Lupus nephritis is a serious manifestation of systemic lupus erythematosus and is a main predictor of poor outcome. The predominance of chromatin-associated autoantigens involved in lupus nephritis point at deficiencies in the processing and clearance of chromatin as central factors in the pathogenesis of the disease.\(^1\)–\(^5\) Enzymatic DNA fragmentation by different endonucleases is significant in apoptotic cell death (reviewed in Samejima and Earnshaw,\(^6\) Kawane and Nagata,\(^7\) and Napirei et al.\(^8\)). In renal tissue, DNaseI represents the main nuclease,\(^9\) and recent findings indicate that transformation of mild lupus nephritis into end-stage disease coincides with renal DNaseI shutdown in (NZBxNZW)F1 mice.\(^10,11\) Down-regulation

Recent findings show that transformation of mild glomerulonephritis into end-stage disease coincides with shutdown of renal DNaseI expression in (NZBxNZW)F1 mice. Down-regulation of DNaseI results in reduced chromatin fragmentation and deposition of extracellular chromatin fragments in glomerular basement membranes where they appear in complex with IgG antibodies. Here, we implicate the anti-apoptotic and survival protein, tumor necrosis factor receptor-associated protein 1 (Trap1) in the disease process, based on the observation that annotated transcripts from this gene overlap with transcripts from the DNaseI gene. Furthermore, we translate these observations to human lupus nephritis. In this study, mouse and human DNaseI and Trap1 mRNA levels were determined by real-time quantitative PCR and compared with protein expression levels and clinical data. Cellular localization was analyzed by immune electron microscopy, IHC, and in situ hybridization. Data indicate that silencing of DNaseI gene expression correlates inversely with expression of the Trap1 gene. Our observations suggest that the mouse model is relevant for the aspects of disease progression in human lupus nephritis. Acquired silencing of the renal DNaseI gene has been shown to be important for progression of disease in both the murine and human forms of lupus nephritis. Early mesangial nephritis initiates a cascade of inflammatory signals that lead to up-regulation of Trap1 and a consequent down-regulation of renal DNaseI by transcriptional interference.

(\textit{Am J Pathol} 2013, 182: 688–700; \url{http://dx.doi.org/10.1016/j.ajpath.2012.11.013})
of DNaseI results in reduced chromatin fragmentation and deposition of extracellular chromatin complexes in glomerular basement membranes where they appear in complex with IgG antibodies. Therefore, loss of DNaseI activity is identified as a central factor that contribute to transformation of mild mesangial into severe diffuse-proliferative lupus nephritis in murine and human forms of the disease. Despite the fact that DNaseI has been known for >50 years, mechanisms that regulate expression and secretion of this enzyme are not clear. The down-regulation of the DNaseI gene in kidneys during progression of lupus nephritis may theoretically be caused by several regulatory pathways. One possibility is a direct effect of early inflammation and proinflammatory cytokines, although no data to support this exist. By inspection of the DNaseI gene organization in the University of California, Santa Cruz genome browser (http://genome.ucsc.edu/cgi-bin/hgTracks?hgsid=309769569), we found an overlap of 59 nucleotides in the annotated transcript with transcripts from the convergently transcribed tumor necrosis factor receptor-associated protein 1 (TRAP1) gene in their 3'-untranslated regions. This gene organization is peculiar and found in only a few percentage of human transcription units (H. Nielsen, unpublished data) and is likely to preclude coexpression of the two genes. Given that transcription proceeds well beyond the 3' end of the mature transcript, the overlap between the transcripts of the two genes is substantial, and it is unlikely that they are transcribed simultaneously. Recent evidences supporting this hypothesis was published by Churchill and Weissman, who demonstrated that the two RNA polymerase II (RNAPII) molecules elongating in opposite directions cannot pass each other, and elongating RNAPII suppresses downstream antisense transcription. RNAPII has been shown to induce chromatin modification through the Set2/Rpd3S pathway. Therefore, silencing of both DNaseI and TRAP1 after activation of one of them may be an expected outcome. Therefore, we consider the DNaseI and TRAP1 genes as a coupled gene pair and use the term transcriptional interference (in a broad sense) to describe the concept that the expression of one gene is affected by the expression of the other at the transcriptional level.

TRAP1 belongs to the heat shock protein 90 family. This protein is a highly conserved chaperone molecule with important roles in signal transduction, protein folding, and protein degradation. Furthermore, it is also important for folding newly synthesized proteins or for stabilizing and refolding denatured proteins after stress. TRAP1 has antioxidant and antiapoptotic functions, probably via involvement of reactive oxygen species. It is up-regulated during stress and functions as a survival protein, whereas DNaseI is a death-associated protein involved in clearance of dead and dying cells. Thus, regulation of DNaseI and TRAP1, respectively, is phenomenologically linked to antagonistic processes associated with stress and inflammation. We, therefore, aimed to determine whether i) increased TRAP1 gene expression during progressive lupus nephritis is a response to inflammation and stress, ii) expression of DNaseI is inversely correlated with expression levels of TRAP1 as predicted, and iii) renal DNaseI and TRAP1 expression patterns in the murine model are paralleled in human lupus nephritis.

