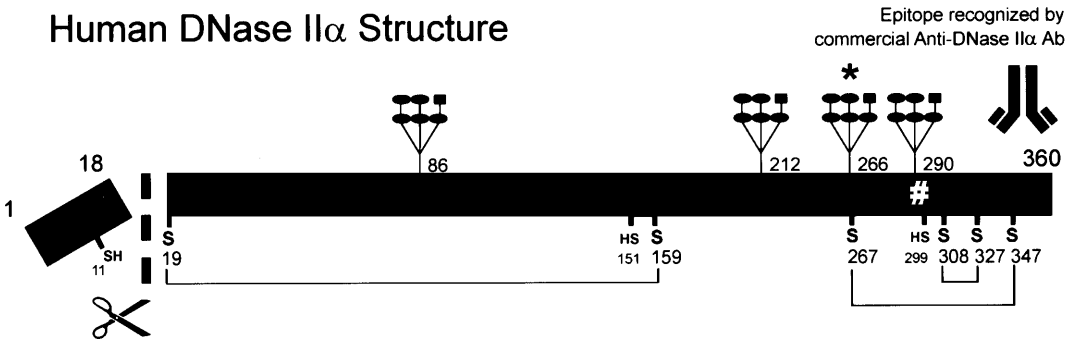

Figure 7 Revised protein structure of DNase IIα

The protein is a single, contiguous polypeptide chain, from which a signal peptide has been cleaved, featuring N-linked sugar moieties at asparagine residues (positions 86, 212, 266 and 290) and disulphide bridges at cysteine residues (positions 19, 159, 267, 308, 327 and 347). A potential conflict between N-glycosylation at Asn^{266} and disulphide bridging at Cys^{267} is indicated with *. The probable catalytic site at His^{295} is indicated with #. Predicted pairing of cysteines is based on [11,24]. Free thiol groups are depicted for cysteine residues (positions 11, 151 and 299) which are unlikely to be involved in disulphide bridging. The site corresponding to the portion of the human protein detected by the commercially available antibody is indicated.

single, contiguous polypeptide containing intrachain, but no interchain, disulphide linkages. There are several very highly conserved cysteine residues in DNase II family members (Table 3), which are potential candidates for participation in bridging. In the present study, we ablated each of these cysteine residues by mutation to alanine in expression constructs. When any of the six very highly conserved cysteine residues (positions 19, 159, 267, 308, 327 or 347) were altered, protein activity was completely destroyed (Figure 4). In contrast, mutants at a non-conserved residue (Cys^{11}) and a residue conserved only in mammalian species (Cys^{299}) retained activity. The mutant form at the non-conserved Cys^{151} residue was not expressed, however, which made it impossible to evaluate its activity. Since protein size and form did not change in the mutant proteins, it underscores our observation that these residues are not involved in interchain bridging of distinct domains, but rather form an intrachain linkage [26].

Results with the new anti-DNase IIα polyclonal antibody, anti-hnD2 [29], are also supportive of the single-chain protein structure model. This antibody was initially developed in the course of experiments demonstrating the inducibility of DNase IIα expression in response to phorbol-ester treatment in HL60 and THP-1 cells [29]. Although we were unable to detect protein in HL60 or THP-1 cells using this antibody (results not shown), it clearly and specifically detects transfected DNase IIα protein in a mouse cell line that lacks endogenous DNase IIα expression (Figure 6). In the original paper using this antibody [29], the authors reported detecting a protein of approx. 38 kDa in HL60 and THP-1 cells. However, we detected a protein of 45 kDa in transfected cells, and a 37 kDa form was only seen when glycosylation was inhibited. This suggests that HL60 and THP-1 cells may exhibit altered glycosylation and/or processing of DNase IIα protein. Further work evaluating deglycosylation and reduction of protein produced in these cell lines will be needed to explain this difference. However, our results with overexpressed protein demonstrate processing of the protein which is identical with that seen with the commercial antibody and consistent with the revised protein structure model.

