Brief Report

A CTG Polymorphism in the CNDP1 Gene Determines the Secretion of Serum Carnosinase in Cos-7–Transfected Cells

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Recently, we demonstrated that a polymorphism in exon 2 of the serum carnosinase (CNDP1) gene is associated with susceptibility to developing diabetic nephropathy. Based on the number of CTG repeats in the signal peptide, five different alleles coding for 4, 5, 6, 7, or 8 leucines (4L–8L) are known. Diabetic patients without nephropathy are homozygous for the 5L allele more frequently than those with nephropathy. Since serum carnosinase activity correlates with CNDP1 genotype, we hypothesized in the present study that secretion of serum carnosinase is determined by the CNDP1 genotype. To test this hypothesis, we transfected Cos-7 cells with different CNDP1 constructs varying in CTG repeats and assessed the expression of CNDP1 protein in cell extracts and supernatants. Our results demonstrate that CNDP1 secretion is significantly higher in cells expressing variants with more than five leucines in the signal peptide. Hence, our data might explain why individuals homozygous for the 5L allele have low serum carnosinase activity. Because carnosine, the natural substrate for carnosinase, exerts antioxidative effects and inhibits ACE activity and advanced glycation end product formation, our results support the finding that diabetic patients homozygous for CNDP1 5L are protected against diabetic nephropathy. *Diabetes* 56:2410–2413, 2007

Diabetic nephropathy is the most frequent cause for end-stage renal disease in the Western world (1) .The incidence of diabetic nephropathy is ~40% in type 1 and type 2 diabetic patients (2). Although major risk factors for development and progression of diabetic nephropathy (e.g., poor glycemic control and hypertension) have been identified, diabetic nephropathy can still develop in diabetic patients with well-controlled blood glucose concentrations (3). Also, in hypertensive diabetic patients, the speed of renal function deterioration can be reduced by appropriate treatment, but treatment as such does not eliminate the susceptibility to develop diabetic nephropathy (4).

A number of epidemiologic studies have suggested that susceptibility to developing diabetic nephropathy is genetically determined (5,6). In a linkage analysis performed on 18 Turkish families with type 2 diabetes and nephropathy, we have previously identified a susceptibility locus for diabetic nephropathy on chromosome 18q22.3–q23. Association between diabetic nephropathy and this locus was subsequently confirmed in Pima Indians (7) and African Americans (8). Recently, we have narrowed down this locus and found that susceptibility to develop diabetic nephropathy was related to the presence of a polymorphism in a single gene, the CNDP1 gene (9), encoding the serum carnosinase protein.

Serum carnosinase, a dipeptidase belonging to the M20 metalloprotease family, is the rate-limiting enzyme for the hydrolysis of carnosine into β-alanine and histidine. Carnosine is an antioxidant (10), inhibits nonenzymatic glycosylation (11), prevents high glucose–induced extracellular matrix accumulation (9), and prevents cross-linking of proteins caused by reactive aldehydes (11). In light of these biochemical properties, it becomes clear that carnosine might be a modulator of hyperglycemia-induced damage. Moreover, since carnosine is also a natural ACE inhibitor (12) and since pharmacologic inhibition of the renin-angiotensin system is known to decline progression of diabetic nephropathy (13,14), carnosine can be considered a protective factor for diabetic nephropathy. It must be stressed, however, that the findings related to carnosine as an ACE inhibitor must be interpreted with some precaution, as the concentration of carnosine to inhibit ACE is ~5.26 mmol/l and thus not in a physiological range (12). Furthermore, although serum carnosine concentrations rapidly increase after ingestion of meat, serum carnosin concentrations are in general very low and decrease after meat ingestion within 5 h to almost undetectable levels (15).

Similar to all secreted proteins, CNDP1 is synthesized as a precursor containing an NH_2-terminal signal peptide sequence. This enables the nascent protein to be targeted to the endoplasmatic reticulum. In general, signal peptides contain a hydrophobic stretch flanked by polar NH_2- and COOH-terminal domains (16). The hydrophobic domain is of utmost importance for the function of the signal peptide (17), i.e., for targeting the protein into the secretory pathway. There is a $(CTG)_n$ polymorphism (D18S880) located within the signal peptide of the human serum

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PNGase, peptide *N*-glycosidase; SNP, single nucleotide polymorphism.

carnosinase. This results in a signal peptide containing a hydrophobic stretch of 4, 5, 6, 7, or 8 leucines (4L–8L alleles). Since diabetic patients homozygous for the 5L allele of CNDP1 are protected from diabetic nephropathy and since serum carnosinase activity is low in these patients, we tested whether carnosinase secretion is determined by the CNDP1 genotype. Another polymorphism within the CNDP1 signal peptide is the arginine to glycine transition in codon 6 (R6G; c.16G>A; rs11151964). The secretion efficiency of this variant, which has not been mentioned in relation to diabetic nephropathy before, was investigated in the present study as well.

