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Molecular Cloning and Expression of a Novel Peptide (LN1) Gene: Reduced Expression in the Renal Cortex of Lupus Nephritis in MRL/lpr Mouse

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A gene has been identified by mRNA differential display whose expression is reduced in the renal cortex of MRL/lpr mouse. The nucleotide sequence of the cDNA contains an open reading frame that encodes a protein of 338 amino acids (termed LN1). In situ hybridization showed that LN1 mRNA is present in glomeruli, and a 39 kDa protein was detected in the kidney by immunoblot. A human LN1 cDNA was also isolated, the deduced amino acid sequence of which is 78% identical to that of mouse LN1. Although the function of LN1 remains to be elucidated, its reduced expression may contribute to the pathogenesis of lupus nephritis.

Lupus nephritis is characterized histologically by the proliferation of mesangial cells [1]. Although pathogenesis of lupus nephritis remains unclear, disease progression of lupus nephritis is associated with the overproduction of various cytokines and growth factors that induce mesangial cell proliferation [2,3]. Because inflammatory cells that have infiltrated into the glomeruli are thought to play a role in the progression of lupus nephritis, chemoattractant factors and adhesion molecules in glomeruli are also implicated [4,5]. MRL/Mp-lpr/lpr (MRL/lpr) mice have been used as an animal model for systemic lupus erythematosus (SLE) [6]. Glomerular dysfunction, as reflected by proteinuria, starts at about three to four months of age. The renal histology, which is similar to human lupus nephritis, is a notable increase in cellularity composed of mesangial cells and macrophages within the mesangial area by the three months of age.

We have now used mRNA differential display, a polymerase chain reaction (PCR)-based method for identifying RNA molecules that are present in one population, but absent from or present in reduced amounts in another, to detect mRNAs that are aberrantly expressed in the renal cortex of the MRL/lpr mouse [7-10]. To detect genes whose expression is reduced in the early stage of lupus nephritis, we examined total RNA from the renal cortices of MRL/lpr mice at 6 weeks of age.

MATERIALS AND METHODS

Animals. Male MRL/lpr mice, MRL/Mp-+/+ (MRL/+/+) mice, BALB/c mice, were obtained from Japan SLC Inc.

mRNA differential display. Total RNA was extracted from kidney cortices of MRL/lpr mice, MRL/+/+ mice, and BALB/c mice at 6 weeks of age using RNA zol B (Tel-Test). Differential display was performed with an RNAmap kit (GenHunter). Briefly, 0.2 μg of total RNA was reverse transcribed into cDNA with MMLV reverse transcriptase and the degenerate oligo(dT) primers, T12MT, T12MC, T12MG and T12MA (where M represents a mixture of dG,

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dA, and dC). The cDNAs were then amplified by PCR in the presence of [α-^{35}S]dATP with arbitrary decanucleotide primers (AP1 to AP15 in the RNAmap kit) and T12MT, T12MC, T12MG and T12MA primers. The amplification profile consisted of denaturation at 94°C for 30 s, annealing at 40°C for 120 s, and extension at 72°C for 30 s. The PCR products were analyzed by electrophoresis on a denaturing 6% polyacrylamide gel. The reproducibility of amplification of selected products was confirmed by repeating procedure at least three times with different preparations of cDNA. Products of interest were recovered from gels and reamplified by 40 cycles of PCR.

Northern blot analysis. Total RNA (10 μg) was subjected to electrophoresis on a 1.2% agarose gel and transferred to a Hybond-N+ filter (Amersham). Probes were generated by random priming with a Megaprime kit (Amersham). After hybridization at 42°C overnight, the filters were washed at 42°C in 0.1 × standard saline citrate (SSC) containing 0.1% SDS and exposed to Kodak X-Omat film.

