Organization of the human carboxypeptidase E gene and molecular scanning for mutations in Japanese subjects with NIDDM or obesity

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Summary Insulin is synthesized in the pancreatic beta cell as a larger precursor molecule proinsulin which is converted to insulin and C-peptide by the concerted action of prohormone convertase 2 (PC2), prohormone convertase 3 (PC3) and carboxypeptidase E (CPE). One of the features of non-insulin-dependent diabetes mellitus (NIDDM) is an elevation in the proinsulin level and/or proinsulin/insulin molar ratio suggesting that mutations in these three proinsulin processing enzymes might contribute to the development of NIDDM. The identification of a mutation in the CPE gene of the fat/fat mouse which leads to marked hyperproinsulinaemia and late-onset obesity and diabetes is consistent with a possible role for mutations in CPE in the development of diabetes and obesity in humans. In order to test this hypothesis, we have isolated and characterized the human CPE gene and screened it for mutations in a group of Japanese subjects with NIDDM and obesity. The human CPE gene consists of 9 exons spanning more

than 60 kb. Primer extension analysis identified the transcriptional start site at –141 bp from the translational start site. Single strand conformational polymorphism analysis and nucleotide sequencing of the promoter and entire coding region of the CPE gene in 269 Japanese subjects with NIDDM, 28 nondiabetic obese subjects and 104 nonobese and nondiabetic controls revealed three nucleotide changes, a G-to-T substitution at nucleotide –53, a G-to-A substitution at nucleotide –144 (relative to start of transcription) in the promoter region and a silent G-to-A substitution in codon 219. None of the nucleotide substitutions were associated with NIDDM or obesity. Thus, genetic variation in the CPE gene does not appear to play a major role in the pathogenesis of NIDDM or obesity in Japanese subjects. [Diabetologia (1998) 41: 701–705]

Keywords Carboxypeptidase E, processing, NIDDM, obesity, genetics.

Insulin is synthesized in the pancreatic beta cell as a larger precursor molecule proinsulin which is processed to insulin by the combined action of prohormone

convertase 2 (PC2), prohormone convertase 3 (PC3) and carboxypeptidase E (CPE) [1–4]. Proinsulin has about 10% of the potency of insulin and its conversion to insulin is necessary for full biological activity. Recent studies have shown that human proinsulin is first cleaved by PC3 to form 32–33 split proinsulin followed by the rapid removal of Arg31Arg32 by CPE to generate des 31, 32 proinsulin, and subsequent processing of this intermediate product by PC2 and CPE generates insulin and C-peptide [5].

Elevated proinsulin level and/or proinsulin/insulin molar ratio is often observed in non-insulin-dependent diabetes mellitus (NIDDM) subjects [6–8]. Recent studies suggest that mutations in PC2, PC3 and/or CPE might be responsible for the hyperproinsu-

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Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus; bp, base pair; PCR, polymerase chain reaction; PC2, prohormone convertase 2; PC3, prohormone convertase 3; CPE, carboxypeptidase E; SSCP, single strand conformational polymorphism.

linaemia, insulin deficiency and NIDDM [9]. A population association study showed a significant difference in the distribution of alleles at a simple tandem repeat DNA polymorphism in intron 2 of the PC2 gene between NIDDM and control groups implicating PC2 gene in the development of NIDDM [10]. However, single strand conformational polymorphism (SSCP) analysis [11] of all the exons of the PC2 gene did not reveal any mutations that could readily explain the observed association [10]. Similar studies of the human PC3 gene revealed two amino acid polymorphisms, Arg/Gln53 and Gln/Glu638, neither of which was associated with NIDDM, proinsulin levels or the proinsulin/insulin ratio [12].

Carboxypeptidase E (CPE) is a member of the metallocarboxypeptidase gene family and is involved in the trimming of paired basic residues at the C-terminus of prohormone-derived peptides [13]. This enzyme is also known as carboxypeptidase H and enkephalin convertase. A mutation in CPE, Ser202Pro, is responsible for the phenotype of the fat/fat mouse, which is characterized by marked hyperproinsulinaemia and develops late-onset obesity and diabetes [14]. Several lines of evidence suggest that this mutation abolishes CPE [15]. Although the relationship between loss of CPE activity and the later appearance of obesity-diabetes syndrome remains unclear, mutations in the CPE gene might contribute to the development of obesity and NIDDM in humans. Here we report on the organization of the human CPE gene and the results of a search for mutations in this gene in Japanese subjects with NIDDM and obesity.

