Identification of the three non-identical subunits constituting human deoxyribonuclease II

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Abstract We purified DNase II from human liver to apparent homogeneity. The N-terminal amino acid sequences of each of three components constituting the purified mature enzyme were then separately determined by automatic Edman degradation. A combination of this chemical information and the previously reported nucleotide sequence of the cDNA encoding human DNase II [Yasuda et al. (1998) J. Biol. Chem. 273, 2610–2626] allowed detailed elucidation of the enzyme's subunit structure: human DNase II was composed of three non-identical subunits, a propeptide, proprotein and mature protein, following a signal peptide. Expression analysis of a series of deletion mutants derived from the cDNA of DNase II in COS-7 cells suggested that although a single large precursor protein may not be necessary for proteolytic maturation, the propeptide region L^{17}–Q^{46} may play an essential role in generating the active form of the enzyme.

Key words: Amino acid sequence; Deoxyribonuclease II; Proteolytic processing; Purification; Subunit structure; Human liver

1. Introduction

Although the physiological role(s) of the ubiquitously distributed enzyme DNase II (EC 3.1.22.1) has not yet been fully elucidated [1,2] and it appears to occur primarily in intracellular lysosomes [3], the enzyme is postulated to be involved in some cases of apoptosis, in which it may play a role in DNA ladder formation [4]. We have previously demonstrated the genetic polymorphism of human DNase II with regard to differences in its activity levels [5]. However, very little information on the molecular structure of DNase II has been obtained: chemical properties reported so far have shown great variability, and even its molecular mass and subunit structure remain to be confirmed [2–4,6]. Recently, we successfully accomplished the first molecular cloning of the cDNA encoding mammalian (human) DNase II [7], and clarified both its chromosomal localization [8] and its genomic structure [9]. The composite nucleotide sequence of this cDNA includes an open reading frame of 1080 bp, which encodes a single polypeptide of 360 amino acid residues. These cDNA data led us to speculate that the translated product consists of three portions, a putative signal peptide, proprotein and mature protein. However, although our cDNA information allowed detailed elucidation of the structural properties of the enzyme, chemical approaches, including expression analysis of the recombinant proteins in eukaryotic cells, were required to finally confirm its molecular structure.

In the present paper, we describe the purification of DNase II from human liver, the identification of the three subunits constituting the enzyme and the expression analysis of the recombinant proteins in COS-7 cells.

2. Materials and methods

2.1. Analytical methods and materials

DNase II activity was assayed by the single radial enzyme diffusion (SRED) method [1]. Protein quantities were determined using a protein assay kit (Bio-Rad, Richmond, CA) with BSA as a standard. The conventional method was used to assay β-D-galactosidase [10]. SDS-PAGE was performed in 15% gels according to the method of Laemmli [11], and the proteins thus separated were visualized by silver staining or immunoblotting [12]. A specific antibody against human DNase II was prepared according to our previously reported method [1].

2.2. Purification of DNase II from human liver

A whole human liver was obtained from a 57-year-old woman at postmortem, 12 h after death due to loss of blood. The liver (ca. 1200 g) was homogenized in 0.1 M sodium phosphate (pH 6.2) containing 10 mM EDTA and 1 mM PMSF. After the homogenate had been adjusted to pH 2.5 with phosphoric acid and centrifuged at $10\,000\times g$ for 30 min, the supernatant was subjected to ammonium sulfate fractionation. The fraction precipitated between 0.3 and 0.9 ammonium sulfate saturation was collected and dialyzed against 50 mM sodium acetate (pH 4.5) containing 5 mM EDTA and 1 mM PMSF (buffer A). The dialysate was applied to a CM-Sepharose CL-6B column (2.8×40 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with buffer A, and the adsorbed materials were eluted with a linear NaCl gradient (0–1.0 M). The fractions showing DNase II activity were dialyzed against 50 mM sodium acetate (pH 5.0) containing 5 mM EDTA and 1 mM PMSF (buffer B), and applied to an SE-53 column (2.8×40 cm; Whatman, Maidstone, UK) pre-equilibrated with buffer B. After the active fractions had been dialyzed against 50 mM sodium acetate (pH 5.0) containing 5 mM EDTA, 3% (v/v) glycerol and 0.5 mM 2-mercaptoethanol (2ME) (buffer C), the dialysate was applied to a Reactive blue 4-agarose column (1.6×12 cm; Sigma, St. Louis, MO) pre-equilibrated with buffer C and eluted with a linear NaCl gradient (0–1.5 M). The active fractions were redialyzed against the same buffer and applied to a heparin-Actigel column (1.0×20 cm; Sterogen Biochemicals, San Gabriel, CA) pre-equilibrated with buffer C. The active fractions were eluted with a linear NaCl gradient (0–1.0 M) and dialyzed against 25 mM potassium phosphate (pH 6.0) containing 5 mM EDTA, 3% (v/v) glycerol and 0.5 mM 2ME (buffer D). The dialysates were applied to a P-11 column (1.0×15 cm; Whatman) pre-equilibrated with buffer D, and eluted with a linear NaCl gradient (0–1.0 M). The active fractions were finally subjected to gel filtration on a Sephadex G-75 column (1.6×100 cm; Amersham Pharmacia Biotech) pre-equilibrated with

