Comparative characterization of rat deoxyribonuclease 1 (Dnase1) and murine deoxyribonuclease 1-like 3 (Dnase1l3)

Markus NAPIREI[1], Swantje WULF, Dirk EULITZ, Hans Georg MANNHERZ and Thomas KLOECKL

Abteilung für Anatomie und Embryologie, Medizinische Fakultät, Ruhr-Universität Bochum, Universitätsstrasse 150, D-44780 Bochum, Germany

Deoxyribonuclease 1 (DNASE1, DNase I) and deoxyribonuclease 1-like 3 (DNASE1L3, DNase γ, DNase Y, LS-DNase) are members of a DNASE1 protein family that is defined by similar biochemical properties such as Ca^{2+}/Mg^{2+}-dependency and an optimal pH of about 7.0 as well as by a high similarity in their nucleic acid and amino acid sequences. In the present study we describe the recombinant expression of rat Dnase1 and murine Dnase1l3 as fusion proteins tagged by their C-terminus to green fluorescent protein in NIH-3T3 fibroblasts and bovine lens epithelial cells. Both enzymes were translocated into the rough endoplasmic reticulum, transported along the entire secretory pathway and finally secreted into the cell culture medium. No nuclear occurrence of the nucleases was detectable. However, deletion of the N-terminal signal peptide of both nucleases resulted in a cytoplasmic and nuclear distribution of both fusion proteins. Dnase1 preferentially hydrolysed 'naked' plasmid DNA, whereas Dnase1l3 cleaved nuclear DNA with high activity. Dnase1l3 was able to cleave chromatin in an internucleosomal manner without proteolytic help. By contrast, Dnase1 was only able to achieve this cleavage pattern in the presence of proteases that hydrolysed chromatin-bound proteins. Detailed analysis of murine sera derived from *Dnase1* knockout mice revealed that serum contains, besides the major serum nuclease Dnase1, an additional Dnase1l3-like nucleolytic activity, which, in co-operation with Dnase1, might help to suppress anti-DNA autoimmunity by degrading nuclear chromatin released from dying cells.

Key words: DNA degradation, DNase I, DNase γ, DNASE1, DNASE1-like 3 (DNASE1L3), nuclease.

INTRODUCTION

Deoxyribonuclease 1 (DNASE1, DNase I, EC 3.1.21.1) is a secretory endonuclease first isolated from bovine pancreas extracts in 1905 [1]. It displays Ca^{2+}- and Mg^{2+}/Mn^{2+}-dependency, an optimal pH at about 7.0, and cleaves double-stranded DNA into 3′-OH/5′-phospho tri- and/or tetra-oligonucleotides [2]. Monomeric actin, Zn^{2+}, as well as chelators of bivalent cations, are known inhibitors of this nuclease [2,3]. The pI of the enzyme is about 4.8. DNASE1 is secreted into most body fluids by a variety of endocrine and exocrine glands, especially those of the gastrointestinal and urogenital tract [4–6]. Its physiological functions are supposed to be the degradation of dietary DNA within the intestinal tract to supply the organism with oligonucleotides and, in addition, to suppress anti-DNA autoimmunity, most probably by degrading chromatin released from dying cells [2,7,8]. Furthermore, DNASE1 was believed to facilitate apoptotic DNA fragmentation in a variety of tissues [3]. However, so far this assumption is not supported by experiments employing *Dnase1* wild-type and knockout mice (M. Napirei and H. G. Mannherz, unpublished work).

Recently, three additional members of a *DNASE1* gene and protein family displaying high similarity in their nucleotide and amino acid sequences to DNASE1 were discovered. These genes were termed deoxyribonuclease 1-like 1, 2 and 3 by Rodriguez et al. [9] (*DNAS1L1–3*). In accordance with the gene and protein nomenclature [10,11] we will term these genes *DNASE1L1–DNASE1L3* in this paper. These genes and their gene products were discovered and characterized in different species, therefore several synonyms were proposed for the same gene and protein. A detailed comparative biochemical analysis of the human DNASE1 protein family was performed by Shiokawa and Tanuma [12], who transiently expressed all four members in HeLa S3 cells.

The first member of the DNASE1 protein family besides the original DNASE1 is DNASE1L1 (DNASE1-like 1), which was first discovered by Parrish et al. in 1995 and termed 'DNL1L' (human DNase I lysosomal-like) [13]. Synonyms for the human protein are Xib [14] and DNase X [15]. The *DNASE1L1* gene is located on the human chromosome Xq28 and was described as being mainly expressed in heart and skeletal muscle [13–15]. Concerning its biochemical properties contradictory data were published. One report described it as an acidic, ion-independent endonuclease [16]. It was also reported that an 18 kDa amino-terminal fragment of the recombinant protein displayed the typical DNASE1-like biochemical properties [17]. In contrast, Los et al. [15] described how the mature 35 kDa DNASE1L1 (DNase X) protein exhibited typical DNASE1-like biochemical properties, and this was also verified by Shiokawa and Tanuma [12].

The second member of the *DNASE1* gene family is the human *DNASE1L2* gene, which was first described by Rodriguez et al. [9] in 1997. They mapped the gene to the chromosomal region 16p13.3, where the human *DNASE1* gene is also located, and verified *DNASE1L2* gene expression in the brain by RT-(reverse transcription) PCR analysis [9]. Recombinant expression of *DNASE1L2* in HeLa S3 cells revealed that the enzyme was (the first known) bivalent-cation-dependent nuclease with acidic pH optimum, biochemical properties which contrast with those typical of DNASE1 [12,18].

The third and best characterized relative of DNASE1 is DNASE1L3 {DNAS1L3 (human) [9], nhDNase (novel human DNase) [19], DNase Y (rat) [20] and LS-DNase (human and

Abbreviations used: DNASE1, deoxyribonuclease 1; DNASE1L3, deoxyribonuclease 1-like 3; GFP, green fluorescent protein; LEC, bovine lens epithelial cells; NLS, nuclear localization signal; PAI-1, plasminogen activator inhibitor 1; rER, rough endoplasmic reticulum; rhDNASE1, recombinant human deoxyribonuclease 1; *ΔSP-Dnase1*, cDNA of *Dnase1* lacking the nucleotide sequence for the N-terminal signal peptide comprising the first 21 amino acids; SRED, single radial enzyme diffusion; RT-, reverse transcription.

[1] To whom correspondence should be addressed (markus.napirei@ruhr-uni-bochum.de).

murine liver/spleen DNase) [21]}. It was first isolated in 1994 from nuclei of rat thymocytes beside two further nuclease activities (DNase α and β) and therefore termed 'DNase γ' [22]. Dnase1l3 has a molecular mass of 32 kDa, a basic pI of 9.5 and a pH optimum of about 7. It is an endonuclease and cleaves double-stranded DNA (and single-stranded DNA [20]) producing 3′-OH/5′-P ends, is Ca^{2+}/Mg^{2+}-dependent and inhibited by Zn^{2+}, but not by monomeric actin [12,20]. Expression of the *DNASE1L3* gene was verified for the spleen, liver, thymus, lymph node, bone marrow, small intestine and kidney by the RNA dot-blot technique [12]. At the cellular level, *DNASE1L3* gene expression was verified for spleen macrophages, kidney, thymus and intestine, as well as for hepatic Kupffer cells, by RNA *in situ* hybridization [21]. Since DNASE1L3 cleaves nuclear chromatin internucleosomally, it has been suggested to be involved in apoptotic DNA fragmentation of thymocytes [23,24], neuronal PC12 cells [25], C2C12 myoblasts [26] and of cell lines expressing the recombinant nuclease [27–29]. It has been suggested that its activity during cell death is regulated by poly(ADP-ribos)ylation [27–29]. Although DNASE1L3 contains an N-terminal signal peptide for its translocation into the rER (rough endoplasmic reticulum), it was described as occurring in the nucleus of non-apoptotic cells and to be chromatin-bound [20,22,25]. This was explained by the occurrence of two functional NLSs (nuclear localization signals) in the mature protein [12,30]. However, others have shown that this nuclease is secreted into the cell culture medium of cells transfected with a *DNASE1L3* expression vector [21].