The histidine residue corresponding to position 295 of the human DNase IIα sequence was originally predicted by alkylation in the porcine protein to be critical for activity [11]. Indeed, when we mutated this residue to an alanine in overexpressed human protein, it was found to lack activity completely [26]. Despite the frequent statement that His^{295} forms the 'active site' of DNase IIα [24,38–41], there is no evidence for the role of this residue in catalysis. Although many endonucleases do feature active-site histidine residues that are involved directly in enzymic activity [31,42–44], His^{295} could exhibit its effect on activity through critical participation in DNA binding, for example. To analyse this possibility, wild-type and H295A mutant proteins were tested for their ability to bind DNA using a method developed for the caspase-activated DNase [31]. We observed that both wild-type and mutant protein could effectively bind DNA (Figure 5), suggesting that His^{295} is not required for DNA binding. This suggests that His^{295} is indeed involved in catalysis. Alternatively, other residues may be required for catalysis and alkylating or mutating His^{295} results in tertiary perturbations of the protein structure and interference with distal residues involved in catalysis.

Our observations of the roles of the signal peptide, N-glycosylation, disulphide bridging and DNA binding provide a new understanding of the human DNase IIα structure–activity relationship, results that are critical for understanding the protein structure of the entire DNase II family of proteins. We summarize our current model of protein structure in Figure 7, noting sugar attachment sites, the purported active site, cysteines that are unimportant for disulphide bridging, and cysteine sites where disulphide bridging is likely, based on our results. The actual pairing of the disulphides depicted is hypothetical, based on results from the purified porcine spleen protein [11,24], which we have shown to contain an incorrect overall domain structure. These pairings need to be confirmed. This collected knowledge of the components of the protein that are necessary to generate active enzyme has an important bearing on the choice of production systems for our subsequent work. It will be critical for examination of DNase IIα as a putative protein therapeutic agent for CF or other disorders [20,27] and will complement future three-dimensional structural analysis.