This study therefore focuses on the relation between DNaseI and TRAP1 at transcriptional and translational levels. Considering the evidence-based information given earlier, we put forth a twofold hypothesis. We hypothesized that the ratio of expression levels of TRAP1/DNaseI to be high after mesangial nephritis and that in end-stage organ disease both genes, but not those surrounding this DNA region, are severely down-regulated, a consequence of the potential for RNAPII to induce chromatin modification.

The results presented in this study indicated that there indeed exists an inverse relation when comparing the expression of the two genes both at the mRNA and protein levels and that, in situations with loss of DNaseI, TRAP1 is relatively overexpressed. Furthermore, both genes are silenced in end-stage organ disease, indicating a down-regulation of both genes because of chromatin modifications.

**Materials and Methods**

**Ethics Statement**

The National Animal Research Authority approved this study (approval ID: 07/11167, ID-178). Coherent analyses on renal biopsies, taken from patients with lupus nephritis, were approved by The Scientific Ethical Committee, Copenhagen [(KF) 01-2006-7214]. Informed written consent was given by each patient.

**Murine and Human Tissue Samples**

Renal tissue was collected from euthanized female (NZBxNZW)F1 mice (The Jackson Laboratory, Bar Harbor, ME) and every second week from the age of 4 weeks until development of end-stage disease, clinically defined when severe proteinuria developed (≥20 g/L). Included in this study are 25 mice from the prenephritic mice (Group 1, having no glomerular deposits of chromatin or IgG), 8 mice with mesangial nephritis (Group 2, having mesangial deposits of chromatin-IgG complexes), and 8 mice with end-stage disease [Group 3, with deposits of chromatin-IgG complexes in mesangium and in glomerular basement membranes (GBMs)]. For baseline details of these mice, see Fenton et al. Tissue was either snap-frozen for protein extraction, preserved in RNAlater (Ambion Inc., Austin, TX) for mRNA level analyses, or embedded in Tissue Tech Optimal Cutting Temperature (OCT) compound for immunofluorescence analyses. Serum and urine samples were collected at 2- to 3-week intervals and stored at −80°C. Data obtained in these mice represent baseline data for comparison with data generated in human lupus nephritis. Renal biopsies from patients with class IV (diffuse-proliferative) lupus nephritis as determined by the International Society of Pathologists.
Nephrology/Renal Pathology Society (ISN/RPS) 2003 guidelines were obtained in parallel with biopsies obtained for diagnostic purposes (ISN/RPS class I–IV). Entry criteria were fulfillment of the classification criteria for systemic lupus erythematosus from the American College of Rheumatology and clinical indication for renal biopsy. Control samples from morphologically normal cortical tissue were sampled from nephrectomy specimens immediately after the procedure to surgically treat ca rens. Paraffin-embedded tissues for IHC analyses and biopsies fixed in 8% formaldehyde in PBS for immune electron microscopy (IEM) were prepared as described. Tissues from non-lupus renal diseases were used for control experiments.

ELISA to Detect Murine Anti-dsDNA Antibodies

Enzyme-linked immunosorbent assay (ELISA) was performed exactly as described previously. Titters were defined as the reciprocal value of the dilution giving 50% of maximum binding to wells, as determined by a reference anti-dsDNA monoclonal antibody. Thus, sera were regarded as positive if a 1:100 dilution reached an optical density at 492 nm, representing 50% of maximum binding of the reference antibody.

RNA Isolation, cDNA Synthesis, Protein Isolation, Western Blot Analysis, and DNaseI Zymography

Total RNA was isolated from RNAlater (Ambion Inc., Austin TX) preserved human kidney tissues with the use of TRizol (Invitrogen, Carlsbad, CA) as described by the manufacturer. Murine samples were isolated from RNAlater-preserved kidneys either by using TRizol or the EZ-1 RNA tissue mini kit (Qiagen, Nordic, Norway). The concentration and quality of extracted RNA were determined spectrophotometrically by NanoDrop (NanoDrop Technologies, Wilmington, DE) and murine samples also by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples were reverse-transcribed with random primers by using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). After RNA was recovered from the aqueous phase by ethanol precipitation, proteins were recovered from the interphase/phenol phase and dissolved in 1% SDS. The protein concentration was determined with bichinchoninic acid assay kit as recommended by the manufacturer (Pierce Biochemicals, Rockford, IL). Protein (6 µg) was loaded onto a 4% to 12% NuPAGE Bis-Tris gel (Invitrogen). SDS/PAGE and Western blot analysis were performed according to standard procedures. Membranes were blocked with 5% (w/v) skimmed milk and probed with the relevant primary rabbit anti-DNaseI/anti-Trap1 antibodies. After incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Invitrogen), binding was assayed by chemiluminescence detection. Determination of molecular weight was done with MagicMark XP molecular weight markers (Invitrogen). An antibody recognizing β-actin was included to ensure equal loading of the samples. DNaseI gel zymography was performed exactly as described.