Here we report a comparative characterization of rat Dnase1 and murine Dnase1l3 fused or not fused to GFP (green fluorescent protein) and transiently expressed in murine NIH-3T3 fibroblasts and LEC (bovine lens epithelial cells). Our results show that both nucleases follow the entire secretory pathway and are not detectable in the nucleus. The secreted nucleases exhibited typical DNASE1-like biochemical properties. However, their substrate preferences were quite different. Whereas Dnase1l3 cleaved chromatin with high, and plasmid DNA with low, activity, Dnase1 exhibited the opposite properties. Furthermore we found that Dnase1l3 cleaved chromatin internucleosomally. In contrast, a high proportion of Dnase1 cleaved it only randomly and needed proteolytic help to achieve internucleosomal chromatin cleavage. We hypothesize that these cleavage and substrate differences result from the extremely different pI values of both enzymes. We also observed that serum of some *Dnase1* knockout mice contained a residual nuclease activity that displayed the characteristics of Dnase1l3.

MATERIALS AND METHODS

Sequence data

The following protein sequences, deposited in the SwissProt database, were used in this work: murine Dnase1 (P49183), rat Dnase1 (P21704), human DNASE1 (P24855), murine Dnase1l3 (O55070), rat Dnase1l3 (O89107) and human DNASE1L3 (Q13609). Furthermore, the following DNA sequences deposited at GenBank® were used in the present work: rat *Dnase1* (X56060) and murine *Dnase1l3* (U00478). For protein and DNA sequence analysis, the DNAstar Lasergene software (DNASTAR Inc., Madison, WI, U.S.A.) was applied.

Animals and serum extraction

Dnase1 knockout (*Dnase1*$^{-/-}$) mice of the inbred strain C57Bl/6 and the outbred strain CD-1 (ICR) were generated by backcrossing 129 × C57Bl/6 F2 mice [7] with wild-type mice of the appropriate strain at least ten times. Animals were bred in our animal facility, were allowed free access to standard laboratory chow and water and were kept on a 12 h:12 h light/dark cycle. Mouse blood was collected from diethyl ether-anesthetized animals by cardiac puncture. After coagulation for 12 h at 4 °C, the blood was centrifuged for 10 min at 600 ***g*** and the serum was transferred into a fresh tube. All animal procedures carried out in this work received prior approval by the local animal protection committee and were in agreement with the Tierschutzgesetz, the German Animal Protection Law.

Cloning of rat *Dnase1* and murine *Dnase1l3* expression vectors

For cloning a rat *Dnase1–GFP* expression vector, the available rat *Dnase1* cDNA cloned into the vector pBSIIKS+ (Stratagene, Amsterdam, Netherlands) [31] was used as a template for amplification of the *Dnase1* cDNA. The complete rat *Dnase1* cDNA was amplified using the N-terminal primer 5′-CCATCTGAGTGGAATTCAGGATGAGG-3′ (primer I) containing an EcoRI restriction site generated by site-directed mutagenesis and the C-terminal primer 5′-TCAATGACATGATATCTTTCTGAGTG-3′ (primer II) containing an EcoRV restriction site as well as a mutated stop codon generated again by site-directed mutagenesis. The *Dnase1* cDNA amplification product was subsequently cloned into the EcoRI/SmaI restriction sites of the vector pEGFP-N3 (Clontech Laboratories Inc., Heidelberg, Germany). For cloning of the rat *Dnase1* cDNA lacking the N-terminal signal peptide comprising the first 21 amino acids (*ΔSP-Dnase1*), we used the forward N-terminal primer 5′-CAACCTGCTGAATTCGGCTATGACTCTGAGAATTGCAGC-3′ (primer III) containing an EcoRI restriction site and a translation start codon generated by site-directed mutagenesis. The C-terminal primer II and the cloning strategy were identical with those used in the cloning of the complete rat *Dnase1* cDNA. For expression of rat *Dnase1* and *ΔSP-Dnase1* without the *GFP* fusion tag, the complete *Dnase1* cDNA was amplified by PCR using primer I and the C-terminal primer 5′-ATGGTGGCTCTAGATCACATCTTTCT-3′ (primer IV) containing a translation stop codon generated by site-directed mutagensis. The rat *ΔSP-Dnase1* cDNA was amplified by PCR using primer II and primer IV. Both cDNAs were subcloned into the vector pBSIIKS+, isolated by EcoRI/NotI cleavage and cloned into the vector pEGFP-N1, from which the *GFP* cDNA was eliminated by EcoRI/NotI cleavage.

The murine *Dnase1l3* cDNA was cloned using spleen cDNA as a template for amplification of the *Dnase1l3* cDNA by PCR. The cDNA was generated by isolation of total RNA using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcription of 2 μg of RNA employing the Omniscript RT Kit from Qiagen and oligo(dT) primers (12–18 nts, Sigma Aldrich, Deisenhofen, Germany). RT-PCR of the *Dnase1l3* cDNA was performed using the N-terminal primer 5′-GAAGTCCCAGGAATTCAAAGATGT-3′ (primer V) and the C-terminal primer 5′-GCGTGATACCCGGGAGCGATTG-3′ (primer VI) containing an EcoRI and SmaI restriction site generated by site-directed mutagenesis. The *Dnase1l3* cDNA amplification product was subsequently cloned into the EcoRI/SmaI restriction sites of the C-terminal GFP protein fusion vector pEGFP-N1 (Clontech). For cloning the murine *Dnase1l3* cDNA lacking the N-terminal signal peptide comprising the first 25 amino acids, spleen cDNA was employed in RT-PCR using the N-terminal primer 5′-CTGGCCATGAGGCTCTGCTCC-3′ (primer VII) containing a translational start codon generated by site-directed mutagenesis and the C-terminal primer VI. The PCR product was subcloned into the vector pBSIIKS+, isolated by EcoRI/SmaI cleavage, and cloned into the EcoRI/SmaI restriction sites of the vector pEGFP-N1. For the expression of murine *Dnase1l3* and *ΔSP-Dnase1l3* without the

GFP fusion tag, the complete *Dnase1l3* cDNA was amplified by PCR using primer V and the C-terminal primer 5′-GCGTGATAC-CTAGGAGCGATTG-3′ (primer VIII) containing a translational stop codon generated by site-directed mutagenesis. The murine *ΔSP-Dnase1l3* cDNA was amplified using primer VII and VIII. Both cDNAs were subcloned into the vector pBSIIKS+, isolated by EcoRI/NotI cleavage and cloned into the vector pEGFP-N1 from which the *GFP* cDNA was eliminated by EcoRI/NotI cleavage.

Cell transfection

Cultivation of NIH-3T3 fibroblasts (ACC59) was performed according to the instructions of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms) (Braunschweig, Germany). LEC were prepared as described previously [32]. Transient transfection of the cells with expression vectors coding for GFP-fusion proteins was performed using the PolyFect Transfection Reagent and protocol (Qiagen). For subcellular localization of the Golgi apparatus, a pECFP-Golgi expression vector supplied by Clontech coding for a cyan fluorescent Golgi marker protein was co-transfected. For generatting cell extracts and collecting cell-culture supernatants, 3×10^5 cells were seeded in six-well plates 1 day before transfection. Transient gene expression was allowed to take place for 24 h. The cells were then scraped off, collected by centrifugation and lysed in 300 μl of SDS sample buffer (see below) without dithiothreitol for producing denaturing SDS/PAGE zymograms or in 300 μl of TET-buffer (10 mM Tris/HCl/1 % (v/v) Triton-X100/ 10 mM EDTA, pH 7.5) for producing native PAGE zymograms or performing plasmid- DNA-digestion assays or SRED (single radial enzyme diffusion) assays. For microscopical investigation of transfected cells, 10^5 cells were seeded on coverslips, transfected and washed with PBS after transient gene expression for 24 h. The cells were fixed for 20 min with 4 % (v/v) paraformaldehyde dissolved in PBS, washed in water and mounted on a microscope slide. Subsequently the cells were examined with an Axiophot fluorescence microscope and photographed with an AxioCam HRc digital camera (Zeiss, Göttingen, Germany). Confocal laser scanning microscopy was performed using a Zeiss LSM 510 microscope.