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Abbreviations: DTT, dithiothreitol; M, mature protein; 2-ME, 2-mercaptoethanol; P, proprotein; PT, propeptide; RT, reverse transcriptase; SRED, single radial enzyme diffusion; S, signal peptide

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Table 1
Purification of DNase II from human whole liver

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	15 300	31 200	2.04	1	100
1. Ammonium sulfate fractionation	1 034	24 500	23.7	11.6	78.5
2. CM-Sepharose CL-6B	234	15 700	67.1	32.9	50.3
3. SE-53	27.4	12 500	456	224	40.1
4. Reactive blue 4-agarose	19.3	11 100	575	282	35.6
5. Heparin-Actigel	7.20	8 750	1220	598	28.0
6. P-11	0.71	3 200	4510	2210	10.3
7. Sephadex G-75	0.69	3 180	4610	2260	10.2

DNase II activity and protein concentration were measured as described in Section 2.

buffer D supplemented with 0.25 M NaCl. The active fractions were collected, concentrated and used for the subsequent analysis.

2.3. N-terminal amino acid sequence analysis

After separation of the purified DNase II (ca. 100 μg) by SDS-PAGE on a 15% gel, the resulting proteins were electro-transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). The portion of the membrane carrying the proteins and the intact DNase II preparation (ca. 20 μg) were subjected to automatic Edman degradation using a protein sequencer (model 494; Perkin-Elmer, Urayasu, Japan).

2.4. Construction of expression vectors and deletion mutagenesis

A DNA fragment containing all the coding sequences for human DNase II was obtained by reverse transcriptase (RT)-PCR amplification of the total RNA derived from the thyroid gland of a 48-year-old man, obtained 12 h after death due to loss of blood, using a set of two primers, DN2-N6 (5′-AAAGGATCCATAGCAGCTATGATCCCGCTGCTGCTG-3′) and DN2-C5 (5′-AAAGCGGCCGCTTAGATCTTATAAGCTCTGCTGGG-3′). RT-PCR was performed according to a previously described method [7]. The fragment was ligated into the pcDNA3.1(+) vector (Invitrogen, San Diego, CA) to construct the expression vector pcDNA3.1-DN2. In the same manner, DNase II cDNA was cloned in its antisense orientation into the pcDNA3.1 vector to produce the vector pcDNA3.1-DN2anti. A series of deletion mutants were constructed by RT-PCR using the following primers, the sequences of which are numbered from the first ATG. Forward primers were: DN2-D7, 5′-AAAGGATCCATAGCAGCTATGCTGACCTGCTACGGG-3′ (49–65 bp), in which the sequence from position −19 to 3 bp was included; DN2-E4, 5′-AGAGGGCTGCAGTACAAGTATC-3′ (139–160 bp); and DN2-E2, 5′-TCTTCCATGCGTGGGCACAC-3′ (322–341 bp). Reverse primers were: DN2-E1, 5′-GCCCACGCATGGAAGAGGCCCCGGGGGGAC-3′ (34–48 bp), in which the sequence from position 322 to 337 bp was included so as to introduce the juxtaposition of amino acids A^{16} and S^{108}; DN2-E3, 5′-GTACTGCAGCCCTCTGGCCCCGGCGGGGAG-3′ (34–48 bp), in which the sequence from position 139 to 160 bp was included so as to introduce the juxtaposition of amino acids A^{16} and R^{47}; DN2-E5, 5′-AAAGCGGCCGCTTAGTCCTGAGCCTTGCTGGG-3′ (304–321 bp) and DN2-E6, 5′-AAAGCGGCCGCTTAACTGCGCCGCCTCCCCGGACC-3′ (119–138 bp), which included the translation termination codon. The deletion mutants ΔM^{1}–A^{16} (ΔS), ΔS^{108}–I^{360} (ΔM) and ΔR^{47}–I^{360} (Δ(P+M)), which lacked part of the signal peptide (S), the mature protein (M), and the proprotein (P)/M, respectively, were produced using the following combinations of primers: DN2-D7/C5, DN2-N6/E5, and DN2-N6/E6, respectively. Splicing by the overlap extension method [13] was also employed to construct another three deletion mutants, ΔL^{17}–D^{107} (Δ(PT+P)), ΔL^{17}–Q^{46} (ΔPT) and ΔL^{17}–Q^{46}/S^{108}–I^{360} (Δ(PT+M)), which lacked part of the propeptide (PT)/P region, PT and PT/M, respectively. This process used the three primer sets DN2-N6/E1/E2/C6, DN2-N6/E3/E4/C6 and DN2-N6/E3/E4/E5, respectively. Each amplified fragment was cloned into the expression vector pcDNA3.1(+). All the mutants were verified by restriction mapping and partial DNA sequencing. Plasmid DNAs for transfection were purified using the Plasmid Midi Kit (Qiagen, Chatsworth, CA).

