Human Prohormone Convertase 3 Gene

Exon-Intron Organization and Molecular Scanning for Mutations in Japanese Subjects With NIDDM

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Proinsulin is converted to insulin by the concerted action of two sequence-specific subtilisin-like proteases termed prohormone convertase 2 (PC2) and prohormone convertase 3 (PC3). PC3 is a type I proinsulin-processing enzyme that initiates the sequential processing of proinsulin to insulin by cleaving the proinsulin molecule on the COOH-terminal side of the dibasic peptide, Arg^{31}-Arg^{32}, joining the B-chain and C-peptide. Thus, PC3 plays a key role in regulating insulin biosynthesis. Expressions of insulin and PC3, but not PC2, are coordinately regulated by glucose, consistent with the important role of PC3 in regulating proinsulin processing. NIDDM is associated with increased secretion of proinsulin and proinsulin-like molecules, suggesting that mutations in the PC3 gene may be involved in the development of this disorder. To examine this hypothesis, we have isolated and characterized the human PC3 gene and screened it for mutations in a group of Japanese subjects with NIDDM. The PC3 gene consists of 14 exons spanning more than 35 kb. The exon-intron organization of PC2 and PC3 genes are conserved, consistent with a common evolutionary origin for the prohormone convertase gene family. Single-strand conformational analysis and nucleotide sequencing of the entire coding region of the PC3 gene in 102 Japanese subjects with NIDDM revealed missense mutations in exons 2 (Arg/Gln^{53}) and 14 (Gln/Glu^{638}), neither of which was associated with NIDDM in this population. These data suggest that genetic variation in the PC3 gene is unlikely to be a major contributor to NIDDM susceptibility in Japanese. *Diabetes* 45:897–901, 1996

Insulin is synthesized in the pancreatic β-cell as a larger precursor molecule proinsulin, which then undergoes limited proteolysis to insulin and C-peptide (1). Proinsulin has ~10% of the potency of insulin, and its conversion to insulin is necessary for full biological activity. Prohormone convertase 2 (PC2) and prohormone convertase 3 (PC3) (also known as PC1) are members of a family of Ca^{2+}-dependent serine proteases that are structurally related to bacterial subtilisin and the yeast protease Kex-2 (2,3). They are expressed specifically in neuroendocrine cells including pancreatic islets, pituitary, and brain. Several lines of evidence suggest that PC2 is responsible for cleavage of the C-peptide/A-chain junction of the proinsulin molecule, whereas PC3 cleaves at the B-chain/C-peptide junction (4–6).

Disproportionately elevated serum proinsulin levels relative to insulin have been reported in NIDDM, one consequence might be a relative insulin deficiency that could contribute to fasting and postprandial hyperglycemia (7–10). The molecular basis for increased proinsulin levels observed in subjects with NIDDM is uncertain, but one possibility is that mutations in the genes encoding PC2 and/or PC3 affect expression of these genes in diabetic subjects or result in the synthesis of proteins with altered biochemical properties (11). 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 suggesting that genetic variation in the PC2 gene might contribute to the development of NIDDM (12–14). However, single-strand conformational polymorphism (SSCP) analysis (15) did not reveal any mutations that could readily explain the possible molecular basis for the observed association.

The contribution of the PC3 gene to NIDDM susceptibility has not been examined. The bulk of the evidence supports a sequential mechanism for human proinsulin processing, whereby PC3 first cleaves proinsulin to generate des 31,32 proinsulin followed by cleavage of the Arg^{65} bond by PC2 (16). Also, when an increased demand is placed on the

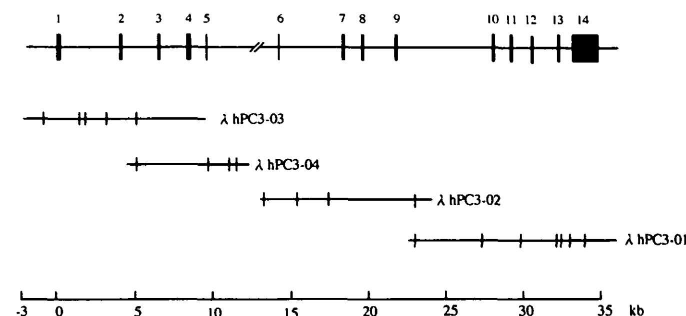

FIG. 1. Map of the human PC3 gene. The 14 exons are indicated. The translational initiation site is the 0 coordinate. The vertical lines in each clone represent natural *Eco*RI sites.

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CRE, cAMP response element; PC2, prohormone convertase 2; PC3, prohormone convertase 3; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism.