Immunoblotting

Western blots were performed on homogenates of 3×10^5 cells transiently transfected with the different expression vectors described above. The cells were scraped off the culture dishes, collected by centrifugation (600 ***g***, 10 min), washed with PBS and lysed in 300 μl of SDS sample buffer [50 mM Tris/HCl/2 % (w/v) SDS/10 % (v/v) glycerol/100 mM dithiothreitol/0.1 % (w/v) Bromophenol Blue, pH 6.8]. Cells were homogenized by rotor-stator treatment [Ultra-Turrax® T8 homogenizer (IKA Labortechnik, Staufen, Germany) at maximal power (level 6)] for 30 s. Subsequently the samples (25 μl) were subjected to a SDS/12%-(w/v)-PAGE, followed by protein transfer on to a PVDF membrane using the Biometra (Göttingen, Germany) semi-dry Fastblot™ system. Immunodetection of proteins on the blotting membranes using the alkaline phosphatase protocol with 5-bromo-4-chloroindol-3-yl phosphate and NitroBlue Tetrazolium as detection substrates was performed as described by Sambrook et al. [33]. As primary antibodies, a polyclonal rabbit anti-GFP antibody (8367-2; BD Biosciences Clontech, Heidelberg, Germany) was employed in a dilution of 1:200. As a secondary antibody, a chicken anti-(rabbit Ig) antibody conjugated with alkaline phosphatase (sc-2967; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) was employed at a dilution of 1:2000.

Nuclease assays

Nuclease zymograms

Native PAGE zymograms and denaturing SDS/PAGE zymograms employing cell-culture supernatants and cell extracts of cells transfected with the different *Dnase1* and *Dnase1l3* expression vectors were produced as described previously [5,7].

Plasmid-DNA digestion assay

To examine the nucleolytic activities of cell-culture supernatants, different dilutions of the supernatants were added to 20 μl of substrate solution (100 ng of pDNA pBSIIKS+ vector dissolved in 20 mM Tris/HCl, pH 7.5, containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$) and incubated at 37 °C for 30 min. Thereafter, the samples were heated to 65 °C for 5 min and subjected to TAE (Tris/acetate/EDTA)/1 %-(w/v)-agarose-gel electrophoresis. The DNA was visualized as described above.

Nuclear-DNA degradation assay

Isolation of MCF-7 cell nuclei was performed as previously described [5]. The cell nuclei were treated with either cell culture supernatants (different amounts) of cells transfected with the *Dnase1* or *Dnase1l3* expression vectors or with 5 % (v/v) serum derived from *Dnase1* wild-type or knockout CD-1 (ICR) and C57Bl/6 mice [8]. Some reaction samples additionally contained either 15 ng/ml murine PAI-1 (plasminogen activator inhibitor 1; Calbiochem Novabiochem, Schwalbach, Germany), 250 i.u./ml heparin (Liquemin©, Hoffmann–La Roche, Grenzach Wyhlen, Germany), different amounts of rhDNASE1 (recombinant human DNASE1; Pulmozym©; Hoffmann–LaRoche), 5 or 10 units/ml thrombin, or 100 units/ml aprotinin (both supplied by Sigma–Aldrich, Deisenhofen, Germany). The degradation assays contained 10^5 cell nuclei diluted in 200 μl of reaction buffer (10 mM Hepes/50 mM NaCl/2 mM $MgCl_2$/2 mM $CaCl_2$/40 mM β-glycerophosphate, pH 7.0). Subsequent to an incubation step at 37 °C, the nuclear DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen) and the DNA was analysed by 1.5%-(w/v)-agarose-gel electrophoresis.

SRED assay

Detection of nuclease activities in cell-culture supernatants was furthermore performed by the SRED assay [34]. For one SRED gel (9 cm^2), 5.1 ml of reaction buffer (0.2 M sodium cacodylate/ 4 mM $MgCl_2$/4 mM $CaCl_2$, pH 6.5), 2.7 ml of calf thymus DNA (2 mg/ml dissolved in water), 54 μl of aq. Ethidium bromide (10 mg/ml) and 2.9 ml of water were mixed and warmed to 55 °C. Agarose was melted in water at a concentration of 2 % (w/v), and 10.8 ml was added to the pre-warmed mixture with stirring at 55 °C. Thereafter the solution was poured on a preheated glass plate, and a hole was punched in the gel while it was solidifying. Different amounts of supernatant were added into the punched wells, and the gel was incubated at 37 °C for 24 h. Thereafter the gel was photographed under UV light and the diameter of the circular dark zones formed around the punched holes as the result of DNA hydrolysis was used to calculate the nuclease activity [7].

RESULTS

Amino acid sequence comparison of DNASE1 and DNASE1L3 from different species

Several studies concerning the structural properties of DNASE1 have been made in the past by amino acid sequencing, crystallographic refinement or site-directed mutagenesis. Employing these

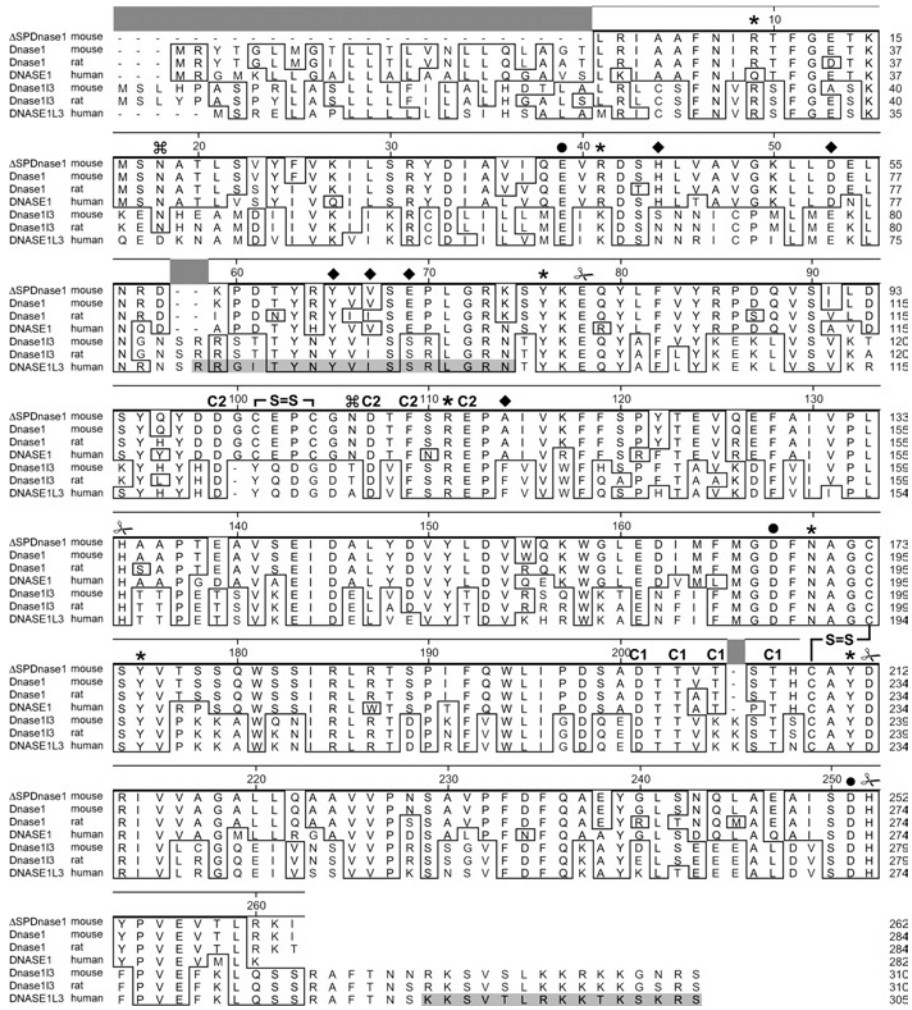

Figure 1 Amino acid sequence comparison of DNASE1 and DNASE1L3 from different species

