Identification of three crucial histidine residues (His¹¹⁵, His¹³² and His²⁹⁷) in porcine deoxyribonuclease II

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DNase II is an acid endonuclease that is involved in the degradation of exogenous DNA and is important for DNA fragmentation and degradation during cell death. In an effort to understand its catalytic mechanism, we constructed plasmids encoding nine different histidine (H)-to-leucine (L) mutants for porcine DNase II and examined the enzyme properties of the expressed mutant proteins. Of the mutants, all but H132L were secreted into the medium of expressing cells. Six of the mutated DNase II proteins (H41L, H109L, H206L, H207L, H274L and H322L) showed enzyme activity, whereas the H115L, H132L and H297L mutants exhibited very little activity. The H115L and H297L mutants were found to undergo correct protein folding, but were inactive. To further examine these mutants, we expressed H115A and H297A

DNase II mutants; these mutants were inactive, but their DNase activities could be rescued with imidazole, indicating that His¹¹⁵ and His²⁹⁷ are likely to function as a general acid and a general base respectively in the catalytic centre of the enzyme. In contrast with the secreted mutants, the H132L mutant protein was found in cell lysates within 16 h after transfection. This protein was inactive, improperly folded and was drastically degraded via the proteosomal pathway after 24 h. The polypeptide of another substitution for His¹³² with lysine resulted in the misfolded form being retained in endoplasmic reticulum.

Key words: acid–base catalysis, deoxyribonuclease, histidine mutation, protein folding and degradation.

INTRODUCTION

DNase II (EC 3.1.22.1), a deoxyribonuclease that hydrolyses the phosphodiester bonds of DNA, is capable of generating 3'-phospho-oligonucleotides under acidic conditions in a metal-ion-independent fashion [1]. The enzyme is ubiquitous, and DNase II activity has been found in a wide variety of animal tissues [2]. During production of endogenous pDNase II (porcine DNase II), the initial translation product is targeted to the lysosome [3], where proteases cleave the 45 kDa DNase II translation product into two fragments of 35 and 10 kDa. These fragments form a non-covalently bonded α/β heterodimer, i.e. the mature DNase II enzyme [4].

In terms of the cellular function(s) of this enzyme, recent studies have shown that DNase II appears to be responsible for destroying the nuclear DNA expelled from erythroid precursor cells [5]. Following phagocytosis, DNase II in phagocytes functions to cleave the DNA of the apoptotic cells [6]. In addition, the apoptotic nuclease (a DNase II homologue) of the nematode worm *Caenorhabditis elegans* is critical for the DNA degradation and laddering associated with apoptosis [7]. These studies strongly indicate that DNase II is likely to be involved in apoptosis.

In terms of a functional mechanism, the strongly nucleophilic histidine residues in nucleases are often involved in catalytic hydrolysis of phosphodiester bonds [8]. Mutation of the conserved His³⁷¹ residue in members of the herpesviral DNase family (H371A) was found to abrogate nuclease activity, even though the mutants retained their DNA binding abilities [9]. Similarly, another apoptotic nuclease, caspase-activated DNase, was found to have several functionally relevant histidine residues [10]. However, no previous biochemical study has examined the catalytic mechanism of DNase II. As with other nucleases, it is very

likely that the histidine residues are catalytically important in DNase II.

In the present study we performed site-directed mutagenesis of the histidine residues in pDNase II. The cDNA of pDNase II [11] was used to construct a series of plasmids encoding DNase II mutants with histidine-to-leucine substitutions at each of the nine histidine residues in pDNase II. The mutants were expressed in HEK-293T (human embryonic kidney 293T) cells, purified and tested for enzyme activity. To examine the basis for the enzyme activity loss in specific mutants, we used chemical rescue of catalytic activity by an exogenous proton donor (i.e. imidazole), which has proved useful for investigating the function of specific histidine residues as proton donors/acceptors [12,13].