Gene Expression Analyses

Real-time quantitative PCR (qPCR) was performed with ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Predesigned carboxyfluorescein-labeled gene expression assays (Applied Biosystems) were purchased, with the following accession numbers for murine and human analyses: DNaseI Mm01342389_g1 and Hs00173736_m1, and Trap1 Mm00446002_m1 and Hs00212476_m1. RPLPO (large ribosomal subunit protein) was used as endogenous control for human samples and β-actin and TBP (TATA box binding protein) for genomic primer/probes for murine samples. The relative expression levels were calculated with the dCT method relative to prenephritic kidneys (for mice) and normal renal tissue (for human samples).

Antibodies

The following antibodies were used in this study: mouse anti-human Trap1 (612345; BD Bioscience, San Jose, CA), rabbit anti-human DNaseI (ab113241; Abcam Inc., Cambridge, MA), rabbit anti-murine DNaseI and rabbit or goat anti-murine Trap1 antibodies (the latter for confocal microscopy of murine tissue), Alexa 595-conjugated anti-rabbit IgG and Alexa 488-conjugated anti-goat IgG secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany).

Immunofluorescence Microscopy

Four-micrometer thick sections of OCT-embedded kidneys were blocked for 30 minutes in 1% bovine serum albumin in PBS, followed by washing and 30 minutes of incubation with rabbit anti-DNaseI or with rabbit anti-Trap1 antibodies (Santa Cruz Biotechnology), both diluted 1:50 in PBS. Slides were washed and incubated further for 30 minutes with Alexa 488-conjugated anti-rabbit F(ab)2 secondary antibodies (Invitrogen). Normal goat and rabbit IgGs were used as negative controls. The sections were analyzed by an Olympus BX51 microscope (Olympus, Tokyo, Japan).

Confocal Microscopy

Four-micrometer thick sections of OCT-embedded kidneys were blocked for 30 minutes in 1% bovine serum albumin in PBS, followed by washing and 30 minutes of incubation with primary rabbit anti-DNaseI and goat anti-Trap1 antibodies (Santa Cruz Biotechnology). Sections were then treated with Alexa 595-conjugated anti-rabbit IgG and Alexa 488-conjugated donkey anti-goat IgG secondary antibodies each for 30 minutes. After each step, the sections were washed in PBS before mounting. The samples were observed in Zeiss-LSM510 Meta confocal microscope (Carl Zeiss GmbH, Jena, Germany).
Renal Trap1 and DNaseI in Lupus Nephritis

IEM to Detect IgG Bound in Vivo in Glomeruli and to Determine Expression Levels and Subcellular Localization of Trap1 and DnaseI in Murine and Human Tubular Cells

For IEM, ultrathin cryosections of kidneys from pre-nephritic (Group 1) or from nephritic (NZBxNZW)F1 mice with pure mesangial nephritis (having chromatin-IgG complexes in the mesangium; Group 2) or with endstage nephritis (having chromatin-IgG complexes in mesangium and in GBM; Group 3), or from human control kidneys and kidneys with ISN/RPS class IV nephritis (having chromatin-IgG complexes in GBM) were prepared and processed exactly as described previously.24,25,31 In vivo-bound glomerular autoantibodies were detected by incubating the human sections with anti-human IgG (RaM IgG) antibodies (ICN/Chappel, Aurora, OH) followed by protein A-conjugated with 5-nm gold particles (PAG-5 nm; University of Utrecht, The Netherlands). Similar analyses of the (NZBxNZW)F1 kidneys included in this study have been published.10

Parallel murine and human sections were stained with antibodies to murine and human Trap1 or DNaseI proteins to trace amount and intracellular localization of the two proteins in healthy and diseased kidneys. These proteins are traced by 5-nm gold particles. Micrographs were taken at total magnification of ×50,000 to 70,000 with the use of a JEM-1010 transmission electron microscope (Jeol, Tokyo, Japan).

IHC Analysis

Immunohistochemical staining of DNaseI and Trap1 was performed as described,4 and Polink-2 Plus horseradish peroxidase with 3,3′ dianinobenzidine kit (Newmarket Scientific, Suffolk, UK) was used as detection system.

Immunoeexpression Score

To evaluate the degree of protein expression by IHC, we blindly quantified the intensity of DNaseI and Trap1 staining in 5- to 10-view fields per human renal biopsy. The immunoeexpression score (IEXP-SC) was adapted from Richardsen et al32 as follows: IEXP-SC = AEX × ISI, where AEX is the percentage cellular area of expression and ISI is the immunostaining intensity (grades 1 to 3).5 The product gives a score from 0 to 300, expressed as follows: negative, IEXP-SC = 0; weakly positive, IEXP-SC = 1 to 100; moderately positive, IEXP-SC = 101 to 200; and strongly positive, IEXP-SC = 201 to 300.