Alignment of the amino acid sequences of murine, rat and human DNASE1 (SwissProt Accession numbers P49183, P21704 and P24855) and DNASE1L3 of all three species (O55070, O89107 and Q13609). Identical amino acids are boxed, and the numbering of each sequence is given on the right. Numbering at the top refers to the murine Dnase1 sequence of the mature protein (ΔSP-Dnase1). The first 21 (DNASE1) and 25 (DNASE1L3, 20 amino acids in the case of human DNASE1L3) N-terminal amino acids represent predicted signal peptides for the directed translocation into the rER. Two NLS sequences (shaded) were identified for the human DNASE1L3 protein [30]. The amino acids building the catalytic centre (✂, [35]) and chelating the Mg^{2+} ions in the catalytic centre (●, [35]) of the DNASE1 protein are highly conserved between DNASE1 and DNASE1L3. The same holds true for five of the seven residues identified as essential for DNA binding (*, [36]), the second disulphide bridge (S═S) and the two Ca^{2+}-ion-binding sites (C1/2, [37]) of DNASE1. Differences between DNASE1 and DNASE1L3 exist for the N-glycosylation sites of DNASE1 at Asn^{18} and Asn^{106} (⌘, [40]), the binding site for monomeric actin (◆, [38]) and for the first disulphide bridge (S═S) of DNASE1, which are not conserved in the DNASE1L3 proteins. In contrast with DNASE1, DNASE1L3 proteins contain an additional C-terminal domain of 21 amino acids rich in the basic amino acids lysine and arginine.

methods, the amino acids participating in DNA-, Ca^{2+}- and bivalent-metal-ion binding, as well as the essential residues of the catalytic centre, were discovered. Furthermore, the amino acids participating in actin binding and two disulphide bridges were identified. Figure 1 gives a comparative presentation of the murine, rat and human DNASE1 and DNASE1L3 amino acid sequences. All nucleases contain a predicted N-terminal signal peptide for the directed translocation of the polypeptide into the rER, which is 22 and 25 amino acids long in DNASE1 and DNASE1L3 respectively. The exception is human DNASE1L3, whose signal peptide is only 20 amino acids long. All nucleases share the same catalytic centre, which is composed of two histidine residues (His^{134} and His^{252}) and their hydrogen-bond partners (Glu^{78} and Asp^{212}) [35]. The acidic residues Glu^{39}, Asp^{168} and Asp^{251}, which may serve as chelators for bivalent cations within the catalytic centre, are also conserved, as also are five of the seven residues identified in human DNASE1 as essential for DNA binding (Tyr^{76}, Arg^{111}, Asn^{170}, Tyr^{175} and Tyr^{211}) [35,36]. Further structural features that are highly conserved are the two Ca^{2+}-binding sites and the essential disulphide bridge formed by Cys^{173} and Cys^{209} [37]. Sequence differences exist for the N-glycosylation sites at Asn^{18} and Asn^{106} and the actin-binding site of DNASE1 (His^{44}, Asp^{53}, Tyr^{65}, Val^{67}, Glu^{69} and Ala^{114} [38]), which are not conserved between DNASE1 and DNASE1L3 in all species analysed. Only the murine Dnase1l3 protein contains a N-glycosylation site at Asn^{283} of the mature protein. In contrast with DNASE1, the DNASE1L3 enzymes of all three species contain an additional C-terminal domain of 21 amino acids that is rich in the basic amino acids lysine and arginine. Shiokawa et al. [30] have shown that this region of the human enzyme contains a monopartite NLS. GFP fused to this domain (KKSVTLRKKTKSKRS; residues 291–305) was shown to translocate into the nucleus when recombinantly expressed. A second NLS, which resembles a classical bipartite NLS, was identified by these authors in the N-terminal region of human DNASE1L3 (RRGITYNYVISSRLGRK; residues 80–96). Again, fusion of this NLS to GFP resulted in accumulation

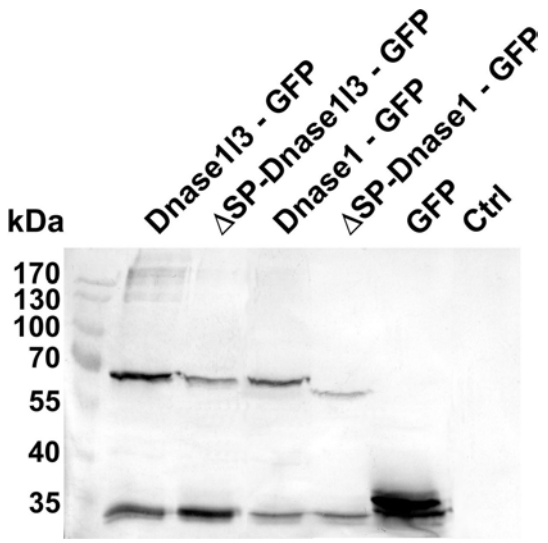

Figure 2 Detection of Dnase1– and Dnase1l3–GFP fusion proteins by Western-blot analysis

Expression of the different *Dnase1–* and *Dnase1l3–GFP* vectors used in the present study was verified by Western-blot analysis of extracts of NIH-3T3 cells using a polyclonal anti-GFP antibody. The N-terminally truncated Dnase1– and Dnase1l3–GFP fusion proteins lacking the N-terminal rER signal peptides of the nucleases were termed ΔSP-Dnase1–GFP and ΔSP-Dnase1l3–GFP respectively. All GFP fusion proteins were synthesized correctly, as evaluated by a postulated molecular mass of approx. 60–63 kDa (GFP ≈30 kDa; Dnase1, ≈30 kDa; Dnase1l3, ≈33 kDa). In addition to the fusion proteins, a protein smaller in size than GFP was detected in all transfection assays. This product seems to be a degradation product of GFP, since it was also detectable in cell transfections with GFP alone.

of GFP in the nucleus of transiently transfected cells. However, four further entries of the human DNASE1L3 protein sequence into the SwissProt database revealed that position 96 is likely not occupied by lysine but by asparagine. Therefore the functionality of this sequence as a NLS remains unsettled. A further and main difference between DNASE1 and its relative DNASE1L3 is their extremely different pI values. Whereas the rat mature Dnase1 has an acidic pI of 4.9, the murine mature Dnase1l3 has a basic pI of 8.9. Thus the charge of rat Dnase1 at pH 7.0 is −9.4, whereas it is +6.7 for the murine Dnase1l3 protein.

Dnase1 and Dnase1l3 are translocated into the rER

To investigate the subcellular localization of rat Dnase1 and murine Dnase1l3, we transiently transfected primary LEC or cells of the established NIH-3T3 fibroblastic line with *Dnase1–* and *Dnase1l3–GFP* expression vectors and investigated their expression after 24 h. LEC possess a large flat cell body and are therefore useful for subcellular-localization studies. Both nucleases contain a predicted N-terminal signal peptide for translocation into the rER. In the case of rat Dnase1 these are the first 21 amino acids and, in the case of murine Dnase1l3, they are the first 25. The transfection experiments were performed with expression vectors coding for GFP fusion proteins of either the complete nucleases or nucleases lacking the postulated N-terminal rER signal peptides (ΔSP-Dnase1–GFP and ΔSP-Dnase1l3–GFP). The nucleases were fused with their C-terminus to GFP. As proof of the correct synthesis of all fusion proteins, we performed a Western-blot analysis of extracts of transfected fibroblasts using a polyclonal anti-GFP antibody (Figure 2). Indeed, all GFP-fusion proteins were synthesized as evaluated by the correct molecular masses of approx. 57–63 kDa (GFP ≈27–30 kDa, Dnase1 ≈30 kDa, Dnase1l3 ≈33 kDa). However, the synthesis of the proteins lacking the N-terminal signal peptide was considerably lower, indicating that, for efficient synthesis the enzymes might need to be translocated into the rER. In addition, a GFP degradation product was discovered by immunoblotting in all transfections.