EXPERIMENTAL

DNase activity and protein assays

The hyperchromicity assay was used for the chromatographic purification of expressed proteins and the calculation of enzymatic specific activities, as previously reported [14]. One unit of activity was defined as the amount of DNase II necessary to cause an increase of 1 A_{260} unit/min in 1 ml of assay solution containing 0.04 mg of calf thymus DNA, 0.01 M EDTA and 0.15 M sodium acetate, pH 4.7. Measurements of the DNase activities in cell medium or lysates (in cases of low activity or complex mixtures) were performed using the metachromatic agar-diffusion assay, as previously reported [15], with the exception that the above-mentioned DNase II assay buffer was used. SDS/PAGE was performed according to the method of Laemmli [16]. The refolding of expressed polypeptides to active DNase II was detected by zymography of DNase II activity in DNA-containing

Abbreviations used: ER, endoplasmic reticulum; HEK, human embryonic kidney; LAMP-1, lysosome-associated membrane protein-1; pDNase, porcine DNase; WT, wild-type.

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gels, as previously described [17]. In cases where the mutant DNase II activities were extremely low, a plasmid-cutting assay was used to measure basal enzyme activity. Briefly, the purified enzyme was incubated in 50 mM sodium acetate, pH 4.7, 15 μ M BSA and 300 ng/ μ l pCRII plasmid DNA (Invitrogen) at 25°C for 15 min. The reaction products were separated by 1%-(w/v)-agarose-gel electrophoresis in Tris/acetate/EDTA buffer. The intensities of the supercoiled form were quantified with the LabWorks software (UVP BioImaging Systems, Cambridge, U.K.). DNase activities were calculated according to the decrease in intensity of the supercoiled form. Protein concentrations were determined using a BCA (bicinchoninic acid) protein assay kit (Pierce), with BSA as the standard.

Mutant clones

The WT (wild-type) DNase II gene was cloned into the pcDNA3 vector (Invitrogen) as previously described [11] and was used as the template for preparation of all DNase II mutants. These mutants were generated using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) or the PCR-overlap-extension method [18]. All mutated genes were confirmed by direct sequencing. The primers used for mutagenesis were as follows:

H41L: 5'-AAGCTGCCGGCCCTCAGCAGTCCCGGAGA-TG-3'

H109L: 5'-ACAGAGGATCTCAGCTCTCTTCCAACCGT-GG-3'

H115L: 5'-TCCAACCGTGGGCTCAGGAAGGGTGTACT-GC-3'

H115A: 5'-TCCAACCGTGGGGCCACGAAGGGTGTACT-GC-3'

H132L: 5'-TTCTGGCTAATCCTCAGCGTTCCAACTT-CC-3'

H132K: 5'-TTCTGGCTAATCAAGAGCGTTCCAACTT-CC-3'

H206L: 5'-GTAGTCAAGGGCCTTCATGTTCTCCAGGAAC-CC-3'

H207L: 5'-GTCAAGGGCCATCTTGTTCTCCAGGAACCCT-GG-3'

H274L: 5'-CTCTGGGGTCCAGCTCGTGCTGGATGTGA-CC-3'

H297L: 5'-AATGCCACAGAAGACCTCTCCAAGTGGTG-TG-3'

H297A: 5'-AATGCCACAGAAGACGCCTCCAAGTGGTG-TG-3'

H322L: 5'-AAAAGAGAGGAGCTTCGGGGTGGGGGCAC-AC-3'

The nucleotide sequences were obtained from GenBank® (accession no. AJ001387); the mutated sites are underlined.

Cell culture and transfection

HEK-293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% (v/v) fetal bovine serum (Invitrogen), 50 units/ml penicillin and 50 μ g/ml streptomycin (Invitrogen). Cells were incubated at 37°C in an atmosphere of air/CO₂ (19:1). Cells in the exponential growth phase were transfected with 30 μ g of each plasmid, using a modified calcium phosphate method [19]. For mutants that

were secreted into the medium, cells were incubated for 16 h post-transfection, washed with serum-free medium, and incubated for an additional 32 h. Cells were then harvested using cell lifters and separated from the medium by centrifugation. For mutants that were not secreted, cells were directly harvested 48 h post-transfection and lysed.