Laser Capture Microdissection of Murine Kidneys

Ten-micrometer thick kidney cryosections were prepared and immediately fixed in zinc buffer (40 mmol/L ZnCl2, 5.8, 7/10). Z

Table 1 Baseline Data for the Female (NZBxNZW)F1 Mice Included in This Study

<table>
<thead>
<tr>
<th>(NZBxNZW)F1 mouse group</th>
<th>Age (weeks)</th>
<th>Anti-DNA Ab titer*</th>
<th>Proteinuria</th>
<th>EDS in glomeruli1</th>
<th>Renal DNaseI mRNA levels</th>
<th>Renal condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 25)</td>
<td>12.7 ± 5.7</td>
<td>5 ± 24</td>
<td>+1 ± 0</td>
<td>1 ± 0</td>
<td>1.51 ± 0.58</td>
<td>Pre-nephritic</td>
</tr>
<tr>
<td>2 (n = 8)</td>
<td>23.7 ± 5.8</td>
<td>710 ± 1027</td>
<td>+1.38 ± 0.7</td>
<td>2 ± 0</td>
<td>1.36 ± 0.50</td>
<td>Mesangial nephritis</td>
</tr>
<tr>
<td>3 (n = 8)</td>
<td>28.8 ± 4.5</td>
<td>453 ± 415.13</td>
<td>+4 ± 0</td>
<td>3 ± 0</td>
<td>0.25 ± 0.12</td>
<td>End-stage kidney disease</td>
</tr>
</tbody>
</table>

These data are taken from a larger number of (NZBxNZW)F1 mice analyzed in another context.9,10 Data given as group average ± SD.

*The titers of the anti-dsDNA antibodies are defined as described in Materials and Methods.

1Indicates as follows: 1, no electron dense structures in glomeruli; 2, electron dense structures solely in the mesangial matrix; and 3, electron dense structures in glomerulus basement membranes.

Table 2 Basic Clinical, Serological and Histological Data on Kidneys from Patients with Lupus Nephritis Subjected to qPCR

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sex/age (years)</th>
<th>ACR criteria*</th>
<th>Disease duration</th>
<th>ISN/RPS class lupus nephritis</th>
<th>Activity/chronicity score</th>
<th>Anti-dsDNA antibody (µmol/L)</th>
<th>S-Creatinine (mg/24 hours)</th>
<th>Proteinuria</th>
<th>Therapy1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN1</td>
<td>F/39</td>
<td>1,4,5,7,9,10,11</td>
<td>2 months</td>
<td>IV</td>
<td>6/1</td>
<td>4001</td>
<td>78 normal</td>
<td>2.4</td>
<td>Prednisolone 25 mg, daily for 2 weeks; H-Chlorochine 400 mg, daily for 4 weeks</td>
</tr>
<tr>
<td>LN2</td>
<td>F/18</td>
<td>5,7,10,11</td>
<td>6 weeks</td>
<td>IV</td>
<td>7/1</td>
<td>&gt;400</td>
<td>68 normal</td>
<td>3.6</td>
<td>Prednisolone 25 mg, daily for 2 days</td>
</tr>
<tr>
<td>LN3</td>
<td>M/22</td>
<td>1,4,7,9,10,11</td>
<td>3.5 years</td>
<td>IV</td>
<td>9/10</td>
<td>&gt;400</td>
<td>357 elevated</td>
<td>9.0</td>
<td></td>
</tr>
</tbody>
</table>

*ACR-criteria: 1, malar rash; 2, discoid rash; 3, photosensitivity; 4, oral ulcers; 5, arthritis; 6, serositis; 7, renal disorder; 8, neurologic disorder; 9, hematologic disorder; 10, immunologic disorder: anti-dsDNA, anti-Sm, and/or anti-phospholipid antibodies; 11, anti-nuclear antibodies (ANA).

1Treatment duration.

2Cut off for this assay is set to 15.

F, female; M, male.
30 mmol/L ZnAc₂, and 600 mmol/L CaAc₂ in 0.1 mmol/L Tris, pH 7.4) for 5 minutes, stained with hematoxylin, dehydrated, air-dried, and overlaid with liquid coverglass (PALMZeiss, Bernreid, Germany). Cortex was collected by laser capture microdissection on a PALM Laser-MicroBeam System. For each sample, tissue from 10 sections were collected, lysed in TRIzol (Invitrogen), and stored at −70°C for further RNA isolation.

**In Situ Hybridization**

*In situ* hybridization to detect DNaseI and Trap1 mRNA molecules was performed as described by Nielsen et al.\(^{33}\) The hybridization temperature was 57°C, and the DNA: locked nucleic acid chimeric probes were labeled with carboxyfluorescein in both 5′ and 3′ ends (incubated at 60 mmol/L) and detected with alkaline phosphatase conjugated anti-fluorescein (Roche, Indianapolis, IN). The sequence of the probes were for human DNaseI 5′-AAGTCGCCCATCAACATGACGT-3′, RNA/27% locked nucleic acid; T\(_{m}\) = 86°C, and the sequence of the Trap1 probe was 5′-TTTCAACACTCCAGAACCAC-3′, RNA/41% locked nucleic acid; T\(_{m}\) = 84°C. The probes were obtained from Exiqon (Vedbaek, Denmark).

**Statistics**

Data are presented as means ± SD. An unpaired *t*-test was performed to test differences between each group, and one-way analysis of variance was performed to compare all groups for each parameter; \( P < 0.05 \) was considered significant.