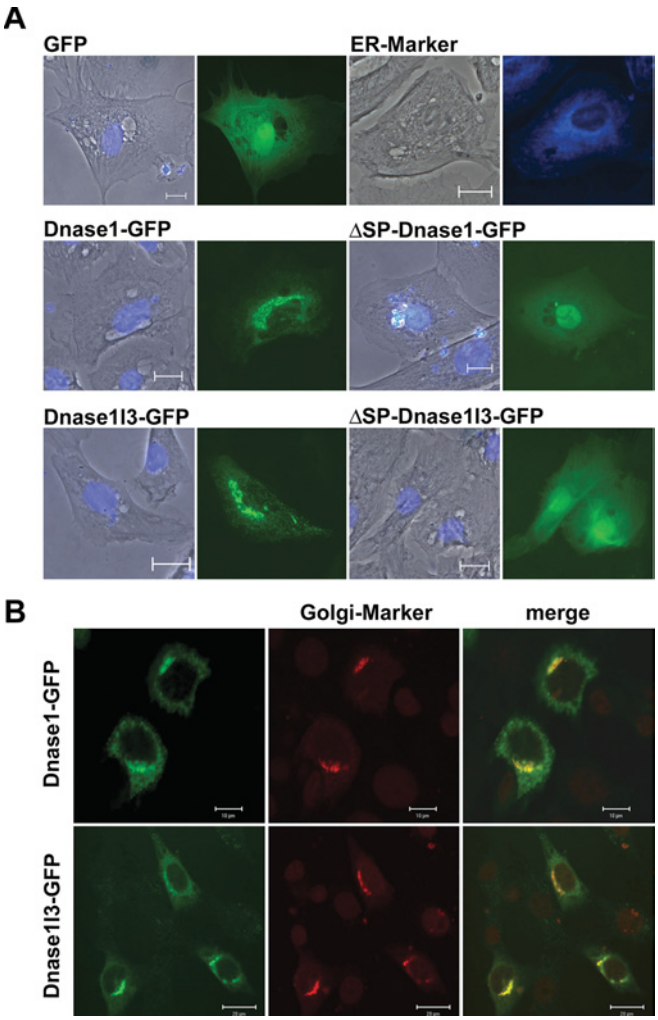

Figure 3 Subcellular localization of the Dnase1– and Dnase1l3–GFP fusion proteins

(**A**) Fluorescence microscopy of bovine LEC transiently transfected with *Dnase1–* or *Dnase1l3–GFP* expression vectors reveals a fluorescence staining of the rER and the Golgi apparatus located as a crescent near the nucleus. The morphology of the rER and Golgi apparatus in these cells was deduced from staining with a marker for the ER ('ER-Marker') and from co-transfection experiments using a vector coding for a Golgi marker protein (see **B**). Deletion of the first 21 (ΔSP-Dnase1–GFP) or 25 (ΔSP-Dnase1l3–GFP) N-terminal amino acids leads to cytoplasmic and nuclear distribution of the fusion proteins comparable with the cellular localization of the GFP protein alone. The bar represents 20 μm. (**B**) Confocal-laser-scanning micrographs of NIH-3T3 fibroblasts co-transfected with a *Dnase1-* or *Dnase1l3-GFP* expression vector in combination with an expression vector coding for a cyan fluorescent Golgi marker protein. The subcelluar localization of the fusion proteins was identical with that of transfected LEC (see **A**). Co-localization of the nuclease–GFP fusion proteins (green fluorescence) with the cyan fluorescent Golgi marker protein (red fluorescence) is demonstrated by the yellow merge of both fluorescence patterns and reveals that the crescent adjacent to the nucleus represents the Golgi apparatus. No nuclear occurrence of the nuclease–GFP fusion proteins was detectable.

Representative examples of transfected LEC investigated by standard fluorescence microscopy are presented in Figure 3(A). Our results reveal that both the Dnase1– and the Dnase1l3–GFP fusion proteins were translocated into the rER as deduced from the

staining pattern obtained by staining with an ER-tracking stain. Both nucleases appeared to be subsequently transported along the entire secretory pathway, as deduced from their localization within the Golgi apparatus (see below) and presumed secretory vesicles (punctate pattern for Dnase1l3–GFP in Figure 3A). Furthermore, both nuclease activities could be verified in cell-culture supernatants of the transfected cells (see below). No nuclear or perinuclear accumulation was observed for either nuclease fused to GFP. By co-localization studies using confocal laser scanning microscopy of NIH-3T3 fibroblasts transfected with the *Dnase1*– or *Dnase1l3–GFP* expression vectors in combination with a vector coding for a cyan fluorescent Golgi marker protein we were able to verify by their co-localization that the intensively stained subcellular crescent structure in close proximity to the nucleus of transfected LEC and NIH-3T3 cells represents the Golgi apparatus (Figure 3B). These results demonstrate again that both nucleases were directed into the secretory compartment under normal cellular physiological conditions. This was supported by the fact that elimination of the N-terminal signal peptide resulted in a cytoplasmic distribution and nuclear accumulation of both fusion proteins (Figure 3A) and a lack of nucleolytic activity in the cell-culture supernatants of the transfected cells (see below). Since Dnase1, by contrast with Dnase1l3 does not contain a postulated NLS, we suppose that the nuclear accumulation of both fusion proteins was not the result of a directed nuclear import. Instead we believe that the nuclear accumulation resulted from passive diffusion into the nucleus and subsequent binding to chromatin. However, in all transfections we observed, in parallel with the expected nuclease–GFP fusion proteins, a GFP degradation product (Figure 2). Assuming that this product still displays fluorescence, we cannot exclude the possibility that it may partly interfere with our localization studies. However, this GFP degradation product will not have influenced the fact that the nucleases initially possessing the signal peptide are directed into the secretory pathway under normal physiological conditions.

Biochemical properties of Dnase1 and Dnase1l3

For the comparative characterization of rat Dnase1 and murine Dnase1l3, which share 92 % identity, we transfected NIH-3T3 fibroblasts with expression vectors lacking the *GFP* nucleotide sequence. First, we performed plasmid-DNA digestion assays with different amounts of cell-culture supernatants of transfected cells and found that the supernatants contained elevated nuclease activity in comparison with supernatants of non-transfected control cells (Figure 4A). Interestingly we found that the supernatant of cells transfected with a *Dnase1* expression vector displayed a higher activity towards plasmid DNA in comparison to the supernatant of *Dnase1l3*-transfected cells. This might be caused either by higher transfection/expression efficiency or different substrate preferences of both nucleases. The supernatants of cells transfected with *ΔSP-Dnase1* and *ΔSP-Dnase1l3* expression vectors did not contain an enhanced nuclease activity in comparison with controls (results not shown). These experiments substantiate our previous findings that both nucleases have to translocate into the rER and to pass through the secretory pathway.

Next we defined the metal-ion dependence of both nucleases secreted into the cell-culture supernatants by employing the plasmid-DNA digestion assay. We found that both nucleases are Ca^{2+}- and Mg^{2+}/Mn^{2+}-dependent and inhibited by Zn^{2+} and chelators of bivalent cations such as EDTA and EGTA (Figure 4B). Interestingly, both nucleases displayed a higher activity in the presence of Mn^{2+} than of Mg^{2+}. In further experiments we analysed the nucleolytic activity of both enzymes in the presence of PAI-1 (plasminogen activator inhibitor 1), the serine protease inhibitor