Purification of the mutated proteins

For two-step purification of secreted proteins, 500 ml of growth medium was collected and dialysed against 50 mM sodium acetate, pH 4.7, for 12 h. Precipitated material was removed by centrifugation. The supernatant was concentrated to less than 10 ml by ultrafiltration and applied to a Cibacron Blue 3-GA agarose (Sigma) column (0.5 cm diameter $\times$ 20 cm long) previously equilibrated with 50 mM sodium acetate, pH 4.7. The proteins were eluted with a gradient of 0–1 M NaCl in 50 mM sodium acetate, pH 4.7, and the DNase II activities were measured in the collected fractions. For mutants without obvious DNase II activity, dot-blots were used to locate the fractions containing the expressed proteins. For the second step, the pooled fractions were dialysed, concentrated, and applied to the ÄKTA purification system (Amersham Biosciences) on a cation-exchange Mini S (PE 4.6/50) column (Amersham Biosciences) previously equilibrated with 50 mM sodium acetate, pH 4.7. Proteins were eluted with a gradient of 0–0.5 M NaCl in the same buffer. A SOURCE 15S (Amersham Biosciences) column (0.5 cm diameter $\times$ 20 cm long) was sometimes used for the second step in place of the Mini S column, with the same elution scheme. In some cases, a second small Cibacron Blue 3-GA-agarose column (0.25 cm diameter $\times$ 10 cm long) was used in a third step to obtain adequate protein homogeneity. The purified DNase II proteins were dialysed against 50 mM sodium acetate buffer, pH 4.7, concentrated, then frozen at -20°C until used.

Electrophoretic-mobility-shift assays for DNA binding

To avoid DNA degradation, the purified WT, H115L and H297L DNase II proteins were first incubated for 15 min at 25°C with 100 mM iodoacetate, which destroyed DNase activities. PCR-generated 300 bp DNA fragments were then added, the mixture was incubated for 10 min at 25°C, and the samples were analysed by 1%-agarose-gel electrophoresis. Instead of the typical basic pH buffer, we used 20 mM sodium acetate, pH 4.7, as the electrophoresis buffer in order to maintain the native DNase II structure for DNA binding. BSA was used in a protein control assay to present the DNA retardation caused by DNase II.

Dot-blots and Western blots

Anti-(pDNase II) antibodies were produced in rabbits using the purified pDNase II as the antigen. Prior to sample application, each PVDF membrane (PerkinElmer) was sequentially preincubated with methanol and a buffer containing 48 mM Tris/HCl, 40 mM glycine, 0.0375% SDS and 20% (v/v) methanol, pH 9.2. For dot-blots, 5 μ l of each sample was spotted on to the membrane and semi-dried. The spots were blocked in PBS containing 5% (w/v) dried non-fat milk for 1 h at room temperature and probed with anti-(pDNase II) antibodies (1:1000) in PBS for 1 h. The membranes were washed with washing buffer (20 mM Tris/HCl, pH 8.0, 400 mM NaCl and 0.05% Tween-20), and then incubated with a horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma; 1:3000) in PBS for 1 h. For Western blots, normalized aliquots of proteins were separated by SDS/12%-(w/v)-PAGE, electrotransferred to PVDF membranes, and then

Figure 1 Purities and DNase activities of the DNase II mutants

(A) SDS/PAGE. Each lane contained 0.25 μ g of protein. The gel was silver-stained. (B) DNase activities measured with the metachromatic agar-diffusion assay. Growth media were collected 48 h after cells were transfected with the WT, H115L and H297L constructs, and 0.25 μ g of protein was applied to each well. (C) Western blots using the anti-(pDNase II) antibody. Each lane was loaded with 0.5 μ g of protein.

detected as described above. The immunopositive proteins were detected using an enhanced-chemiluminescence reagent (PerkinElmer), and the chemiluminescence was visualized using Biomax MR film (Kodak).

Cell lysates and subcellular fractionation

The harvested cells were washed twice with cold PBS, resuspended in cold PBS containing a protease-inhibitor cocktail (Sigma) and then lysed by three freeze–thaw cycles. Samples were centrifuged, and the supernatants were collected as cell lysates. Subcellular fractions were prepared by differential centrifugation at 4°C, as previously described [3], with slight modifications. Briefly, cells were harvested 48 h post-transfection, centrifuged, then washed twice with PBS on ice. The cells were then suspended in 3 cell volumes of suspension buffer (Hepes, pH 7.4, containing 0.33 M sucrose and a protease-inhibitor cocktail) and disrupted by being passed through a narrow-gauge (no. 27) needle. The samples were centrifuged at 1020 *g* for 15 min, the pellets were resuspended in 3 cell volumes of suspension buffer and the centrifugation step was repeated. The supernatants were pooled and centrifuged at 3300 *g* for 15 min and the new supernatant was centrifuged at 11 700 *g* for 35 min. The pellet from this step was retained as the lysosomal fraction. The supernatant was centrifuged at 100 000 *g* for 4 h, and the pellet from this step was retained as the ER (endoplasmic reticulum)/Golgi fraction.