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**Figure 1** Renal expression levels of DNaseI and Trap1 in (NZBxNZW)F1 mice from Groups 1 to 3. A: The mRNA levels of DNaseI and Trap1 are determined in kidneys of individual (NZBxNZW)F1 mice in prenephritic mice (Group 1), and in mice with mesangial nephritis (Group 2) or with diffuse-proliferative nephritis (Group 3). Inset shows means ± SD of DNaseI and Trap1 mRNA levels. Data show that both Trap1 and DNaseI vary considerably within and between the three groups of mice, and especially in Group 2 and Group 3. Group 2 had significantly higher Trap1 mRNA levels, whereas in Group 3, DNaseI mRNA was severely and significantly reduced (inset). B: The ratio of Trap1/DNaseI mRNA levels is shown for individual mice. The inset shows means ± SD of the Trap1/DNaseI ratio that is significantly higher in Group 3 than in the other groups. C: Typical DNaseI zymography and Trap1 Western blot data are shown in mice belonging to the different groups. The zymography and Western blot selections are unpaired and randomly selected within each group of mice. *\( P < 0.05 \), ***\( P < 0.0001 \).
Results

Classification of Lupus Nephritis in (NZBxNZW)F1 Mice from Groups 1 to 3 and in Biopsies from Patients with Lupus Nephritis Included in This Study

To determine renal expression levels of DNaseI and Trap1 during progression of lupus nephritis, 4- to 40-week old (NZBxNZW)F1 mice were analyzed by qPCR. Baseline data from these mice are presented in Table 1 and show that the mice can be grouped into three groups according to the presence of glomerular deposits of immune complexes; 25 pre-nephritic mice (Group 1, having no glomerular deposits of chromatin or IgG), 8 mice with mesangial nephritis (Group 2, having mesangial deposits of chromatin-IgG complexes), and 8 mice with end-stage disease (Group 3, with deposits of chromatin-IgG complexes in mesangium and in GBM). Baseline renal data on the three human patients included in the qPCR analyses are presented in Table 2 and show that all are classified as ISN/RPS class IV. That is, they have chromatin-IgG complex deposits in both the mesangium and GBM, similar to deposits in Group 3 (NZBxNZW)F1 mice. Control samples (n = 3) from morphologically normal cortical tissue were sampled from nephrectomy specimens because of surgical treatment of ca rensis. Furthermore, three murine kidneys (one from each of Groups 1–3) and six human kidney biopsies (one control and five from ISN/RPS class IV lupus nephritis) were subjected to IEM to analyze at high resolution for Trap1 and DNaseI expression profiles.

To quantify the intensity of DNaseI staining, 26 human renal biopsies were used (n = 5 controls, n = 4 ISN/RPS class II–III, and n = 17 ISN/RPS class IV lupus nephritis). For quantification of Trap1 staining, 21 human renal biopsies were used (n = 4 controls, n = 4 ISN/RPS class II–III, and n = 13 ISN/RPS class IV lupus nephritis), because of lack of material that allowed quantification of Trap1 in five biopsies.
Renal Expression Patterns of DNaseI and Trap1 mRNA and Proteins in (NZBXNZW)F1 Mice from Groups 1 to 3

We have demonstrated before\textsuperscript{5,10} that DNaseI was expressed in prenephritic kidneys shown in Group 1 (Figure 1A) and in kidneys from mice with mesangial nephritis shown in Group 2 (Figure 1A), whereas DNaseI expression was dramatically reduced in kidneys from Group 3 (NZBxNZW)F1 mice (Figure 1A) as evidenced by qPCR analysis. In comparison, Trap1 mRNA levels varied within and between the three groups of (NZBxNZW)F1 mice. However, three types of expression could be discerned. The first type comprised 24 of 25 mice in Group 1 and 5 of 8 mice in Group 2 (Figure 1A). Here, the mRNA levels of DNaseI and Trap1 were roughly equal. The second type comprised a single mouse from Group 1 and 3 of 8 mice from Group 2 and was characterized by a threefold to sixfold increase in Trap1 mRNA levels with a reduction in DNaseI expression. Finally, the third type comprised all of the eight mice with end-stage disease (Group 3) and was characterized by very low DNaseI mRNA levels and normal-to-low Trap1 mRNA levels. Thus, the overall impression that is left was that of a transient up-regulation of Trap1 mRNA levels around the time of development of mesangial nephritis followed by very low levels of both mRNAs at end-stage disease. Although the expression types did not coincide with the grouping-based IgG complex deposition, the general trends were evident from inserts in Figure 1A that show the mRNA levels within the three groups and Figure 1B that depicts the mRNA ratios. Note that the very high Trap1/DNaseI ratios in Group 3 are based on low expression levels. In Figure 1C, typical DNaseI zymography and Trap1 Western blot analyses are shown in randomly chosen mice belonging to the different groups.

To exclude the fact that the observed differences were due to differential expression in the compartments of the kidney, analysis of mRNA levels of the DNaseI and Trap1 transcripts in microdissected cortex were compared with whole kidney in a mouse with diffuse-proliferative nephritis (Group 3). This analysis showed similar mRNA levels in the two samples. Furthermore, analysis of expression of the proteins by IHC showed that the down-regulation in Group 3 mice was found similar in cortex and whole kidney (data not shown; see Zyková et al\textsuperscript{12}). Thus, the down-regulation of DNaseI and Trap1 in Group 3 (NZBxNZW)F1 mice did not seem to be a focal process.