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REFERENCES

1 Catcheside, D. G. and Holmes, B. (1947) The action of enzymes on chromosomes. Symp. Soc. Exp. Biol. **1**, 225–231
2 Bernardi, G., Appella, E. and Zito, R. (1965) Studies on acid deoxyribonuclease. III. Physical and chemical properties of hog spleen acid deoxyribonuclease. Biochemistry **4**, 1725–1729
3 Bernardi, G. and Griffe, M. (1964) Studies on acid deoxyribonuclease. II. Isolation and characterization of spleen-acid deoxyribonuclease. Biochemistry **3**, 1419–1426
4 Liao, T.-H., Liao, W.-C., Chang, H.-C. and Lu, K.-S. (1989) Deoxyribonuclease II purified from the isolated lysosomes of porcine spleen and from porcine liver homogenates. Comparison with deoxyribonuclease II purified from porcine spleen homogenates. Biochim. Biophys. Acta **1007**, 15–22
5 Barry, M. A. and Eastman, A. (1993) Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. Arch. Biochem. Biophys. **300**, 440–450
6 Yasuda, T., Nadano, D., Awazu, S. and Kishi, K. (1992) Human urine deoxyribonuclease II (DNase II) isoenzymes: a novel immunoaffinity purification, biochemical multiplicity, genetic heterogeneity and broad distribution among tissues and body fluids. Biochim. Biophys. Acta **1119**, 185–193
7 Yasuda, T., Nadano, D., Sawazaki, K. and Kishi, K. (1992) Genetic polymorphism of human deoxyribonuclease II (DNase II): low activity levels in urine and leukocytes are due to an autosomal recessive allele. Ann. Hum. Genet. **56**, 1–10
8 Yasuda, T., Sawazaki, K., Nadano, D., Takeshita, H., Nakanaga, M. and Kishi, K. (1993) Human seminal deoxyribonuclease I (DNase I): purification, enzymological and immunological characterization and origin. Clin. Chim. Acta **218**, 5–16
9 Krieser, R. J., MacLea, K. S., Longnecker, D. S., Fields, J. L., Fiering, S. and Eastman, A. (2002) Deoxyribonuclease IIα is required for DNA digestion during the phagocytic phase of apoptosis and its loss causes perinatal lethality. Cell Death Differ. **9**, 956–962
10 Kawane, K., Fukuyama, H., Kondoh, G., Takeda, J., Ohsawa, Y., Uchiyama, Y. and Nagata, S. (2001) Requirement of DNase II for definitive erythropoiesis in the mouse fetal liver. Science **292**, 1546–1549
11 Liao, T.-H. (1985) The subunit structure and active site sequence of porcine spleen deoxyribonuclease. J. Biol. Chem. **260**, 10708–10713
12 Oosthuizen, M. M. J., Myburgh, J. A. and Schabort, J. C. (1984) Lysosomal acid deoxyribonuclease from vervet monkey livers – I. Purification and physico-chemical characterization. Int. J. Biochem. **16**, 1207–1215
13 Rajagopal, S. V. and Rao, K. S. (1992) Purification and properties of a deoxyribonuclease (acid DNase) from rat brain. Biochem. Arch. **8**, 135–142
14 Lesca, P. (1976) Protein inhibitor of acid deoxyribonucleases. Improved purification procedure and properties. J. Biol. Chem. **251**, 116–123
15 Torriglia, A., Perani, P., Brossas, J. Y., Chaudun, E., Treton, J., Courtois, Y. and Counis, M. F. (1998) L-DNase II, a molecule that links proteases and endonucleases in apoptosis, derives from the ubiquitous serpin leukocyte elastase inhibitor. Mol. Cell. Biol. **18**, 3612–3619
16 Torriglia, A., Chaudun, E., Chany-Fournier, F., Jeanny, J., Courtois, Y. and Counis, M. (1995) Involvement of DNase II in nuclear degeneration during lens cell differentiation. J. Biol. Chem. **270**, 28579–28585
17 Malferrari, G., Mazza, U., Tresoldi, C., Rovida, E., Nissim, M., Mirabella, M., Servidei, S. and Biunno, I. (1999) Molecular characterization of a novel endonuclease (Xib) and possible involvement in lysosomal glycogen storage disorders. Exp. Mol. Pathol. **66**, 123–130
18 Shiokawa, D., Ohyama, H., Yamada, T., Takahashi, K. and Tanuma, S. (1994) Identification of an endonuclease responsible for apoptosis in rat thymocytes. Eur. J. Biochem. **226**, 23–30
19 Tanuma, S. and Shiokawa, D. (1994) Multiple forms of nuclear deoxyribonuclease in rat thymocytes. Biochem. Biophys. Res. Commun. **203**, 789–797
20 Krieser, R. J., MacLea, K. S., Park, J. P. and Eastman, A. (2001) The cloning, genomic structure, localization, and expression of human deoxyribonuclease IIβ. Gene **269**, 205–216
21 Krieser, R. J. and Eastman, A. (1998) The cloning and expression of human deoxyribonuclease II: a possible role in apoptosis. J. Biol. Chem. **273**, 30909–30914