Immunofluorescence and confocal microscopy

HEK-293T cells were grown on 10-mm-diameter glass coverslips and transfected as described above. After transfection (24 h), the cells were fixed with 4% (w/v) paraformaldehyde and then permeabilized with 0.1% Triton X-100 in PBS. Both primary- and secondary-antibody incubations were carried out for 30 min at room temperature with PBS containing 10% (v/v) fetal bovine serum. Rabbit anti-(pDNase II) antibodies were purified from the antiserum and used in 1:2000 dilution. Mouse anti-calnexin antibody (1:200), FITC-conjugated goat anti-mouse IgG (1:1000) and Rhodamine-conjugated goat anti-rabbit IgG (1:1000) were purchased from Chemicon. During the last 15 min of secondary antibody staining, Hoechst 33258 (5 μ g/ml; Sigma) was applied. After extensive washing, the coverslips were mounted on slides. Confocal microscopy was performed with TCS SP2 Spectral Confocal System (Leica) using a 63 $\times$ magnification/1.32-numerical-aperture oil-immersion lens. Double-label immunofluorescence signals were sequentially collected using

single-fluorescence excitation and acquisition settings to avoid cross-over.

CD measurements

The spectra were recorded on a Jasco J-715 spectropolarimeter between 250 and 190 nm in a cylindrical cuvette of 0.1 cm pathlength in 50 mM sodium phosphate buffer, pH 5.8. The baseline given by the CD spectrum of 50 mM phosphate buffer, pH 5.8, was recorded and subtracted from the CD spectra of each sample. Protein concentrations were based on A_{280} . The far-UV CD spectra were analysed using SELCON3 algorithms [20], using a basis set that included the CD spectra of 56 proteins, as obtained from the CDPPro website [21].

RESULTS

Six of nine histidine residues are dispensable for pDNase II catalytic activity

The nine histidine residues in pDNase II were separately mutated to leucine in a series of plasmids, which were used to express the mutant proteins in HEK-293T cells. The expressed proteins were collected from the medium and purified. The purity of each expressed DNase II mutant protein was examined by SDS/PAGE (Figure 1A), except for the H132L mutant, which was not secreted into the medium. The bands observed on the gel were blurred. This is most likely due to different degrees of glycosylation at the six glycosylation sites in pDNase II [11] and seems to indicate that the various histidine sites had different effects on the glycosylation states of the expressed proteins. The specific enzyme activities of the expressed WT, H41L, H109L, H206L, H207L, H274L and H322L proteins ranged from 138 to 331 units/mg (Table 1), which is comparable with that of native pDNase II [4]. This seems to indicate that H41, H109, H206, H207, H274 and H322 are dispensable for the catalytic action of pDNase II.

The H115L and H297L mutant enzymes are inactive

A sensitive metachromatic agar-diffusion assay revealed that the H297L DNase II mutant protein showed almost no DNase II activity (Figure 1B). In addition, the mutation of His¹¹⁵ to leucine drastically abrogated pDNase II activity (Figure 1B). Western-blot analysis demonstrated that these proteins were successfully expressed in our system (Figure 1C), indicating that the lack

Table 1 Specific enzyme activities* of the expressed DNase II proteins

Clone	Specific enzyme activity (units/mg)	Column used sequentially†
WT	321 ± 6	Blue, Source, Blue
H41L	220 ± 5	Blue, Mini
H109L	287 ± 5	Blue, Mini
H206L	219 ± 4	Blue, Source
H207L	138 ± 4	Blue, Source
H274L	172 ± 7	Blue, Mini
H322L	331 ± 6	Blue, Mini, Blue
Native‡	185	—

* Values are means ± S.D. for three independent determinations.