2.5. Transfection of the expression vectors into COS-7 cells

COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 1 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% (v/v) fetal calf serum (Gibco BRL) at 37°C under 5% CO_2. The cells were transiently transfected using LipofectaminPlus reagent (Gibco BRL) according to the manufacturer's instructions. Transfection efficiencies were estimated by cotransfecting with the pSV-β-galactosidase vector (Promega, Madison, WI) and subsequently assaying aliquots of cell lysates for β-D-galactosidase activity. Two days after transfection, the medium was recovered and cell lysates were prepared by performing five freeze-thaw cycles in 0.25 M Tris-HCl (pH 7.5) containing 1 mM PMSF. DNase II activities in both the cell lysates and medium were assayed by the SRED method.

3. Results and discussion

3.1. Purification of DNase II from human liver

The seven-step purification procedure resulted in approximately 2300-fold purification with a 10% yield, and about 700 μg DNase II was obtained from the whole human liver (Table 1). Human DNase II has previously been only partially purified from the gastric mucosa, uterine cervix [14], urine [1] and lymphoblasts [15]. Therefore, this is the first report of the purification of DNase II to apparent homogeneity from a human source. When the enzyme was subjected to SDS-PAGE after denaturation by dithiothreitol (DTT) at 100°C, only two bands were detected at corresponding positions by both silver staining and immunostaining (Fig. 1). The molecular masses of these proteins were estimated to be 8–10 kDa and 32 kDa. However, without denaturation, the purified enzyme yielded only a single 45 kDa band (Fig. 1). These find-

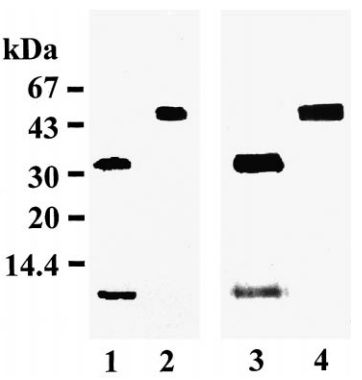

Fig. 1. SDS-PAGE patterns of purified human DNase II, with and without DTT treatment. The purified enzyme (about 5 μg, lanes 1 and 2; 2 μg, lanes 3 and 4) was either heated to 100°C in the presence of 25 mM DTT (lanes 1 and 3) or not exposed to heat/DTT (lanes 2 and 4), and was then subjected to SDS-PAGE on a 15% gel according to the method of Laemmli [11]. Afterwards, the proteins were visualized by silver staining (lanes 1 and 2) or immunostaining with anti-human DNase II (lanes 3 and 4).