22 Krieser, R. J. and Eastman, A. (2000) Deoxyribonuclease II: structure and chromosomal localization of the murine gene, and comparison with the genomic structure of the human and three *C. elegans* homologs. Gene **252**, 155–162
23 MacLea, K. S., Krieser, R. J. and Eastman, A. (2003) A family history of deoxyribonuclease II: surprises from *Trichinella spiralis* and *Burkholderia pseudomallei*. Gene, in the press
24 Wang, C. C., Lu, S. C., Chen, H. L. and Liao, T. H. (1998) Porcine spleen deoxyribonuclease II. Covalent structure, cDNA sequence, molecular cloning, and gene expression. J. Biol. Chem. **273**, 17192–17198
25 Takeshita, H., Yasuda, T., Iida, R., Nakajima, T., Hosomi, O., Nakashima, Y., Mori, S., Nomoto, H. and Kishi, K. (1998) Identification of the three non-identical subunits constituting human deoxyribonuclease II. FEBS Lett. **440**, 239–242
26 MacLea, K. S., Krieser, R. J. and Eastman, A. (2002) Revised structure of the active form of human deoxyribonuclease IIα. Biochem. Biophys. Res. Commun. **292**, 415–421
27 Eastman, A. and Krieser, R. (2001) Deoxyribonuclease II proteins and cDNAs. U.S. Pat. 6,184,034
28 Shak, S., Capon, D. J., Hellmiss, R., Marsters, S. A. and Baker, C. L. (1990) Recombinant human DNase I reduces the viscosity of cystoic fibrosis sputum. Proc. Natl. Acad. Sci. U.S.A. **87**, 9188–9192
29 Chou, S. F., Chen, H. L. and Lu, S. C. (2002) Up-regulation of human deoxyribonuclease II gene expression during myelomonocytic differentiation of HL-60 and THP-1 cells. Biochem. Biophys. Res. Commun. **296**, 48–53
30 Yasuda, T., Takeshita, H., Nakazato, E., Nakajima, T., Hosomi, O., Nakashima, Y. and Kishi, K. (1998) Activity measurement for deoxyribonucleases I and II with picogram sensitivity based on DNA/SYBR Green I fluorescence. Anal. Biochem. **255**, 274–276
31 Sakahira, H., Takemura, Y. and Nagata, S. (2001) Enzymatic active site of caspase-activated DNase (CAD) and its inhibition by inhibitor of CAD. Arch. Biochem. Biophys. **388**, 91–99
32 Marshall, R. D. (1972) Glycoproteins. Annu. Rev. Biochem. **41**, 673–702
33 Miletich, J. P. and Broze, Jr, G. J. (1990) β Protein C is not glycosylated at asparagine 329. The rate of translation may influence the frequency of usage at asparagine-X-cysteine sites. J. Biol. Chem. **265**, 11397–11404
34 Bause, E. (1983) Structural requirements of N-glycosylation of proteins. Studies with proline peptides as conformational probes. Biochem. J. **209**, 331–336
35 von Heijne, G. (1990) The signal peptide. J. Membr. Biol. **115**, 195–201
36 Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10**, 1–6
37 Yasuda, T., Takeshita, H., Iida, R., Nakajima, T., Hosomi, O., Nakashima, Y., Mori, S. and Kishi, K. (1999) Structural requirements of a human deoxyribonuclease II for the development of the active enzyme form, revealed by site-directed mutagenesis. Biochem. Biophys. Res. Commun. **256**, 591–594
38 Shiokawa, D. and Tanuma, S. I. (1998) Cloning of cDNAs encoding porcine and human DNase II. Biochem. Biophys. Res. Commun. **247**, 864–869
39 Shiokawa, D. and Tanuma, S. (1999) DLAD, a novel mammalian divalent cation-independent endonuclease with homology to DNase II. Nucleic Acids Res. **27**, 4083–4089
40 Lyon, C. J., Evans, C. J., Bill, B. R., Otsuka, A. J. and Aguilera, R. J. (2000) The *C. elegans* apoptotic nuclease NUC-1 is related in sequence and activity to mammalian DNase II. Gene **252**, 147–154
41 Wu, Y. C., Stanfield, G. M. and Horvitz, H. R. (2000) NUC-1, a *Caenorhabditis elegans* DNase II homolog, functions in an intermediate step of DNA degradation during apoptosis. Genes Dev. **14**, 536–548
42 Ito, K., Akiyama, D. and Minamiura, N. (1994) Evidence for an essential histidine residue on active site of human urinary DNase I: carboxymethylation and carbethoxylation. Arch. Biochem. Biophys. **313**, 126–130
43 Friedhoff, P., Kolmes, B., Gimadutdinow, O., Wende, W., Krause, K. L. and Pingoud, A. (1996) Analysis of the mechanism of the *Serratia* nuclease using site-directed mutagenesis. Nucleic Acids Res. **24**, 2632–2639
44 Meiss, G., Scholz, S. R., Korn, C., Gimadutdinow, O. and Pingoud, A. (2001) Identification of functionally relevant histidine residues in the apoptotic nuclease CAD. Nucleic Acids Res. **29**, 3901–3909

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