TA cloning and sequence analysis. Reamplified cDNA fragments were cloned into the plasmid vector pCRII with a TA cloning kit (Invitrogen). Both strands of the cDNA insert were sequenced with a Taq Dye Primer Cycle sequencing kit and a model 373A DNA sequencer (Applied Biosystems).

cDNA library screening. A BALB/c mouse kidney λgt10 cDNA library (Clontech) was screened with a subcloned PCR-derived fragment to obtain a larger cDNA clone. The DNA fragment was labeled with [α-^{32}P]dCTP with the use of a Megaprime kit. Approximately 400,000 phages were plated at a density of 20,000 plaque-forming units per 150-mm dish containing *Escherichia coli* C600 Hfl. Duplicate filters of the resulting plaques were hybridized and washed as described [10]. Positive phages were purified by an additional round of screening. Purified clones were amplified in *E. coli* C600 Hfl and DNA was isolated with a lambda trap plus kit (Clontech). The cDNA inserts were released from the λgt10 vector by digestion with EcoRI and subcloned into pUC18.

Several nondegenerate oligonucleotide primers, based on the internal sequence of the mouse cDNA, were used in conjunction with primers for the phage arms to screen a human kidney λgt10 cDNA library (Clontech) by PCR. The insert of a positive clone was isolated and sequenced.

In situ hybridization. In situ hybridization was performed with a digoxigenin-labeled cDNA probe as previously described [11]. Frozen tissue specimens were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), treated with HCl and proteinase K (Sigma), and washed in PBS. Prehybridization was performed in a buffer containing 4 × SSC, 5 × Denhardt's solution, 0.2 mg/ml of salmon testis DNA (Sigma), 0.2 mg/ml of yeast tRNA (Sigma), and 50 mM sodium phosphate (pH 8.0). cDNA was labeled with digoxigenin-dUTP by random priming with the use of a DNA labeling kit (Boehringer Mannheim). Sections were drained, hybridized overnight with the labeled cDNA probe in prehybridization buffer, and washed at room temperature with 2 × and then 1 × SSC. Digoxigenin-labeled probes were visualized by immunohistochemical staining as described previously [12]. In situ hybridization was performed on triplicate sections with identical results.

The specificity of signals was determined by performing three control experiments: (1) Sections were treated with RNase (1.0 mg/ml) (Boeringer Mannheim) at 37°C for 30 min after proteinase K digestion. (2) Hybridization was performed in the presence of a 100-fold excess of unlabeled probe. (3) Hybridization was performed with male germ cell-associated kinase (mak) cDNA probe [13].

Production of polyclonal antibodies and immunoblot. Polyclonal antibodies were prepared by immunizing New Zealand White rabbits with synthetic peptides, peptide 108-122 (KQDYMPSDRMVSKIQ) or peptide 160-173 (NKPPDVGKKTSRHP) in complete Freund's adjuvant. The antibody for each peptide was purified by affinity chromatography with the corresponding peptide conjugated Sepharose 4B (Pharmacia) column. For immunoblot, protein extracts were electrophoresed in SDS-4/20% polyacrylamide gel and the resolved proteins transferred electrophoretically to a

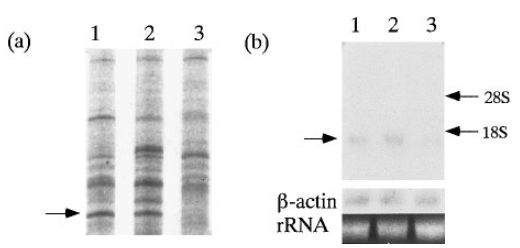

FIG. 1. (a) Differential display of cDNA fragment whose expression was reduced in MRL/lpr mouse. Intensity of the band (arrow) was weak in the renal cortex of MRL/lpr mouse (lane 3), compared with that of BALB/c mouse (lane 1) or MRL/+/+ mouse (lane 2). (b) Northern blot analysis of gene expression detected by differential display. Total RNA was obtained from renal cortex of BALB/c mouse (lane 1), MRL/+/+ mouse (lane 2), and MRL/lpr mouse (lane 3). Decreased expression in MRL/lpr mouse was confirmed.

FIG. 2. Nucleotide and deduced amino acid sequences of LN1. The translation stop codon is indicated by an asterisk. A putative polyadenylation signal is underlined.

nitrocellulose membranes (Amersham). Membranes were immunoblotted using affinity-purified antibodies, goat anti-rabbit IgG conjugated to horseradish peroxidase and the ECL detection system (Amersham).