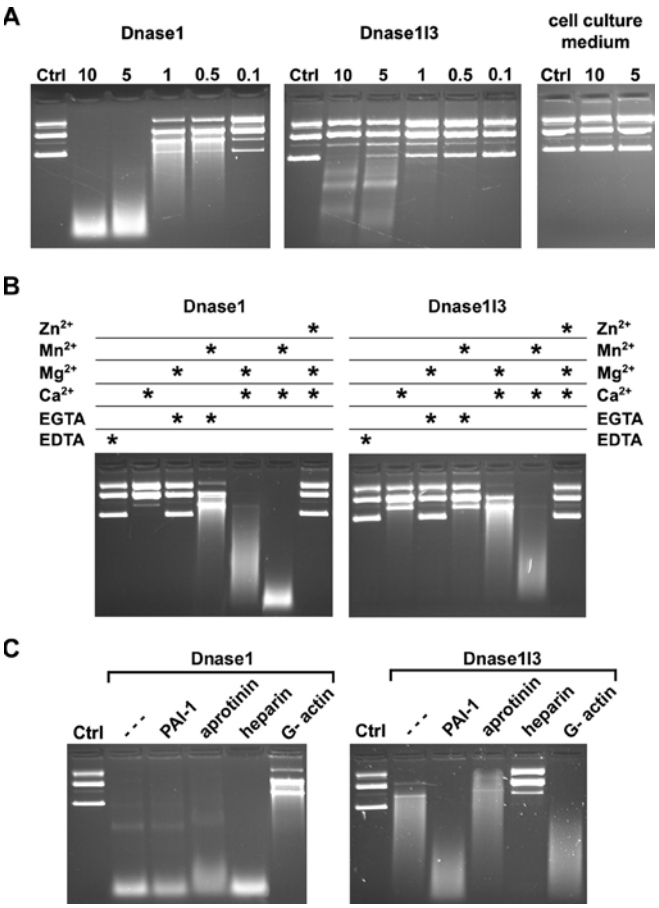

Figure 4 Properties of Dnase1 and Dnase1l3 in plasmid-DNA degradation assays

(**A**) Cell-culture supernatant of *Dnase1*-expressing cells displays a higher nucleolytic activity towards plasmid DNA than that of *Dnase1l3*-expressing cells. The cell-culture medium of non-transfected cells does not contain any nuclease activity. Values are in μl of supernatant employed (1 h of incubation). (**B**) The ion-dependence of Dnase1 (5 μl of supernatant, 0.5 h of incubation) and Dnase1l3 (10 μl of supernatant, 3 h of incubation) were the same. Both nucleases are Ca^{2+}- and Mg^{2+}/Mn^{2+}-dependent. However, in the presence of Ca^{2+} and Mn^{2+}, the nucleolytic activity of both nucleases is enhanced in comparison with Ca^{2+} and Mg^{2+}. (**C**) In contrast with Dnase1 (5 μl of supernatant, 0.5 h of incubation), Dnase1l3 (10 μl of supernatant, 3 h of incubation) was inhibited by the addition of the anticoagulant heparin (250 units/ml), whereas Dnase1 was inhibited by monomeric ADP-ribosylated α-actin (G-actin, 0.25 mg/ml). The serine-protease inhibitor aprotinin (0.5 units/ml) and PAI-1 (0.13 mg/ml) had no inhibitory effect on either nuclease. Abbreviation: Ctrl, plasmid DNA incubated without any medium.

aprotinin, the anticoagulant heparin as well as skeletal muscle α-actin (G-actin), ADP-ribosylated in order to maintain it monomeric even at high ionic strength. Employing the plasmid-DNA digestion assay, we found that heparin selectively inhibited Dnase1l3, whereas G-actin, as predicted from their sequence comparison, only inhibited Dnase1 (Figure 4C). A slight activation of Dnase1l3 by addition of PAI-1 and G-actin was detectable. The reason for this observation remains unknown.

Comparative analysis of Dnase1 and Dnase1l3 activity by different nuclease assays

We also attempted to detect the secreted nucleases by nuclease assays other than the plasmid-DNA digestion assay. To this aim, we employed cell-culture supernatants and cell extracts in three further detection systems. First we employed the SRED assay (Figure 5A). However, only Dnase1 was detectable by this

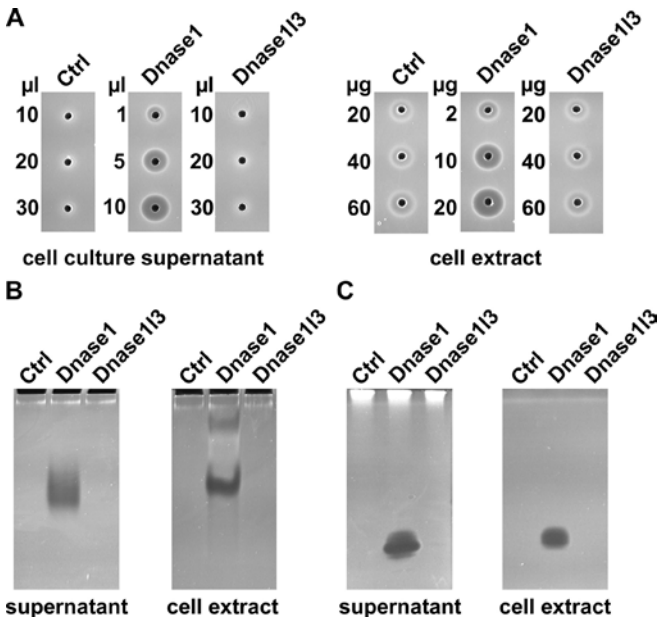

Figure 5 Detection of Dnase1 and Dnase1l3 by different nuclease assays

(**A**) SRED assay employing cell-culture supernatants and cell extracts of NIH-3T3 cells transiently transfected with *Dnase1* and *Dnase1l3* expression vectors. Nuclease activity was only detected in supernatants and extracts of cells expressing *Dnase1* by this method. The same was true for the native-PAGE (**B**) and denaturing-PAGE (**C**) zymogram methods. Abbreviation: Ctrl, supernatant and extract of non-transfected control cells.

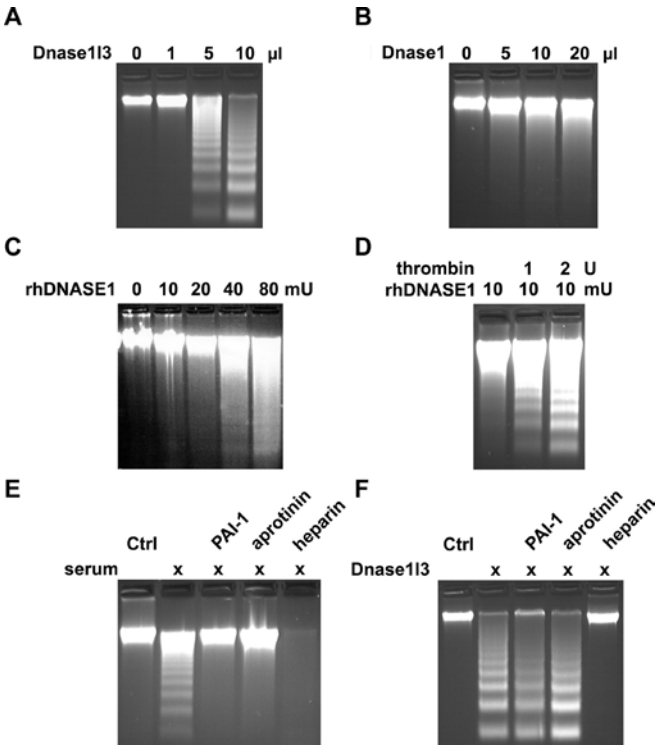

Figure 6 Properties of Dnase1 and Dnase1l3 in nuclear-DNA degradation assays

Isolated MCF-7 cell nuclei were incubated with increasing amounts of cell-culture supernatants of NIH-3T3 cells transiently expressing *Dnase1l3* (**A**) or *Dnase1* (**B**). Only Dnase1l3 cleaved the chromatin in an internucleosomal mode, whereas the amounts of Dnase1 employed gave only a weak chromatin-degrading effect. (**C**) Incubation of cell nuclei with increasing amounts of rhDNASE1 revealed that rhDNASE1 cleaves native chromatin in a random mode, producing a DNA smear in the agarose gel. (**D**) Addition of the serine protease thrombin to the nuclear-DNA degradation assays employing rhDNASE1 revealed that the chromatin-degradating ability of rhDNASE1 was enhanced and switched from a random to an internucleosomal cleavage pattern. (**E**) The internucleosomal mode of chromatin cleavage induced by 5% (v/v) murine wild-type serum is facilitated by Dnase1 in co-operation with the plasminogen system and therefore inhibited by the serine-protease inhibitor aprotinin (0.25 munits/ml) and by PAI-1 (0.013 mg/ml), as shown previously [8]. Heparin (250 units/ml) accelerated the serum-induced chromatin breakdown, most probably due to hyperactivation of the plasminogen system. (**F**) In contrast with the serum Dnase1/plasminogen system, Dnase1l3 induced internucleosomal chromatin breakdown in the absence of proteases and therefore was not inhibited by aprotinin or PAI-1. Comparable with the plasmid-DNA digestion assays (Figure 4C), heparin inhibited the activity of Dnase1l3 towards nuclear DNA. Abbreviation: Ctrl, DNA of cell nuclei incubated in Ca^{2+}/Mg^{2+}-containing buffer.