† Blue: Cibacron Blue 3-GA agarose column; Source, SOURCE 15S column; Mini, Mini S (PE 4.6/50) column.

‡ Derived from our previous data [4].

of catalytic activity was a result of the mutation, not lack of expression.

Characterization of pDNase mutants at His¹¹⁵ and His²⁹⁷

To determine whether the H115L and H297L mutants were correctly folded, we compared the elution profiles of the WT, H115L and H297L mutant DNase II proteins. All three proteins could be eluted from Cibacron Blue 3GA-agarose columns at the same conductivity (60 mS/cm; Figure 2, arrows), indicating that physical properties on the surface of these proteins are the same. To assess the secondary structures of the WT and mutant proteins, we examined their CD spectra. Our results revealed that the WT, H115L and H297L proteins all had nearly identical secondary structures (Table 2). Finally, gel-shift assays for DNA binding ability revealed that the purified WT, H115L and H297L proteins caused similar gel-shifts of a 300 bp DNA fragment (Figure 3). The protein-DNA complexes migrated more slowly as the protein concentrations increased, indicating that the H115L and H297L DNase II molecules retained WT-level DNA binding abilities. Collectively, these results suggest that the mutants were similar to the WT protein in terms of structure and DNA binding ability, indicating that their lack of enzyme activity was probably due to the effect of the mutated histidine residue rather than incorrect protein structure.

Chemical rescue of the H115A and H297A DNase II activities

Since alanine has a smaller side chain, which may accommodate imidazole more readily than the leucine side chain, we tested the ability of imidazole to chemically rescue the enzyme activity of H115A and H297A mutant pDNase II proteins. As described for the H115L and H297L mutants, the H115A and H297A mutant proteins were constructed, expressed, purified and characterized. Examination of the eluted conductivity, DNA binding ability (results not shown) and CD spectra (Table 2) of H115A and H297A revealed that these mutants were structurally consistent with the WT protein. The H115A and H297A mutants were able to hydrolyse DNA to a slight extent, exhibiting basal DNase activity levels 2300- and 8900-fold lower respectively than that of WT DNase II (results not shown). However, when imidazole was included in the assay mixtures, the two mutated DNase II proteins showed substantial increases in DNase activity (Figure 4). Addition of 100 mM imidazole increased the DNase II activity of H115A by approx. 7-fold (Figure 4B, right panel) and that of H297A by approx. 11-fold (Figure 4C, right panel). Addition of higher imidazole concentrations further increased the DNase activity, as shown by smear patterns on the gel (Figures 4B and

Figure 2 Cibacron Blue 3GA-agarose chromatography

The numbers at the top of the dot blots represent the fraction number marked in the chromatographic charts. Asterisks in the H115L and H297L panels represent the significant responses shown in the dot blots.

Table 2 Proportions of different secondary structure found in the WT and the His¹¹⁵- and His²⁹⁷-mutated proteins

DNase II	Amounts of different secondary structure (%)					
	Regular helix	Distorted helix	Regular strand	Distorted strand	Turns	Unordered
WT	0.28	0.15	0.11	0.06	0.17	0.24
H115L	0.25	0.13	0.13	0.07	0.17	0.24
H297L	0.28	0.15	0.11	0.06	0.17	0.24
H115A	0.30	0.15	0.10	0.05	0.16	0.24
H297A	0.29	0.16	0.12	0.05	0.15	0.24

4C, left panel). These findings provide evidence suggesting that H115 and H297 are directly involved in the catalytic mechanism of DNase II.

Figure 3 Electrophoretic-mobility-shift assays for DNA binding

The protein concentrations are listed above each panel. ST, DNA length standard.

The chemical rescue of H115A and H297A DNase II activity is pH-dependent

To examine the catalytic roles of His¹¹⁵ and His²⁹⁷ residues, we assessed the chemical rescue of H115A and H297A in buffers with different pH values. The resulting pH-activity profiles showed nearly bell-shaped curves, with the maximum imidazole-induced activity enhancement of H115A increased by 5.2-fold at pH 4.70 and that for H297A increased by 6.3-fold at pH 4.95 (Figure 4D). The midpoints of the two curves were pH 5.0 for H115A and pH 5.3 for H297A, indicating that the optimum pH for the imidazole-based restoration of catalytic activity was more acidic for H115A than for H297A. This indicates that His¹¹⁵ tended to gain a proton more easily than His²⁹⁷, prompting us to theorize that, during pDNase II enzyme activity, His¹¹⁵ most likely acts as a general acid, while His²⁹⁷ acts as a general base.