Subjects and methods

Subjects. We recruited 269 Japanese subjects with NIDDM and 28 obese subjects without diabetes mellitus (simple obesity) from the Hospital of Wakayama University of Medical Science. We chose 104 non-obese, non-diabetic control subjects

Table 1. Clinical features of study population

	n (M/F)	age	BMI (kg/m ²)	HbA _{1c} (%)
NIDDM				
with obesity	146 (75/71)	54.0 ± 1.4	31.5 ± 0.3	7.9 ± 0.2
without obesity	123 (79/44)	62.6 ± 0.9	21.8 ± 0.2	7.2 ± 0.1
Simple obesity	28 (18/10)	51.2 ± 2.3	29.2 ± 0.6	5.3 ± 0.1
Control	104 (51/53)	61.4 ± 1.3	22.0 ± 0.3	5.3 ± 0.1

(Means ± SEM)

using the following criteria: aged over 40 years, no family history of diabetes mellitus, and a random plasma glucose of less than 6.7 mmol/l. The clinical features of the subjects are summarized in Table 1. Obesity was defined as body mass index (BMI) more than 26 kg/m². Among the NIDDM subjects 53% had a family history of diabetes; i.e. a sibling, parent, grandparent, aunt or uncle with diabetes. At the time of recruitment, informed consent was obtained from each subject.

Isolation of the human CPE gene. Approximately 3×10^9 plaque-forming units of the human genomic library obtained from Clontech (catalogue no. HL1067J; Palo Alto, Calif., USA) were screened by hybridization using the human CPE cDNA (American Type Culture Collection, Rockville, Md., USA) as a probe. Positive clones were replated and rescreened with the same cDNA probes, and this procedure was repeated until all the plaques on the plate hybridized with the cDNA probes. DNA was isolated from the clones, and Southern blots were performed after digestion with various restriction enzymes. DNA was sequenced using the dideoxynucleotide method with either universal or specific primers after subcloning various restriction fragments of each genomic clone into pGEM-3Z, 5Z or 7Z (Promega, Madison, Wis., USA). The positions of exon-intron junctions were determined by comparison of the genomic and cDNA sequences.

Identification of transcriptional start site by primer extension analysis. For primer extension analysis, a 28-residue oligonucleotide (5'-CTCTTTTGTCTGCCCGGCCCTGGGCTTA-3') complementary to nucleotides -47 to -74 bp relative to the translational start site of the human CPE gene was labelled using T4 polynucleotide kinase and [γ -³²P]ATP, annealed to 2 µg of human pituitary gland polyA⁺ RNA (Clontech, catalogue no. 6584-1), and extended with avian myeloblastosis vi-

Table 2. Primers used to amplify the promoter region and exons of the human CPE gene

Exon	Forward primer	Reverse primer	Product (bp)	MgCl ₂ (mmol)	Annealing temp. (°C)
Pro1	ATTTGGCTGTTGACGTGGTT	ACCTTCCCCGGCTGAATGAGTGT	216	1.5	67
Pro2	TCCTCGCAGTGTTTCTCCT	GCGGGCGGCCTCTTTTGTCT	222	4	63
1a	TAGAGGCTGGTGCGGAACCT	CACAGAGCCAGCAGCGCGCT	156	4	63
1b	AAGAGGCCGCCGCGTAGGA	CGCAGCTCGGGGTAGCGGTGGTACT	229	4	61
1c	CGGCATCTCCTTCGAGTACC	CGGGATCGCCAGGGCTGTC	202	4	61
2	AAACATCTCCATGCTATCTA	CACGCCTGATCTGAGCTGCC	259	1.5	50
3	AAGTGACATAATAATAAGCA	ATTTCTTTAGGCTAGAGACG	270	1.5	55
4	CAAGTGATCATACCAATGAT	GTTATAAACTTTGAACCAAT	220	1.5	50
5	GTCTGTGTCTGTTTCTTTAA	ACAATCACATAATCAGAGAG	260	1.5	55
6	TCTTGATTGAGAAGACTAGC	TGAATGCTTGTAACCTTGC	264	2	55
7	ATTTAAGGCCATTCTATAA	ATAACGTAGAGCCTCCAATT	200	1.5	55
8	TTGTGATTTGATATTCTGCC	ACTTCAGTATGCCTGACAAT	220	1.5	55
9	AACACATGCGATTTGATAAT	ATAGATAGATTAAAGCAGC	180	2	55

Pro1 and Pro2 amplify the promotor region.

Fig. 1. Map of the human carboxypeptidase E gene. The 9 exons are indicated. The transcriptional start site is the 0 coordinate. The vertical lines in each clone represent natural EcoRI sites

rus reverse transcriptase. The products were separated by electrophoresis in 5 % polyacrylamide/8 mol urea gel.

SSCP analysis of the human CPE gene. Genomic DNA was extracted from peripheral leucocytes [16]. PCR primers were selected to amplify promoter region and each of the 9 exons and flanking intron sequences of the human CPE gene (Table 2). The PCR was conducted in a volume of 100 µl containing one µg of DNA, 30 pmol of each fluorescence-labelled primer, 2 mmol/l of each dNTP, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 to 4 mmol/l MgCl₂ and 0.2 µl of Taq polymerase (Perkin-Elmer, Norwalk, Conn., USA). The denatured PCR products were separated by 6 % polyacrylamide gel at two different temperatures (4 °C and 22 °C) using an ALFred DNA sequencer (Pharmacia Biotech, Uppsala, Sweden). When shifted bands were observed, the sequence of the PCR product was determined directly and after subcloning into pGEM-3Z.