To analyze whether the mRNA qPCR results were paralleled in the expression of the proteins, DNaseI- and Trap1-specific immunofluorescence analyses were performed. Kidneys from prenephritic Group 1 mice (Figure 2A) showed a similar expression level of the DNaseI and Trap1 proteins; whereas, in kidneys with mesangial nephritis, expression of Trap1 was increased (Figure 2A). A quite different result was observed in end-stage lupus nephritis. In mouse L49, Trap1 appeared robustly expressed, whereas DNaseI was almost undetectable as determined both at the mRNA and the protein levels (Figure 2A). In mouse H9, by contrast, both Trap1 and DNaseI mRNAs and protein expression were weak (Figure 2A). These expression profiles suggested that the end-stage organ disease could be characterized by two different patterns (both with very low DNaseI protein expression, while one with and one without Trap1 expression) and potentially also by two different processes that reduce DNaseI expression (see Discussion). The immunofluorescence pattern in the kidneys from mice L4, L36, L49, and H9 correlated with qPCR (Figure 2B) and results of zymography (for DNaseI) and Western blot analysis (for Trap1) as shown in Figure 2C.

In Figure 3, IEM analyses showed that in Group 1 (mouse L5) and Group 2 (mouse L31) mice, Trap1 (A and C for L5 and L31, respectively) and DNaseI (B and D for L5 and L31, respectively) were simultaneously expressed in individual tubular cells at intermediate levels. In Group 3 mouse (Mouse L40), Trap1 was present at high levels in mitochondrial (E), whereas DNaseI was hardly traced in tubular cells at all (F).
To evaluate whether individual cells express both the Trap1 and DNaseI genes in Group 3 kidneys or whether they display a reciprocal expression pattern, confocal microscopy analyses were performed (Figure 4). As an example, the results from a mouse with end-stage lupus nephritis (mouse H9) with chromatin fragment IgG deposits in the GBM are presented (Figure 4A–C). In this severely diseased kidney, a faint staining of both proteins was observed by confocal microscopy (Figure 4A), consistent with the low mRNA levels found by qPCR (Figure 4B). In the merged image, it is evident that cells are either stained green (for Trap1) or red (for DNaseI). Thus, Trap1 and DNaseI expression seem mostly to be nonoverlapping at the level of single cells. Expression levels of the two proteins compare well with mRNA levels. The merged image is enlarged and shows that DNaseI and Trap1 are not coexpressed in different areas. Mean intensity green channel = 47; mean intensity red channel = 37.

Renal Expression Pattern of DNaseI and Trap1 in Mice with End-Stage Lupus Nephritis (Group 3) Determined by Confocal Microscopy

Next, we translated the murine data into analyses of renal expression levels of Trap1 and DNaseI in human diffuse-proliferative lupus nephritis. Human kidney samples were initially divided into two groups according to kidney morphology: normal controls (n = 3) and patients with diffuse-proliferative lupus nephritis ISN/RPS class IV (n = 3).

The qPCR analysis showed significantly and dramatically reduced DNaseI mRNA levels in kidneys from patients with lupus nephritis compared with control samples. In contrast, Trap1 mRNA levels remained at levels comparable with those in the control kidneys or slightly less (Figure 4A). Western blot analysis of renal proteins were consistent with the qPCR results because renal DNaseI expression was hardly detectable in the lupus nephritis group, whereas Trap1 was strongly expressed in the same samples (Figure 5A). Thus, DNaseI gene expression in the nephritic kidneys was selectively...
silenced, because Trap1 remained high in all samples. As a consequence, the ratio of Trap1/DNaseI mRNA levels was significantly higher in kidneys from patients with lupus nephritis than from the control group (Figure 5B).

Immunohistochemical analysis of human kidney sections showed normal expression in normal kidneys (Figure 6A) and in ISN/RPS class II kidneys (Figure 6B), but there was barely detectable staining for DNaseI in ISN/RPS class IV nephritis (Figure 6C). In contrast, Trap1 staining was relatively equal in controls and lupus nephritis (Figure 6, D–F).

To verify these results, we analyzed further biopsies for expression of both Trap1 (n = 21) and DNaseI (n = 26) by IHC. We quantified the intensity of DNaseI and Trap1 staining by IEXP-SC as shown in Figure 6, G and H, respectively. Renal DNaseI expression was markedly reduced in patients with diffuse-proliferative nephritis ISN/RPS class IV compared with control samples and ISN/RPS class II–III lupus nephritis (Figure 6G). No difference was observed in Trap1 expression between controls and lupus nephritis (Figure 6H).

In parallel analyses, we determined expression of Trap1 and DNaseI mRNA levels in two human ISN/RPS class IV kidneys by in situ hybridization. The results correlated well with results from qPCR because DNaseI mRNA remained almost undetectable in both biopsies (Figure 6, I and K), whereas Trap1 mRNA could easily be detected in both (Figure 6, J and L), and were furthermore consistent with protein expression levels as determined by Western blot analysis (Figure 5A) and IHC (Figure 6, C and F).