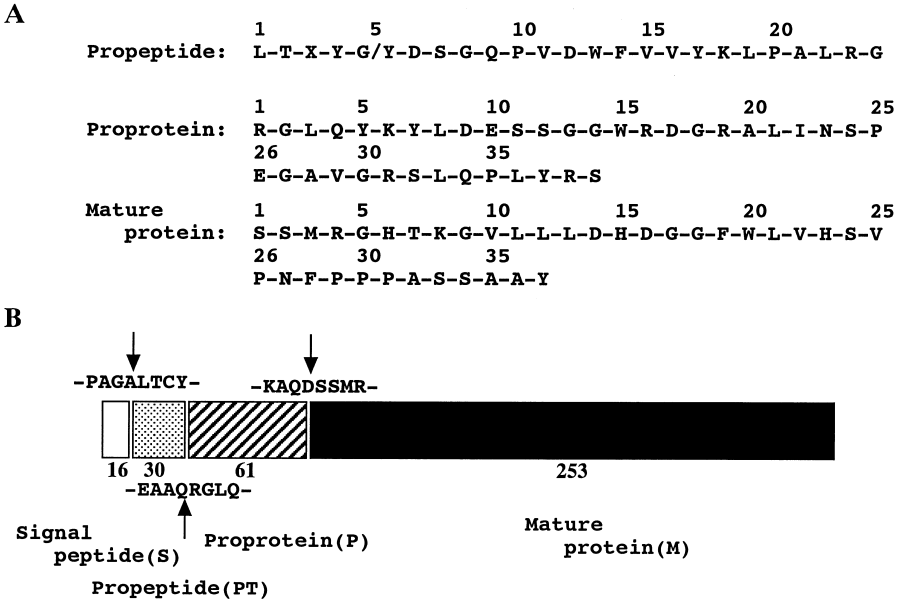

Fig. 2. N-terminal amino acid sequences of the three non-identical subunits constituting mature DNase II (A) and the molecular organization of the enzyme (B). A: The N-terminal amino acid sequences of each subunit are shown. Although the 5th residue of the propeptide may be identical to the corresponding ones of the proprotein or mature protein, we assumed the 5th residue of the propeptide to be Gly, based on our cDNA data. X indicates an undetermined position. B: The structural organization of human DNase II, as determined in this study, is shown. The signal peptide (M^1–A^{16}), propeptide (L^{17}–G^{46}), proprotein (R^{47}–D^{107}) and mature protein (S^{108}–I^{360}) of the enzyme are indicated by open, dotted, hatched and solid boxes, respectively. The numbering of the amino acid residues for each subunit is indicated below the corresponding segment. The amino acid sequences surrounding putative processing sites are shown above or below each site and arrows designate the precise processing site within the indicated sequence.

ings suggest that human DNase II is composed of non-identical subunits.

3.2. Identification of the three non-identical subunits constituting human DNase II

We have previously determined the N-terminal amino acid sequence of the 32 kDa portion of human DNase II, up to the 30th residue [7]. However, the structures of the other components remained to be determined. Edman degradation of the 8–10 kDa and 32 kDa components separated by SDS-PAGE allowed the N-terminal amino acid sequences to be identified up to the 39th and 37th residues, respectively (Fig. 2A). The 8–10 kDa component was initially predicted to be the L^{17}–D^{107} region of DNase II; however, its N-terminal sequence as determined chemically was R^{47}–S^{85}, and this exactly matched the sequence deduced from the cDNA data. Therefore, the Q^{46}–R^{47} bond of DNase II was demonstrated to be an additional cleavage site involved in proteolytic processing (Fig. 2B). In order to ascertain whether the L^{17}–Q^{46} portion was associated with the mature protein, the intact purified enzyme was directly subjected to Edman degradation. Three different phenylthiohydantoin-amino acid derivatives were detected during each degradation cycle. These contained the N-termini of the three components, two of which were identified as the 8–10 kDa and 32 kDa components. The N-terminus of the other component was determined up to the 24th residue and

Table 2
Catalytic activities of DNase II deletion mutants expressed in COS-7 cells