Results

Isolation and characterization of the human CPE gene. The human CPE gene consists of 9 exons spanning more than 60 kb (Fig.1, Table 3; the sequence has been deposited in the GenBank data base with accession numbers AB006890-AB006898). The

Fig.2. Identification of the transcriptional start site by primer extension analysis. The first four lanes represent the sequence of the genomic DNA obtained using the same primer for primer extension. The transcriptional start site is indicated by the arrow

boundaries of the exon-intron junctions conform to the GT-AG rule [17]. The exon-intron organization of the human CPE gene is identical to that of the rat [18] and the nucleotide sequence is identical with that of the previously reported human cDNA [19]. Primer extension analysis identifies the transcriptional start site at nucleotide -141 bp from the translational start site (Fig.2). The transcriptional start sites of the human and rat genes are in similar locations (Fig.3) [18]. The putative promoter region of the human CPE gene is GC rich, containing 70 % GC resi-

Table 3. Exon-intron organization of the human CPE gene

Exon no.	Exon size bp	Sequence at exon-intron junction		Intron size kb	Amino acid interrupted
		5'splice donor	3'splice acceptor		
1	250	AGC CTG gtaagg	ttttag GTG AGC	> 20	Gly 103
2	197	TCT CAG gtgagt	ttttag CCT GGT	2.0	Gly 168
3	164	ACA AAG gtagtg	gaacag CTT GCT	> 22	Lys 224
4	118	GGA GTG gtagtg	ttctag GTA GTA	2.0	Gly 264
5	183	CTG GAG gtgagt	tggcag GAT GCA	2.5	Gly 324
6	140	GAG CAG gtaaac	tcacag ATA CAC	> 15	Asn 371
7	100	CAT CCG gtgggt	tcacag ATA CAC	3.0	Ala 405
8	119	GCT GGG gtaagt	ttttag GTT GAT	2.5	Gly 444
9	> 500				Phe 476

The sites at which introns interrupt the mRNA and protein sequence are indicated. Exon sequences are in capital letters, and intron sequences are in lowercase letters. The sizes of introns were estimated by restriction mapping

Fig. 3. Comparison of the sequences of the promoter regions of human and rat [18] carboxypeptidase E genes. The H and R represent human and rat sequences, respectively. Gaps in the alignment are indicated by dashes. Column means identical nucleotides. The putative transcriptional start sites (*) are shown. The location of the oligonucleotide used for primer extension analysis is shown by underlining. The GC box-like sequence found only in the rat gene is boxed. Two cyclic AMP responsible elements are bolded

duces over nucleotides -1 to -150 bp relative to the transcriptional start site and like the rat gene does not contain a TATA motif. Like PC2 and PC3, however, the promoter region of the human CPE gene contains two cyclic AMP responsible elements (CREs) [12, 20]. There is 60% identity between the human and rat promoter sequences.

Genetic variation in the human CPE gene. We scanned the CPE gene of 269 Japanese NIDDM patients, 28 obese subjects without diabetes mellitus and 104 non-obese, non-diabetic control subjects for mutations using SSCP analysis. Three variants were observed : a G -to-T substitution at nucleotide -53, a G-to-A substitution at nucleotide -144 in the promoter region and a silent G-to-A substitution in codon 219 of exon 4. There was no association between these three sequence variants in the CPE gene and NIDDM or obesity (Table 4).

Carboxypeptidase E (CPE) is involved with the biosynthesis of many peptide hormones and neurotransmitters, removing C-terminal basic amino acids remaining after endoproteolytic processing [13]. CPE has been localized on chromosome 4q28 by fluorescence in situ hybridization [21]. A loss of function mutation in CPE is responsible for the phenotype of the fat/fat mouse which includes diabetes and obesity [14]. Recently it was reported that CPE is a sorting receptor and hyperproinsulinaemia seen in the fat/fat mouse was derived from missorting of proinsulin to secretory pathways [22]. Although the relationship between the loss of CPE activity and obesity-diabetes syndrome is unclear, the loss of CPE activity does cause defects in the processing of prohormone forms of neuropeptides associated with the control of satiety, such as α -MSH and GLP-1. Therefore the mutations in the CPE gene may be responsible for the development of NIDDM or obesity in humans. To examine this hypothesis, we first identified the human CPE gene which will facilitate molecular genetic studies of NIDDM, obesity and other metabolic disorder. The results of the SSCP analysis presented here indicate that structural changes in the CPE gene is not a major factor contributing to the development of NIDDM and obesity in Japanese subjects. However an abnormal CPE expression may play a role in the development of NIDDM or obesity. To

Table 4. Sequence variation in the human CPE gene

	promotor region nucleotide – 53		promotor region nucleotide – 144		exon 4 codon 219	
	G	T	G	A	G	A
NIDDM						
with obesity	0.997	0.003	1.0	0	0.976	0.024
without obesity	1.0	0	0.996	0.004	0.984	0.016
Simple obesity	1.0	0	1.0	0	0.964	0.036
Control	0.995	0.005	1.0	0	0.981	0.019

clarify this issue, familial linkage analysis and association studies with polymorphism located near to CPE will be necessary. Moreover the results here do not preclude a role for this gene in rare forms of diabetes and obesity as seen in the fat/fat mouse, or in patients with hyperproinsulinaemia and obesity [23, 24].

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