RESEARCH DESIGN AND METHODS

Construction of the CNDP1 gene variants. A cDNA clone of the CNDP1 gene (entry no. BX094414, Deutsches Ressourcenzentrum für Genomforschung Library 983) containing six CTG repeats was kindly provided by Dr. M. Moeller (Department of Nephrology, Rheinisch-Westfälische Technische Hochschule, Aachen, Germany) and used as a template. In three sequential PCR steps, CNDP1 variants containing 4, 5, 6, 7, or 8 CTG repeats were constructed. PCRs were performed according to standard methods; sequences of the primers used in each step are depicted in Table 1. The 6L variant was constructed with either adenine (A) or guanine (G) at position +16. All CNDP1 constructs were cloned into the pCS2 + MT vector to generate an myc-tagged carnosinase protein. Transformation was performed in competent *Escherichia coli* (TOP10F'; Invitrogen, Karlsruhe, Germany) using blue-white screening (Sigma, Steinheim, Germany).

Cell culture and transfection. Cos-7 cells (Invitrogen) were transiently transfected using Nanofectin (PAA Laboratories, Pasching, Austria) as recommended by the manufacturer. Cell supernatants were collected, and cell lysates were prepared 48 h after transfection.

Western blot analysis. Samples were denatured, separated by an 8% SDS-PAGE, and transferred to a polyvinylidine fluoride membrane (Roche, Mannheim, Germany) by means of semidry blotting. Three different antibodies were used for the detection of carnosinase: anti-carnosinase K18K polyclonal antibody (kindly supplied by M. Teufel, Sanofi Synthelabo, Recherche, Strasbourg), anti-carnosinase polyclonal (R&D Systems, Wiesbaden-Nordenstadt, Germany), or anti-myc (Abcam, Cambridge, U.K.). After incubation with appropriate secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany), antibody binding was visualized by chemiluminescence (PerkinElmer, Boston, MA). Intensity of the bands was measured by densitometry using ImageJ 1.36b software. In some experiments, aliquots of the protein samples were deglycosylated with peptide *N*-glycosidase (PNGase) F (New England Biolabs, Frankfurt, Germany) before SDS-PAGE.

Statistical analysis. Statistical analysis was performed with SAS software (version 8.02; SAS Institute, Cary, NC). To investigate the influence of leucine adjusted for time on the percentage of carnosianse, a two-way ANOVA has been used. The difference of secretion efficiency of ≤5L to ≥6L was shown by planned linear contrasts using the SAS GLM procedure together with contrast statements. Test results with $P < 0.05$ were considered to be significant.

RESULTS

To test the hypothesis that the length of the hydrophobic leucine stretch in the signal peptide of serum carnosinase influences the efficiency of carnosinase secretion, we constructed myc-tagged CNDP1 gene variants differing in the number of CTG repeats in the signal sequence. Constructs encoding 4, 5, 6, 7, or 8 leucines, representing all thus far known alleles for CNDP1, were cloned into Cos-7 cells.

In cell extracts of CNDP1-transfected cells, immune-reactive bands were detected in Western blot analysis with apparent molecular weights of 86 and 88 kDa, respectively. In supernatants of the transfected cells, a single-dominant band of ~92 kDa was present. Similar data were obtained when monoclonal anti-myc or polyclonal anti-carnosinase antibodies were applied to the Western blot. In cells transfected with the empty vector, no immune-reactive bands were detected (Fig. 1). After PNGase treatment of

TABLE 1
Signal sequence of the CNDP1 cDNA clone 5′ATGGATCCCAAACTCAGGAGAARGGCTGCGTCCCTGCTGGCTGTGCTGCTGCTGCTGCTGCTGGAGCGCGGCATGTTCTCC **3′**

Step 1: PCR forward primer	Step 2: PCR forward primer	Step 3: PCR forward primer/Tm
5′(D)GAGCGCGGC3′	5′ATGGATCCCAAACTCAGGAGAATGGCTGCGTCCCTGCTGGCTGTG(D)GAGCGCGGC3′	5′CAGCCCATCGATATGGATCCCAAACTC(II)GGAGA3′

Reverse primer for all steps: 5′AGCTTTAAATCGATGGAGCTGGCCATCTCTAA3′. (I), length of leucine repeat: for 4L, CTGCTGCTGCTG; for 5L, add CTG; for 6L, add CTGCTG; for 7L, add CTGCTGCTG; and for 8L, add CTGCTGCTGCTG. (II), A/G variant: for the arginine polymorphism, add A at this position; add G for the glycine polymorphism.