RESULTS AND DISCUSSION

A total of 15 arbitrary primers were used in combination with each of four T12MN primers for differential display analysis. Each differential display lane yielded 100 to 200 discrete

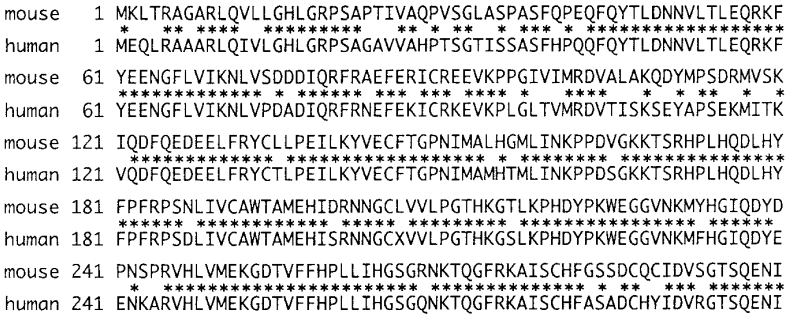

FIG. 3. Comparison of the deduced amino acid sequence of the human LN1 cDNA with that of the mouse LN1. Identical amino acids are indicated by asterisks.

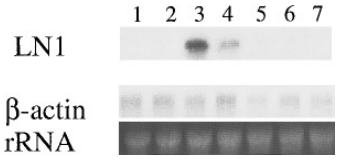

FIG. 4. Tissue-specific expression of LN1 mRNA. Transcripts of LN1 gene were undetectable or present at low concentrations in brain (lane 1), heart (lane 2), lung (lane 5), spleen (lane 6), and skeletal muscle (lane 7). LN1 mRNA was present at a relatively high concentration in kidney (lane 3) and liver (lane 4).

bands, allowing evaluation of >10,000 RNA species. Four bands showed marked, reproducible differences between kidney cortices of MRL/lpr mice and control mice. The intensity of one of these bands was reduced and that of the other three bands was increased in MRL/lpr mice. Northern blot analysis with the probe prepared from the band whose intensity was reduced in MRL/lpr mice also revealed the reduced expression of the corresponding gene (Fig. 1a and 1b). Repression of this gene expression was further confirmed by Northern analysis of five different MRL/lpr mice (data not shown).

The cDNA fragment corresponding to this gene was subcloned into the pCRII vector and sequenced. The cDNA contained a typical polyadenylation signal 14 to 19 bp upstream of the poly(A) tail. To obtain a full-length cDNA, we screened a BALB/c mouse kidney λgt10 cDNA library with the subcloned fragment. The full-length nucleotide sequence contained an open reading frame that encodes a protein of 338 amino acids with a predicted molecular mass of 39 kDa (Fig. 2). The presumed initiation site is the first methionine codon and is flanked by a consensus sequence for translation initiation [14]. A computer search of nucleotide and