Expressed H132L DNase II is not secreted, but instead is degraded inside the cell

In contrast with the other mutant DNase II proteins, which were secreted into the media in our expression system, H132L was

Figure 4 Rescue of H115A and H297A DNase activity by imidazole

DNA was treated with imidazole alone (A) or in the presence of the H115A DNase II (0.15 μ M) (B) or H297A DNase II (0.6 μ M) (C), and DNase activities were measured from the decreases in intensity of supercoiled DNA. (D) The pH-activity profiles for the imidazole-induced rescue of H115A and H297A DNase II activity. The concentration of imidazole was 50 mM. DNase activities were measured from the intensity decreases of supercoiled DNA on gels. Fold changes in DNase activity were obtained from the DNase activity measured in the presence of imidazole divided by that in the absence of imidazole. The enhanced DNase activities were given as the mean values from three independent determinations. Forms of DNA: sc, supercoiled; lin, linear; oc, open circular. ST, DNA length standard.

retained within the cell. Western-blot analysis revealed that cellular levels of the H132L mutant increased to a relatively low maximum level within the first 16 h after transfection, and drastically decreased after 24 h post-transfection (Figure 5A). Northern-blot analysis revealed that the mRNA expression level of the H132L construct was comparable with the expression levels of the other constructs (results not shown), indicating that low mRNA expression did not account for the relatively low protein expression level. These findings suggest that the expressed H132L DNase II proteins are degraded inside the cell prior to secretion.

Figure 5 Western blots of H132L and H132K DNase II proteins

(A) Expression of H132L DNase II at various time intervals. Each lane contained 70 μ g of total cellular proteins. (B) H132K DNase II expressed in the cell lysate and medium. Left: each lane contained 70 μ g of total cellular proteins. Right: media were collected 48 h post-transfection and gels were loaded with 0.5 μ g of protein per lane. (C) H132K and WT DNase II proteins expressed in the lysosomal and ER fractions. Each lane was loaded with 70 μ g of protein. Actin was used as the loading control, and was probed with an anti-actin antibody. The lysosome-specific marker, LAMP-1, was probed with an anti-LAMP-1 antibody as an indicator of the lysosomal fraction.

H132K DNase II is retained within the cell

To further examine the importance of His¹³², we constructed an H132K mutant and assessed the importance of having a positive charge at residue 132. Consistent with H132L, the H132K mutant was not secreted into the media (Figure 5B, right panel). However, unlike the H132L mutant enzyme, which was degraded after 24 h post-transfection, we observed the complete 45 kDa form of H132K in cell lysates at both 16 and 48 h post-transfection, but did not observe the 35 kDa form (Figure 5B, left panel). Expressed WT DNase II could be detected as both the 35 and 45 kDa forms, presumably representing lysosome-processed and unprocessed DNase II proteins respectively. It seems logical to speculate that the exogenously overexpressed protein may have overloaded the lysosome pathway so that a portion of the unprocessed proteins were shunted to the secretory pathway and expressed into the medium. Interestingly, our results suggest that the H132K mutant may not enter the lysosomal pathway and undergo proteolytic

Figure 6 Confocal immunofluorescence analysis of the localization of expressed pDNase II molecules

H293T cells were transfected with WT (A–D) or H132K constructs (E–H). At 24 h after transfection, the cells were fixed, permeabilized and stained with anti-(pDNase II) antibodies, followed by Rhodamine-conjugated secondary antibodies (A and E), and anti-calnexin antibodies followed by FITC-conjugated secondary antibodies (B and F). The expressed WT pDNase II proteins were distributed throughout the entire cell, except the nucleus (A); however, H132K pDNase II proteins were concentrated in the perinuclear region (E). The merged images (C and G) show the co-localization of pDNase II and calnexin (ER marker). The nuclei (D and H) can be visualized with Hoechst 33258.