method, although we employed large amounts of Dnase1l3-containing supernatants, which displayed considerable enzymatic activity in the plasmid-DNA digestion assay (Figure 4B). Secondly, we employed supernatants and cell extracts in native PAGE zymograms. Again Dnase1l3 was not detectable, in contrast with Dnase1 (Figure 5B). However, owing to the alkaline pI (8.9) of murine Dnase1l3, it might not have migrated into the gel. Finally, we employed cell extracts in denaturing SDS/PAGE zymograms. Although Dnase1l3 detection by this method has been described previously [23], we only successfully detected Dnase1 (Figure 5C). In summary, these results indicate that Dnase1 and Dnase1l3 might harbour different substrate or biochemical features causing different detection sensitivities.

Dnase1 and Dnase1l3 exhibit different abilities to degrade native chromatin

In order to investigate the ability of Dnase1 and Dnase1l3 to degrade native chromatin, we employed the cell-culture supernatants of transfected cells in nuclear-DNA degradation assays. Although the Dnase1l3 in comparison with the Dnase1-containing supernatant displayed a lower nucleolytic activity in the plasmid-DNA digestion assay, in nuclear-DNA degradation assays its nucleolytic activity was much higher than that of the Dnase1 supernatant (cf. Figure 4A with Figures 6A and 6B). These results point to different nucleolytic substrate specificities of Dnase1 and Dnase1l3 as detected by the plasmid- and nuclear-DNA degradation assays. Since the plasmid- and nuclear-DNA degradation assays were performed in different buffer systems (see the Materials and methods section), we examined whether the buffer systems themselves influenced the substrate specificity or nuclease activity. No influence of the buffer systems on the substrate specificity was observed (results not shown). However, in the presence of the Hepes/NaCl/β-glycerophophate buffer routinely used for nuclear-DNA degradation assays, the nuclease activities of both nucleases was reduced independently of the substrate employed (results not shown). The substrate differences between both nucleases was substantiated by the observation that Dnase1l3 cleaved nuclear chromatin in an internucleosomal manner (Figure 6A), whereas even high amounts of rhDNASE1 cleaved nuclear chromatin only in a random manner, resulting in a DNA smear in the agarose gel (Figure 6C). In a previous study we have shown that serum Dnase1 needs additional proteolytic activities able to proteolyse DNA binding proteins such as histone H1 in order to achieve internucleosomal chromatin degradation [8]. It was demonstrated that the serum plasminogen system can fulfil this proteolytic function [8]. Plasminogen is activated at nuclear binding sites, generating active plasmin within the nucleus [8]. Our new data demonstrate that thrombin, too, in combination with rhDNASE1, can support considerably lower amounts of rhDNASE1 in internucleosomal chromatin degradation (cf. Figures 6C and 6D). Therefore proteolytic activity clearly enhances the chromatin degradation activity of rhDNASE1. The

nucleosomal-chromatin-degrading activity of murine Dnase1 secreted into serum was inhibited by PAI-1 or aprotinin (Figure 6E). The internucleosomal-DNA-degrading activity of Dnase1l3-containing supernatants was not inhibited by PAI-1 nor the general serine-protease inhibitor aprotinin (Figure 6F). These results demonstrate that Dnase1l3, in contrast with Dnase1, is not dependent on serine-protease activities to facilitate internucleosomal DNA degradation. In agreement with the plasmid-DNA digestion assays, we found that heparin also inhibited Dnase1l3 in the nuclear-DNA degradation assay (Figure 6F), whereas it enhanced the activity of serum Dnase1, most probably by hyperactivating the plasminogen system (Figure 6E). It is known that tissue plasminogen activator is hyperactivated by heparin whereas it had no direct effect on Dnase1 alone (see Figure 4C) [8]. Hyperactivation of the plasminogen system might result in an enhanced cleavage of chromatin-bound proteins and thus in an increased accessibility of nucleosomal linker regions to nucleolytic attack by Dnase1.

Dnase1l3-like nucleolytic activity in murine sera

We showed previously [8] that murine serum derived from *Dnase1* wild-type (*Dnase1*$^{+/+}$) mice contain considerable amounts of Dnase1. The chromatin of necrotic cells or isolated cell nuclei incubated in the presence of this serum is degraded internucleosomally by the concerted action of Dnase1 and the plasminogen system (see above). In contrast, serum from *Dnase1* knockout mice (*Dnase1*$^{-/-}$ serum) was found to no longer induce chromatin degradation [8]. In further experiments we then observed that sera derived from some individual *Dnase1*$^{-/-}$ mice still harboured a residual nuclease activity which cleaved the chromatin of isolated cell nuclei in an internucleosomal mode but with considerably less efficiency than *Dnase1*$^{+/+}$ sera (Figure 7A). This residual serum nucleolytic activity detectable in some sera derived from *Dnase1*$^{-/-}$ mice displayed characteristics of Dnase1l3. Thus it was completely inhibited by heparin, but not by PAI-1 or aprotinin, demonstrating its independence of proteolytic activities to achieve internucleosomal DNA degradation (Figure 7B).

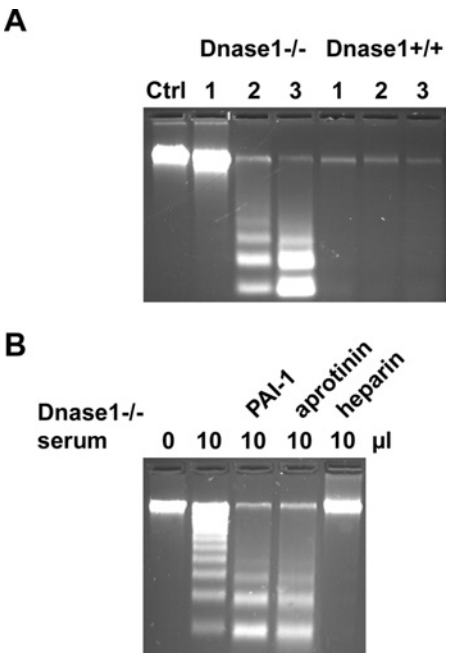

Figure 7 Dnase1l3-like nucleolytic activity in murine sera

(**A**) Two of the three *Dnase1*$^{-/-}$ sera employed in nuclear-DNA degradation assays displayed a residual nuclease activity different from that of Dnase1. However, the overall nucleolytic activity of the three *Dnase1*$^{+/+}$ sera tested was considerably higher in comparison with the two *Dnase1*$^{-/-}$ sera harbouring the additional nuclease activity. Nuclear DNA was degraded to completion by the wild-type serum. (**B**) The additional nucleolytic activity present in a serum of a *Dnase1*$^{-/-}$ mouse displayed the characteristics of Dnase1l3. It was found to be blocked by heparin and not dependent on a proteolytic activity such as that exhibited by the plasminogen system to achieve internucleosomal DNA degradation, since it was not inhibited by PAI-1 nor aprotinin.

DISCUSSION

Clearance of cell debris after or during cell death has been shown to be an important process for the prevention of autoimmunity towards cellular, especially nuclear, antigens. Our recently generated *Dnase1* knockout mouse has proved that a decrease in, or deficiency of, this secretory nuclease results in an enhanced prevalence of anti-nucleosomal antibodies as well as immune-complex-induced glomerulonephritis in mice with the mixed 129 × C57Bl/6 genetic background. These symptoms resemble the human autoimmune disease systemic lupus erythematosus. In cell-culture systems we demonstrated that serum Dnase1 participates in the degradation of nuclear chromatin during necrosis, with high efficiency when co-operating with the proteolytic plasminogen system. Nucleolytic and proteolytic activities together facilitate a fast and complete removal of DNA and DNA-binding proteins, and thus appear to suppress autoimmunity. This process is obviously disturbed when serum from *Dnase1*$^{-/-}$ mice is employed. Interestingly we found that, when we used sera from *Dnase1*$^{-/-}$ mice, some murine sera harbour an additional nuclease activity was able to degrade chromatin in an internucleosomal mode without proteolytic co-operation.