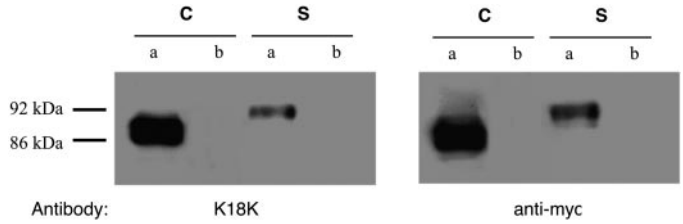

FIG. 1. Western blot analysis of cell protein (C) and supernatant (S) of Cos-7 cells, transfected either with 6L (*a*) or empty vector (*b*). Immunostaining with anti-CNPD1 antybody (K18K) and anti-myc antibody is shown. Immunostaining with anti-CNDP1 of R&D showed similar results (blot not shown).

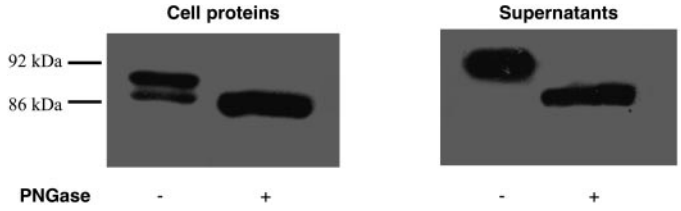

FIG. 2. Analysis of *N*-glycosylation of carnosinase expressed in cell extracts and supernatants. Aliquots of cell extract and supernatant of transfected cells (6L) either were (+) or were not (−) treated with PNGase to remove *N*-glycosyl residues. Note that after PGNase treatment, the molecular weights of carnosinase in supernatants and cell extracts were equal, whereas this was not found in untreated samples.

supernatant and cell extracts, a single band of ~86 kDa was found (Fig. 2), suggesting differences in *N*-glycosylation between both samples.

The percentage of carnosinase secreted in the supernatants of all transfectants was assessed by densitometry (Fig. 3*A*). In Cos-7 cells expressing CNDP1 containing a 4L or 5L stretch, more carnosinase was found in the cell extracts than in the corresponding supernatants (≤5L vs. ≥6L, $P < 0.0001$). In contrast, Cos-7 cells expressing gene variants of CNDP1 with more CTG repeats clearly secreted carnosinase much better. With increasing length of the CTG repeat, the percentage of secreted carnosinase increased (Fig. 3*B*). In cells transfected with a CNDP1 construct lacking the complete signal peptide, no carnosinase could be detected in supernatants (Fig. 3*A*).

To test if the A to G polymorphism at position +16 (R6G) was also relevant for carnosine secretion, CNDP1 6L constructs were generated with either adenine or guanine at position +16. No difference in secretion efficiency of carnosinase was found for these variants (6A = 47.4 ± 6.0 and 6G = 46.1 ± 5.2%, $P = 0.2707$; data not shown).

DISCUSSION

Recently, we have demonstrated that a polymorphism situated in the signal peptide of CNDP1 is associated with susceptibility to developing diabetic nephropathy (9). Association of this polymorphism with diabetic nephropathy has recently been confirmed in European Americans (18). Five different variants of the signal peptide, which all differ in the length of the hydrophobic leucine stretch, were identified in population screenings. A null allele with an insertion of five nucleotides within the leucine repeat was also detected, but because of a frame shift this allele does not code for a proper carnosinase protein (19).

The importance of the $(CTG)_n$ repeat located in the hydrophobic core of the signal peptide is that it influences the efficiency of carnosinase secretion, as demonstrated in the present study. While carnosinase encoded by CNDP1 containing the $(CTG)_4$ and $(CTG)_5$ repeat is poorly secreted, a more efficient secretion occurs when the CNDP1 variants contain more than six CTG repeats. Our results are in line with theoretical calculations based on the G. von Heijne scores. Moreover, our data provide experimental evidence explaining the association of the CNDP1 genotype and serum carnosinase activity (9).