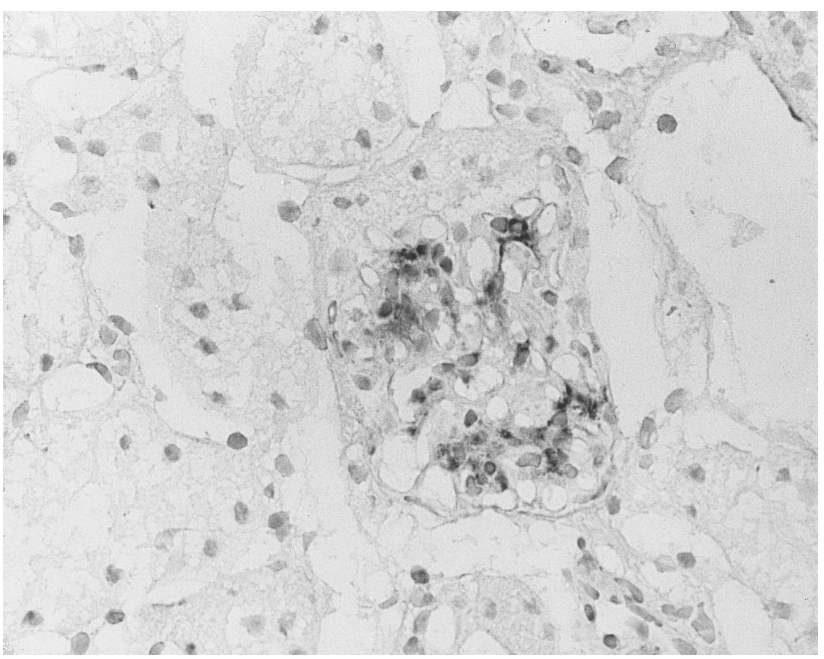

FIG. 5. In situ hybridization of LN1 mRNA in the kidney. In glomeruli, probably mesangial areas were stained for LN1 mRNA, while only nuclei were stained by methylgreen in Bowman's capsule and tubular epithelial cells.

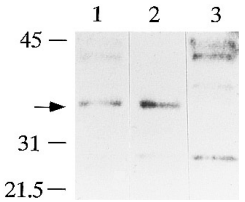

FIG. 6. Immunoblot of LN1 in the kidney. 39 kDa band was detectable with polyclonal antibodies prepared against peptide 108-122 (lane 1) or peptide 160-173 (lane 2). The band was undetectable with control antibody (lane 3).

protein databases did not reveal any homology to previously reported genes of known function. We termed the encoded protein lupus nephritis-associated peptide (LN1).

Sequence analysis of human LN1 cDNA revealed that the nucleotide sequence of the open reading frame is 80% identical to that of the mouse cDNA; the deduced amino acid sequence is 78% identical to that of mouse LN1 (Fig. 3). This conservation indicates the functional importance of this protein.

Transcripts of LN1 were undetectable or present at low concentrations in brain, heart, lung, spleen, and skeletal muscle; in addition to kidney, the transcripts were present at a relatively high concentration in liver (Fig. 4). In situ hybridization revealed the presence of LN1 mRNA in glomeruli, mostly localized to mesangial cells; no signal was detected in Bowman's capsule or the interstitium (Fig. 5). Hybridization signals were not detected when tissue sections were treated with RNase before hybridization or when hybridization was performed with mak cDNA probe or in the presence of a 100-fold excess of unlabeled probe. Polyclonal antibodies prepared against synthetic peptides detected 39 kDa protein on immunoblot analysis of protein extracts prepared from BALB/c mouse kidney (Fig. 6).

The LN1 gene is reduced in the renal cortex of MRL/lpr mice during the early stage of glomerulonephritis. The possibility that the reduced LN1 expression is a consequence of glomerular injury is not completely excluded in this study. However, we have further demonstrated that the expression level of LN1 is relatively high in kidney and LN1 mRNA is present in glomeuli, mostly localized to mesangial cells. These findings suggest that LN1 produced in resident glomerular cells directly affect the pathophysiology of lupus nephritis by contributing to processes such as mesangial cell proliferation, mesangial matrix expansion, or the infiltration of inflamatory cells into glomeruli. A mutation in the Fas apotosis gene is the lpr mutation, suggesting that autoreactive T cells, which escape from a normal apoptotic process, have the increased adhesiveness to the renal tissue and promote renal injury [15]. The lpr gene was on the mouse chromosome 19, whereas over 50% of the variance in nephritis is attributable to quantitative trait loci on mouse chromosomes other than 19 by analyzing the backcross mice [16]. Despite massive lymphoproliferation in several syngenic mice with the lpr gene (C3H, B6, AKR, and NZB), only MRL-lpr mice have severe renal injury [17]. Therefore, some unknown factors specifically associated with the progression of lupus nephritis in MRL/lpr mice exisit.

In summary, we have isolated a novel peptide, LN1, expressed exclusively in glomeruli. Repression of LN1 expression may affect the progression of lupus nephritis. We are concerned with the relation between human LN1 and the severity of renal injury in the course of SLE. This point will avail further characterization of LN1.

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