We therefore analysed further the nucleases belonging to the DNase1 protein family and performed a detailed comparative analysis of rat Dnase1 and murine Dnase1l3, which was initially isolated from nuclei of rat thymocytes and termed 'DNase γ' [22]. Recently this nuclease has been characterized in a number of reported studies. However, contradictory reports exist about its exact intracellular localization under normal physiological conditions [20–22,25]. Here we demonstrate that recombinant expression of *Dnase1l3* fused with its C-terminus to the *GFP* cDNA mirrored in all aspects *Dnase1–GFP* expression. Both fusion proteins were translocated into the rER, transported along the entire secretory pathway, as verified by fluorescence and laser-scanning microscopy and became detectable in cell-culture supernatants. We found no indication of a directed transport of Dnase1l3 into the nucleus, although it has been reported that the enzyme contains two NLSs, one monopartite in the N-terminal region and one bipartite in the C-terminal prolongation domain, which is unique to DNASE1L3 and not present in DNASE1 [30]. However, an uncertainty exists for the monopartite NLS sequence. In contrast with the findings of Shiokawa et al. [30], four recent entries into SwissProt protein sequence databank demonstrated that residue 96 of the complete human DNASE1L3, and further species, is asparagine and not basic lysine. Our own sequence data for murine Dnase1l3 confirm asparagine at position 96 (M. Napirei and T. Klöckl, unpublished work). Therefore the NLS functionality of this sequence remains unsettled. In addition, it remains doubtful whether the second C-terminal NLS is functional under normal physiological conditions, since we did not find an import of the Dnase1l3–GFP fusion protein into the nucleus of transiently transfected NIH-3T3 fibroblasts or LEC. However, our data do not rule out the possibility that, under special conditions such as cell death, Dnase1l3 might diffuse or be actively transported into the nucleus, since under these conditions the intracellular membranes of the rER, secretory vesicles or of

the nuclear envelope may become leaky. Indeed, detection of Dnase1l3 in the nuclei of different cell types undergoing apoptosis has been described [23–29]. Deletion of the rER signal peptides, i.e., the first 21 (Dnase1) or 25 (Dnase1l3) N-terminal residues, resulted in a cytoplasmic distribution and nuclear accumulation of the GFP fluorescence and loss of endonucleolytic activity in the cell-culture supernatants, proving that both nucleases are primarily secretory. The cytoplasmic and nuclear accumulation of ΔSP-Dnase1l3–GFP might be interpreted as evidence for the functionality of the presumed NLSs of Dnase1l3. Since, however, ΔSP-Dnase1–GFP also exhibited a similar distribution without possessing a NLS, this reasoning looses plausibility. In addition, a degradation product of the GFP protein occurred in all transfections, and fluorescence of this product could not be ruled out. Therefore it is not possible to decide whether the NLS of Dnase1l3 is responsible for the transport of the ΔSP-Dnase1l3–GFP protein into the nucleus, since, alternatively, the fusion protein may have been retained in the cytoplasm and only the small GFP-degradation product being passively diffused into the nucleus. In addition to the finding that both nucleases are secretory, we found that transport through the rER and Golgi apparatus seems to be necessary to synthesize functional Dnase1 and Dnase1l3, since we also did not find nuclease activities in extracts of cells transiently transfected with *ΔSP-Dnase1* and *ΔSP-Dnase1l3* expression vectors (results not shown).

For a biochemical comparison, we transiently expressed both nucleases without the GFP fusion tag, since we could not rule out the possibility that the fusion might influence their nucleolytic abilities. We found that both nucleases were secreted into the culture supernatant. Both displayed identical metal-ion-dependencies, as previously reported [12], i.e., both were Ca^{2+}- and Mg^{2+}/Mn^{2+}-dependent; however, in the presence of Mn^{2+}, their nucleolytic activities were enhanced. As predicted from the amino acid sequence comparison, Dnase1l3 was not inhibited by monomeric actin, in contrast with Dnase1.

An interesting finding in the present study was that both nucleases display completely different substrate specificities. We found that Dnase1 degrades plasmid DNA with high activity, whereas it cleaves nuclear DNA with low activity. High amounts of Dnase1 degraded chromatin randomly; only in the simultaneous presence of proteases did it efficiently catalyse internucleosomal DNA degradation. By contrast, Dnase1l3 cleaved plasmid DNA with low activity, and nuclear DNA with high activity. Thereby nuclear DNA was cleaved in an internucleosomal and protease-independent manner, pointing to a basically different DNA binding or cleavage property of Dnase1l3. This might be also the reason why we were not able to detect Dnase1l3 in nuclease assays suitable for the detection of Dnase1 activity, which are based on protein-free and not highly condensed DNA. Since both nucleases share a highly conserved catalytic centre and DNA-binding region, we suppose that their divergent pI values might be the cause of their different substrate specificities. Whereas rat Dnase1 has an acidic pI of 4.9, the murine mature Dnase1l3 protein has a basic pI of 8.9. Therefore Dnase1 has a charge of -9.4, whereas it is $+6.7$ for Dnase1l3 at a pH of 7.0. These charge differences might explain the fact that Dnase1l3, in contrast with Dnase1, was inhibited by the polyanion heparin. Furthermore, it is conceivable that Dnase1l3 is directed to internucleosomal DNA regions, owing to repulsion by the positively charged histones or able to compete with histone H1 for the DNA linker region. This might result in a rapid dissolution of the DNA solenoid structure owing to loss of contact between the nucleosomes. In contrast, Dnase1 might be attracted by histones and preferentially cleave DNA wound around the nucleosomal core. Since the Dnase1-cleaved DNA is still bound to the nucleosomal core and the internucleosomal regions remain uncleaved, the integrity of the DNA solenoid structure might be conserved for a longer time. Therefore only large amounts of Dnase1 are able to degrade chromatin. However, when Dnase1 is supported by proteases such as plasmin or thrombin, very small amounts of Dnase1 suffice to achieve fast and complete chromatin degradation. Furthermore the cleavage pattern of Dnase1 changed from random to internucleosomal in the presence of proteases, since the degradation of histone H1 renders the internucleosomal regions accessible to an attack by Dnase1 [8].

Our results also demonstrate that Dnase1l3 occurred in the serum of a small number of *Dnase1* knockout mice. Previously we identified Dnase1 as the major serum nuclease, which, in cooperation with the plasminogen system, guarantees fast and effective degradation of chromatin within, or released from, necrotic cells [8]. Since the nucleolytic activity of Dnase1 dominates in murine serum, the presence of a further endonuclease has been overlooked in these assays. Nevertheless, our more detailed analysis here revealed the presence of an additional nucleolytic activity in some murine sera. This endonucleolytic activity displayed properties typical of Dnase1l3, as verified by internucleosomal DNA cleavage without proteolytic aid and inhibition by heparin. Indeed, it has been demonstrated that Dnase1l3 is secreted by macrophages present in diverse organs [21]. Since the removal of cellular debris, especially of chromatin, is essential for the prevention of autoimmunity against nuclear constituents, identification of enzymes participating in its clearance is of paramount importance with respect to potential therapeutic strategies. Our results demonstrate that chromatin clearance in the circulation is probably a concerted action of Dnase1, Dnase1l3 and the plasminogen system. Indeed, an impaired Dnase1l3 activity was demonstrated for lupus-prone MRL and NZB/W F1 mice, supporting a function for Dnase1l3 in chromatin clearance [39]. Future studies will aim to elucidate the circumstances under which Dnase1l3 is released into the serum. In addition, our study may give a framework for a reinvestigation of the properties of the remaining Dnase1-like nucleases.

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