Transfected construct	DNase II activities ($\times 10^4$)	
	Cell lysates (units/μg protein)	Medium (units/μl)
Control (non-transfected)	15.7 ± 3.50	3.68 ± 0.383
pcDNA-DN2	185 ± 38.1	43.5 ± 6.86
pcDNA-DN2anti	11.5 ± 2.65	3.71 ± 0.375
ΔSP	18.7 ± 0.975	3.33 ± 0.512
ΔPT	16.6 ± 7.17	3.84 ± 0.256
Δ(PT+P)	15.1 ± 4.36	3.69 ± 0.431
ΔM	17.6 ± 5.87	3.64 ± 0.358
Δ(P+M)	16.2 ± 5.41	3.96 ± 0.384
Δ(PT+M)	15.5 ± 3.45	3.46 ± 0.360
Δ(PT+P)/ΔM	53.3 ± 4.66	10.5 ± 0.383
Δ(PT+P)/Δ(PT+M)	16.2 ± 1.10	3.83 ± 0.371
ΔPT/Δ(P+M)	14.6 ± 3.88	3.18 ± 0.103
Δ(PT+P)/Δ(P+M)/Δ(PT+M)	14.0 ± 2.45	3.48 ± 0.151
pcDNA3.1(+)	14.7 ± 5.51	3.76 ± 0.384

A series of DNase II deletion mutants were constructed by using RT-PCR as described in the text. Cell lysates were prepared from COS-7 cells transfected with the various mutants. These were assayed for DNase II and β-D-galactosidase activities and protein contents. The DNase II activities of the culture media were also determined. Values are means ± S.D. of determinations for at least five transfections.

corresponded to the L^{17}–G^{40} portion of DNase II. These findings show that mature DNase II is composed of three non-identical subunits derived from a single DNase II precursor, which is encoded by a single gene (Fig. 2B). Our experimental results, obtained from a combination of cDNA and chemical analyses, strongly indicate that mammalian DNase II exhibits a three-chain structure formed by three non-identical polypeptides. In agreement with these results, during the preparation of this article, porcine spleen DNase II was reported to consist of three non-identical subunits [16].

3.3. Expression of human DNase II cDNA and its deletion mutants in COS-7 cells

The entire coding region was cloned into a mammalian expression vector (pcDNA3.1-DN2) and transiently expressed in COS-7 cells (Table 2). The DNase II activities of both the lysates and the culture medium from the transfected cells increased 12-fold, compared with activity levels in non- and mock-transfected cells containing endogenous DNase II, whereas no significant enhancement of activity was observed in the cells transfected with the antisense construct (pcDNA3.1-DN2anti). These elevations in activity were completely abolished by the anti-human DNase II antibody. The analyses of cDNA expression indicated that it encoded human DNase II, and that the enzyme was in part diverted to the secretory pathway. Some lysosomal enzymes are known to exit the cell via the constitutive secretory pathway [17].

A series of deletion mutants derived from the human DNase II cDNA were constructed and expressed in COS-7 cells (Table 2). To allow correct intracellular sorting and processing of lysosomal enzymes, these proteins would be expected to need the signal peptide and potential *N*-glycosylation sites in which the carbohydrate could be phosphorylated [17,18]. However, the cells transfected with the ΔS construct showed no significant elevation of DNase II activity in their lysate or culture medium. Therefore, the signal peptide was indispensable for the expression of intact DNase II, as well as other lysosomal enzymes. None of the cells transfected with the four deletion mutants Δ(PT+P), ΔM, Δ(P+M) or Δ(PT+M) showed any increase in enzyme activity. These findings indicate that the individual subunits (PT, P and M) exhibit no catalytic activity. The propeptide regions of cathepsin L [19] and β-hexosaminidase [20] have been reported to be essential for the folding process. The cells transfected with the ΔPT construct, as well as those transfected with the three sets of constructs ΔPT/Δ(P+M), Δ(PT+P)/Δ(P+M)/Δ(PT+M) and Δ(PT+P)/Δ(PT+M), showed no increase in the enzyme activity of either their cell lysates or culture media. On the other hand, the cells transfected with Δ(PT+P)/ΔM exhibited a significant increase in activity which was completely abolished by the antibody. When the effect of the amount of ΔM cotransfected with a constant amount of Δ(PT+P) on DNase II expression was examined, activity increased in parallel with the amount of ΔM. This increase occurred up to an approximately equivalent molarity, then reached a plateau at high amounts, indicating a stoichiometric association between these components (data not shown). These findings suggest that, although a single large DNase II precursor (PT+P+M) may not be necessary for proteolytic maturation, the PT region L^{17}–Q^{46} may play an essential role in generating the active form of the enzyme.

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