In the signal peptide of CNDP1, there is an additional single nucleotide polymorphism (SNP) present, resulting in either adenine or guanine at position +16 (R6G). Because the A/G SNP and $(CTG)_n$ repeat are not in disequilibrium, all possible allelic combinations of A/G and $(CTG)_n$ do occur. We now also show that the A/G SNP does not influence carnosinase secretion efficiency.

Posttranslational modification of serum carnosinase has previously been demonstrated and includes both *N*- and *O*-glycosylation (20). Three putative *N*-glycosylation sites are found in the carnosinase sequence. Secreted and nonsecreted carnosinase differ in the extent of *N*-glycosylation, as could be demonstrated by PNGase treatment. This therefore explains the difference in apparent molecular weight between both. We are aware that *N*-glycosylation can influence protein secretion (21). However,

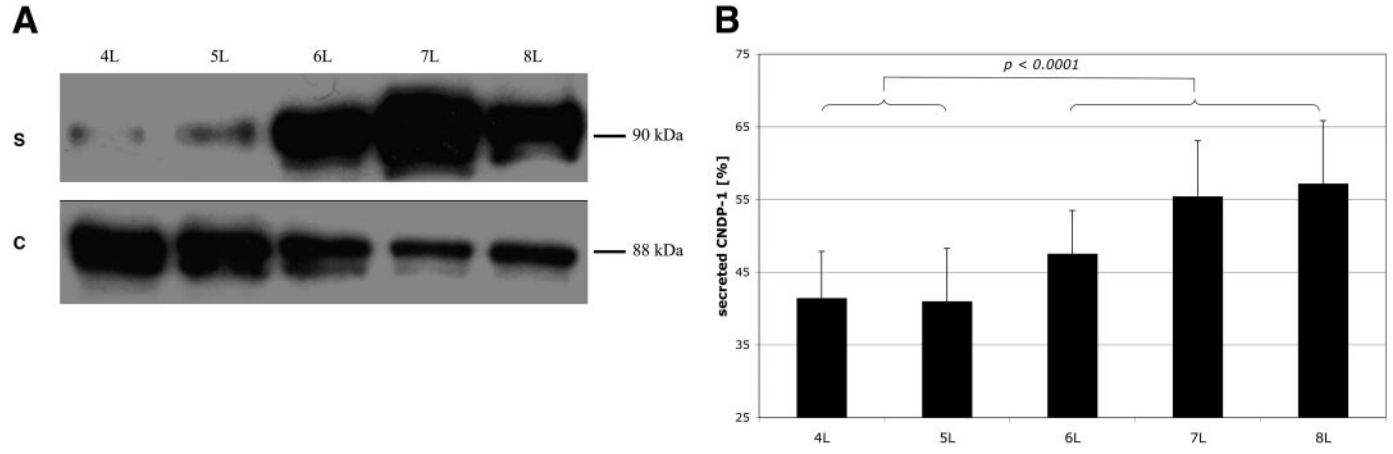

FIG. 3. *A*: Representative Western blot analysis of supernatant (S) and cell protein (C) obtained from Cos-7 cells transfected with CNDP1 variants containing 4L–8L repeats. *B*: Analysis of secretion efficiency. The expression of carnosinase in supernatant and cell extracts was measured by densitometry. The results are expressed as mean percentage secretion ± SD. The numbers of transfections used in this analysis for each of the CNDP1 variants were $n = 7, 8, 8, 7$, and 3 for 4L, 5L, 6L, 7L, and 8L, respectively.

secreted carnosinase from all different CNDP1 variants was to a similar extent *N*-glycosylated, as no difference in molecular weight was detected between the variants. The importance of *N*-glycosylation for secretion efficiency of carnosinase per se was not tested in this study because genetic evidence for the association of serum carnosinase activity and *N*-glycosylation of carnosinase is lacking.

Serum carnosinase deficiency is associated with mental retardation and sensory peripheral neuropathy (22,23). Also, alcoholics with abnormal muscle biopsy findings have low serum carnosinase activity (24); however, it is unclear whether the $(CTG)_n$ polymorphism also plays a role in serum carnosinase activity in this group of individuals.

In conclusion, we show that the $(CTG)_n$ polymorphism in the signal peptide of the CNDP1 gene is functional, determining secretion efficiency of serum carnosinase. Our data also explain why serum carnosinase activity in individuals homozygous for CNDP1 5L is low, resulting in relatively high levels of the renoprotective dipeptide carnosine.

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