processing (45 kDa form; Figure 5B, H132K panel). This hypothesis was further substantiated by subcellular-fractionation experiments, which revealed that the complete 45 kDa form, but not the processed 35 kDa form, was present in ER/Golgi fractions from H132K-expressing cells, whereas neither form was observed in the lysosomal fraction (Figure 5C, left panel). By contrast, cells expressing the WT protein had detectable levels of both the 35 and 45 kDa forms in the lysosomal fraction (Figure 5C, right panel). The WT samples show some of the 35 kDa form in the ER/Golgi fraction, probably due to contamination of the ER/Golgi fraction with some lysosomes (Figure 5C, anti-LAMP-1 panel, where LAMP-1 is lysosome-associated membrane protein-1). The confocal immunofluorescence staining was used to examine cellular distribution of the expressed WT and H132K DNase II proteins. The expressed WT DNase II proteins were distributed throughout the entire cell, except the nucleus (Figures 6A–6D). In contrast, the expressed H132K DNase II proteins were seen within ER (Figures 6E–6H). Altogether these results indicate that the H132K polypeptide was not targeted to lysosomes or secreted into the medium, but rather retained in the ER.

Characterization of H132-mutated DNase II degradation and activity

The H132L mutant was not secreted from cells, because it was consistently degraded prior to secretion. To examine the nature of this degradation, we incubated cells with a proteasome inhibitor and tested for protein expression into the medium. Following proteasome inhibition, the expressed H132L DNase II was secreted into the media (see Supplementary Figure S1B, 'Western' panel, at <http://www.BiochemJ.org/bj/398/bj3980177add.htm>), indicating that the protein was degraded via the proteosomal pathway. We then used a zymogram analysis to test the enzyme activity and refolding ability of the non-degraded H132L mutant. Our results revealed that, whereas expressed WT DNase II could renature *in situ*, restoring DNase activity, the H132L DNase II mutant was unable to refold and regain its enzymatic activity (see Supplementary Figures S1A and S1B, 'Zymogram' panel, at <http://www.BiochemJ.org/bj/398/bj3980177add.htm>). Similarly, the H132K DNase II mutant did not show any DNase activity in a zymogram analysis (results not shown).

DISCUSSION

Our present results suggest that His¹¹⁵, His¹³² and His²⁹⁷ play crucial roles in the function of pDNase II. This is consistent with the absolute evolutionary conservation of these residues, as shown by multiple sequence alignments of DNase II molecules from various species (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/398/bj3980177add.htm>). By contrast, the other six histidine residues in pDNase II, which were found to be dispensable for its activity, are not completely conserved.

Of the three functionally relevant histidine residues in pDNase II, the His²⁹⁷ residue was regarded as being in the active site of pDNase II [4]. Moreover, a previous report indicated that His²⁹⁵ of human DNase II (equivalent to His²⁹⁷ of pDNase II) is involved in catalysis [22]. Here, we identified that another histidine residue, namely His¹¹⁵, is crucial for pDNase II catalysis. In other nucleases, the proton donors/acceptors are almost always histidine residues. For example, bovine RNase A is thought to have a catalytic mechanism in which His¹² acts as a general base and His¹¹⁹ acts as a general acid [23,24]. Similarly, in bovine DNase I, His¹³⁴ and His²⁵² are thought to act as the general acid and general base respectively with the aid of metal ions [25]. Therefore it seems very likely that His¹¹⁵ and His²⁹⁷ may act as a general acid/base pair in pDNase II.

A modelled human DNase II structure based on phospholipase D [26] suggested that His¹¹³ (equivalent to His¹¹⁵ in pDNase II) acts as the first nucleophile by attacking the phosphorus atom, whereas His²⁹⁵ (equivalent to His²⁹⁷ in pDNase II) acts as the second nucleophile by activating a nearby water molecule. However, the results of our pH-activity profiling for the H115A and H297A mutants rescued by imidazole (Figure 4D) indicated that H115 tended to be acidophilic, whereas H297 was more basophilic, tending to counter the predictions of the previous computational model.