When analyzing human nephritic kidneys by IEM, we obtained results highly similar to those obtained in murine IEM analyses (Figure 3). In a non-nephritic control biopsy, no deposits of electron dense structures (chromatin-fragment-IgG complexes10,26) in the mesangial matrix or in the GBM could be observed (Figure 7A). For the five kidneys with ISN/RPS class IV nephritis available for this type of analysis, large electron dense structures containing IgG (traced by 5-nm gold particles) were observed in the mesangial matrix (not shown; see Fenton et al10) and in the GBM (Figure 7, D, G, J, M, and P). In the control kidney, Trap1 was present in the tubular cells (Figure 7B), and in the ISN/RPS class IV Trap1 tended to be expressed at high levels (Figure 7, E, H, K, N, and Q). Although DNaseI was clearly expressed in the control kidney (Figure 7C), DNaseI was hardly detected in the ISN/RPS class IV kidneys (Figure 7, F, I, L, O, and R). These IEM-based data are highly similar to those obtained in the murine kidneys analyzed by qPCR (Figure 1) and by IEM (Figure 3). These data are further consistent with results from immunohistochemical analyses of ISN/RPS class II to III nephritic kidneys versus ISN/RPS class IV nephritic kidneys.

Discussion

The biological and pathophysiological consequences of shutdown of renal DNaseI gene expression on kidney function is immense, mainly because of retention of large undigested chromatin fragments in glomerular membranes and matrices26 and because the chromatin fragments by
themselves impose several inflammatory processes by interaction of TLR7-9 and the inflammation-related Clec4e receptor. Therefore, it is an important issue to understand why this main renal endonuclease is lost in the kidney so that we can develop new therapeutic strategies.

In the present study, we analyzed the expression profiles of the DNaseI and Trap1 genes in parallel during progression of lupus nephritis in a mouse model and in humans. We rationalized the observation of overlap and convergent transcription of the two genes and their involvement in cell death and cell survival, respectively. By qPCR analysis, we observed a systematic change in expression in all compartments of the kidney during disease progression. In pre-nephritic mice, the mRNA levels of both were moderate. At the stage of deposition of IgG complexes in the mesangium, there appeared to be a transient up-regulation of Trap1 mRNA levels, followed by shutdown of DNase1 mRNA. In the end-stage disease characterized by IgG deposits in both mesangium and GBM, even Trap1 mRNA levels are reduced and expression may eventually be completely lost. As a consequence, we observed low levels of both mRNAs in end-stage disease, but a substantially increased ratio of Trap1/DNaseI mRNA levels in Group 3 mice with severe nephritis. Thus, even though the amount of transcriptionally active chromatin is reduced, the high ratio of Trap1/DNaseI mRNA levels in the remaining active chromatin favor the model of transcriptional interference as a main cause for renal DNaseI gene shutdown. The same observations were made in ISN/RPS class IV human lupus nephritis, thus substantiating that nephritis in the (NZBxNZW)F1 model may be a valid model to understand central processes in the human disease.

The levels of DNaseI and Trap1 proteins in whole kidney were assessed by semiquantitative analysis based on Western blot analysis and DNaseI zymography and were found to

Figure 6 Immunohistochemical analyses of DNaseI and Trap1 expression in control kidneys and in kidneys from patients with lupus nephritis. By immunohistochemical analyses of human kidney sections, strong staining intensity of the DNaseI protein was observed in normal control kidneys (A) and in ISN/RPS class II kidneys (B). C: In the ISN/RPS class IV kidney, DNaseI staining was barely detectable. D–F: Immunohistochemical analyses of Trap1 showed an equal expression in control and lupus nephritis sections. G and H: Intensity of cell staining for DNaseI (G) and Trap1 (H) was quantified by IEXP-SC = AEX × ISI, where AEX is percentage cellular area of expression and ISI is immunostaining intensity. The values given are the means ± SD of IEXP-SC in 5 to 10 view fields, where 0 represents negative IEXP-SC; 1 to 100, weakly positive IEXP-SC; 101 to 200, moderately positive IEXP-SC; and 201 to 300, strongly positive IEXP-SC. Expression of DNaseI was markedly reduced in patients with ISN/RPS class IV lupus nephritis, whereas DNaseI was strongly expressed in controls and in ISN/RPS class II-III lupus nephritis. No significant difference was observed in the expression of Trap1 when comparing controls and patients with lupus. I–L: In parallel in situ hybridization analyses, expression of Trap1 and DNaseI mRNA in two human ISN/RPS class IV kidneys. The results correlated well with results from immunohistochemical analysis because DNaseI mRNA remained almost undetectable in both biopsies (I and K), whereas Trap1 mRNA could easily be detected in both (J and L, blue). Thus, the expression levels of DNaseI and Trap1 mRNA as detected by qPCR and by in situ hybridization correlated well with each other and with protein expression in situ as determined by IHC. ***P < 0.0001.
follow the steady-state level of mRNAs based on qPCR, both in the mouse model and in humans. In general, mRNA and protein levels correlate for only a fraction of expressed genes. In a study of two mouse hematopoietic cell lines representing distinct lineages of differentiation, as well as in perturbed livers, it was observed that differential expression of mRNA could only account for at most 40% of the variation of protein expression. Our observation of parallel changes in mRNA and protein levels for both DNaseI and Trap1 suggests that these genes are primarily regulated at the level of transcription, rather than posttranscriptionally.