On the basis of our results and comparisons with the catalytic reaction scheme of phospholipase D [27], we propose the following model for pDNase II (Scheme 1). In this model, His²⁹⁷ acts as an electron donor by attacking the phosphorus atom as the first nucleophile, whereas His¹¹⁵ donates a proton to the fission of the P–O bond (Reaction I). After the first product (ROH) is released (Reaction II), the His¹¹⁵ activates a water molecule to yield the second nucleophile. This deprotonated water attacks the phosphorus atom, which then inverts its configuration (Reaction III). The final reaction involves fission of the N–P bond,

which results in release of the second product (Reaction IV), leading to an overall retention of configuration. Our proposal that the His²⁹⁷ imidazole ring acts as a nucleophile is further supported by a previous report of the iodoacetate modification of His²⁹⁷ in pDNase II [4]. In this modification reaction, the imidazole of His²⁹⁷ was found to act as the electron donor by attacking the iodo-carbon bond and covalently alkylating His²⁹⁷ (Scheme 1B).

In addition to the two histidine residues making up the apparent general acid/general base pair, we identified a third crucial histidine residue in pDNase II. In eukaryotes, the protein quality control systems do not facilitate degradation of all mutated proteins. Only mutations that markedly perturb folding have been shown to trigger rapid degradation [28] via enhanced transcription of genes encoding chaperones and protein-degradation enzymes, which co-operate to degrade the unfolded proteins through ubiquitin-mediated proteosomal pathways [29,30]. Our results strongly suggest that H132 is critical for proper folding of the nascent polypeptide. Since the neighbouring residues around H132 are relatively hydrophobic (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/398/bj3980177add.htm>), replacement of His¹³² by a hydrophobic residue (i.e. leucine) is likely to result in stronger regional hydrophobicity and an unfolded polypeptide. Our results suggest that, when the unfolded H132L DNase II polypeptides accumulated in the ER lumen, the unfolded protein response was triggered and the unfolded proteins were translocated to the cytoplasm, where they were ubiquitinated and subjected to proteosomal degradation (see Supplementary Figure S3, 'H132L' pathway, at <http://www.BiochemJ.org/bj/398/bj3980177add.htm>).

Because the expressed H132K DNase II was misfolded, it was retained in the ER, as shown by co-localization of calnexin and the misfolded H132K DNase II (Figure 6G). However, the confocal immunofluorescence analysis shows not only the co-localization, but also a fused pattern, indicating the interaction of the two proteins. The quality-control system in the ER suggests the calnexin cycle is the major pathway leading to the transport of the correctly folded glycoproteins into Golgi. Moreover, the misfolded proteins are recycled through the interaction with calnexin again for another round of folding [31,32]. The fused pattern (Figure 6G) is probably the result of accumulation of the calnexin–H132K DNase II complex in the ER due to this recycling process.

Lastly, we examined the processing of pDNase II and its mutant forms. Endogenous pDNase II is processed by lysosomal proteases to yield mature DNase II, which is a non-covalently bound α - β heterodimer comprising a 35 kDa α subunit and a 10 kDa β subunit [4]. By contrast, the expressed DNase II proteins collected from the medium consisted exclusively of the complete, uncleaved 45 kDa form. Most likely, due to high-level intracellular accumulation of expressed DNase II, the cells were unable to direct all of the proteins to lysosomes, triggering a secretory pathway (see Supplementary Figure S3, the 'WT' pathway, at <http://www.BiochemJ.org/bj/398/bj3980177add.htm>). A similar situation was previously reported following overexpression of histidine-tagged human DNase II, which was detected in the cell medium [33]. A previous report theorized that DNase II is not actually cleaved in the cell, and proposed instead that this cleavage is an artificial by-product of experimental conditions [34]. Here, we report the first cleavage-defective DNase II mutant (H132K) and show that it is retained in the cell. This provides compelling evidence that DNase II cleavage is not an artifact, but rather is a true cellular process.

In conclusion, we herein show, for the first time, that the His¹¹⁵ and His²⁹⁷ residues in pDNase II act as a general acid/base pair during catalysis, whereas residue His¹³² is involved in proper protein folding. Although additional work, such as crystal

Scheme 1 Model reaction schemes for catalysis by DNase II

(A) A proposed catalytic mechanism for DNase II. (B) Chemical modification of His²⁹⁷ in pDNase II by iodoacetate.

structure solution, will be required to fully confirm these findings, our novel results provide important new insights into the catalytic mechanism and protein folding of DNase II.

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