The cellular localization of expression of DNaseI and Trap1 is widespread, and in kidneys the dominant cell type that expresses DNaseI is the tubular cells, whereas mesangial cells also express this enzyme in a normal situation. By microdissection of kidney cortex followed by qPCR and Western blot analysis, we showed that there was no overall regional difference in expression of the two genes when comparing cortex with unselected kidney tissue. Double labeling and confocal microscopy analysis of a kidney from a mouse with diffuse-proliferative nephritis, we showed that the staining pattern was mostly nonoverlapping at the single-cell level. Finally, our analyses of human tissue sections by IHC and in situ hybridization analyses distinctly showed different staining patterns. These observations argue for a model in which only one of the two genes in the gene pair (DNaseI or Trap1) is expressed in individual cells, although this model needs to be tested more rigorously.

Early lupus nephritis develops secondary to production of anti-dsDNA antibodies in (NZBxNZW)F1 mice. This is followed by immune complex formation and binding of chromatin-IgG complexes to mesangial cells, which may promote mesangial matrix proliferation. If the deposition of immune complexes exceeds the clearance capacity of the mesangial cells, the complexes may bind in glomerular matrices and activate phagocytes and the complement system. This may create the first mesangial inflammation in context of lupus nephritis. Mesangial inflammation is assumed to impose DNaseI shutdown as a consequence of up-regulation of the survival protein Trap1. This is the critical renal event responsible for progression of the early nephritic process into end-stage kidney disease. DNaseI accounts for >80% of the total renal endonuclease activity and is, together with caspase-activated DNase, the most important of the endonucleases accounting for chromatin fragmentation in context of apoptosis (reviewed in Samejima and Earnshaw and Kawane and Nagata). Without such endonucleases, chromatin remains largely unfragmented in dying cells and will be exposed in situ where the fragments are released. This has been shown in lupus nephritis and in various forms of endonuclease knockout mice (reviewed in Fismen et al). Thus, early mesangial nephritis may induce DNaseI shutdown by two theoretical pathways; either by direct down-regulation of renal DNaseI through the effect of proinflammatory cytokines or by up-regulation of the convergently encoded Trap1.

Figure 7 IEM to determine in vivo binding of IgG antibodies in electron dense structures and level and subcellular localization of DNaseI and Trap1 in human control and nephritic kidneys. Six human kidney biopsies were selected because of morphology as determined by presence of IgG and glomerular lori for deposits of electron dense structures. Results are shown from one control kidney biopsy with no sign of inflammation (A–C) and five kidney biopsies with ISN/RPS class IV lupus nephritis (D–R). No IgG or electron dense structures were observed in the control biopsy (A), whereas in the five kidney biopsies from patients with lupus nephritis ISN/RPS class IV electron dense structures were present in GBM and contained IgG (D, G, J, M, and P). These electron dense structures are recently characterized and shown to consist of chromatin fragments and IgG. Staining of Trap1 in the control kidney showed intermediate expression levels (B). Compared with the control mouse, Trap1 was much stronger expressed in the nephritic kidneys (E, H, K, N, and O). In contrast, and in harmony with data from qPCR and IHC, DNaseI was expressed in the control kidney (C), whereas DNaseI was hardly detected in the nephritic kidneys (F, I, L, Q, and R). Thus, in ISN/RPS class IV kidneys, characterized by chromatin-IgG complexes in GBM (seen as electron dense structures in the membranes, previously characterized to be constituted of chromatin fragments) DNaseI was lost. These data harmonize with the data presented on IEM analyses in murine lupus nephritis (Figure 3).
that by transcriptional interference down-regulate DNaseI. These pathways are currently under investigation in our laboratory.

Thus, although we have established that shutdown of renal DNaseI expression is a key event in progression of lupus nephritis, the regulation of the DNaseI gene is still not fully understood. On the basis of the peculiar organization of the DNaseI and Trap1 genes (the overlap of 59 nucleotides) and by data generated in this study, we have presented evidence that is consistent with regulation of both genes by transcriptional interference. Furthermore, our analyses of expression levels and cellular localization of the two proteins are consistent with the general observation in published expression databases that these two genes have a complementary expression pattern. It is quite possible that the two genes are independently regulated and that the expression patterns are a consequence of the two gene products being involved in opposing cell fates. However, the overlap between the annotated transcripts of the two genes strongly suggests that transcription of one will affect the other. Mechanistic evidence supports such a model that is based on chromatin modification (see Churchman and Weissman14), and our observations are equally consistent with such a model. Our analyses of Trap1 expression implicates this gene as a candidate gene in lupus nephritis, and our parallel analyses of the murine and human forms of lupus nephritis shows that the (NZBxNZW)F1 mouse model, in which our hypothesis can be experimentally addressed, is a valid model for these particular aspects of disease pathogenesis and progression.

Insights into processes accounting for progression of lupus nephritis at the epigenetic level,42,43 and particularly for renal DNaseI shutdown, may provide new and fundamental understanding and novel therapy modalities to lupus nephritis.

Acknowledgment

We thank Boye Schnack Nielsen (Bioneer A/S, Hørsholm, Denmark) for his help in the mRNA in situ hybridization analyses.

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