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TABLE 1
Exon-intron organization of the human PC3 gene

Exon	Exon size (bp)	Sequence at exon-intron junction: 5′ splice donor	Sequence at exon-intron junction: 3′ splice acceptor	Intron size (kb)	Amino acid interrupted
1	390	GGT CAG gtaaga.	.ttatag ATT GGT	3.7	Gln[33]
2	105	GAT CGT gtaagt.	.ttgtag GTG ATA	3.0	Arg[68]
3	111	TAC TTG gtaagt.	.taccag CAA GAT	2.2	Leu[105]
4	147	AAC TAT gtaagt.	.ctttag GAT CCA	1.3	Tyr[154]
5	77	AAC AA gtgagt.	.cctcag A CAC	>4.0	Lys[180]
6	89	GGA G gtaaaa.	.tctcag GC ATA	3.7	Gly[210]
7	173	AAA CAG gtaaga.	.ctttag GGG AGA	1.5	Gln[267]
8	212	AGA ATC gtatgt.	.atccag ACG AGC	2.5	Ile[338]
9	101	GCA AA gtaagg.	.ctccag C CCA	4.0	Asn[372]
10	234	CCC AG gtaagt.	.atttag A GCC	0.8	Arg[450]
11	158	GCT G gtaaat.	.tcatag GA ACT	1.6	Gly[503]
12	134	GAC ATG gtaagt.	.tgttag TCT GGA	2.4	Met[547]
13	162	GGG GAG gtctgt.	.ttttag GAG CAG	1.5	Glu[601]
14	>3,000				

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

proinsulin-processing mechanism by a glucose-stimulated increase in proinsulin biosynthesis, there is a coordinate increase in PC3 levels, but not in those of PC2 (17), suggesting that PC3 is the controlling protease regulating proinsulin processing. In this study, we report the exon-intron organization of the human PC3 gene and analysis of this gene for mutations in Japanese subjects with NIDDM.

RESEARCH DESIGN AND METHODS

Subjects. A total of 102 Japanese subjects with NIDDM, diagnosed according to the World Health Organization (18), were recruited from the Hospital of Wakayama University of Medical Science. A total of 100 nondiabetic control subjects were chosen using the following criteria: age >40 years, no family history of diabetes, and a random plasma glucose of <6.7 mmol/l. A positive family history was defined as diabetes diagnosed in siblings, parents, grandparents, aunts, and uncles. Informed consent was obtained from each subject at the time of recruitment. About half of the NIDDM subjects had a family history of diabetes.

Isolation of the human PC3 gene. A human genomic library obtained from Clontech (catalog no. HL1067J; Palo Alto, CA) was screened by hybridization using the mouse PC3 cDNA as a probe. DNA was sequenced using the dideoxynucleotide method with either universal or specific primers (19) after subcloning fragments of the gene into pGEM-3Z (Promega, Madison, WI). The positions of exon-intron junctions were determined by comparison of the genomic and cDNA sequences.

SSCP analysis. Genomic DNA was extracted from peripheral leukocytes by standard methods (20). Polymerase chain reaction (PCR) primers were selected to amplify each of the 14 exons of the human PC3 gene and flanking sequences including exon-intron junctions (Table 2). PCR was conducted in a volume of 10 μl containing 50 ng of DNA, 10 pmol of each primer, 0.2 μl of [α-^{32}P] dCTP (3,000 Ci/mmol), 2 mmol/l of each dNTP, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, and 0.2 μl of *Taq* polymerase (Perkin-Elmer, Norwalk, CT). PCR included an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 2 min. PCR products were diluted 1:10 with loading dye, heated at 94°C for 4 min, and 4 μl was separated on a 5% nondenaturing polyacrylamide gel with or without 10% glycerol. After electrophoresis at 4°C or room temperature at a constant power of 40 W, the gel was dried and exposed to X-ray film for 24 h. When shifted bands were observed, the sequence of amplified DNA was determined both directly and after subcloning into pGEM-3Z.

Biochemical assays. Blood glucose levels were determined using the glucose oxidase method (Beckman Analyzer, Beckman Instruments, La Brea, CA). Serum insulin levels were determined with a Insulin Riabead II kit (Dainabot, Tokyo, Japan)—this kit does not cross-react with standard human proinsulin. Serum proinsulin was measured by the Mitsubishi Yuka Bio Clinical Laboratory (Tokyo, Japan) using their proinsulin-specific antiserum. Cross-reaction studies with derivatives of human proinsulin indicated that this antiserum chiefly recognized a determinant in the region of the junction between the B-chain and C-peptide.

TABLE 2
Primers used to amplify exons of the human PC3 gene

Exon	Forward primer	Reverse primer	Product (bp)
1	ACTCTTGTTCAAGCGAGTG	GTTTCTTGAAAGTGGAAACT	232
2	ATACTTGTTTGGGAACGTGG	TAAGCTAGAGTATTGGTTTG	196
3	GAGGAAGTATACAGAGGTAG	CATAGTCCTTCTGTAGGTAC	196
4	GGTTGGAACTGAAGTGCCCA	AGTCACAGTCATGAGAAGGCA	253
5a	AGGGTAAGAGGCTTGGGAAG	ACACAAATGCATATTTACTC	180
5b	AATGCTGCCACAGTGTTATA	ACACAAATGCATATTTACTC	136
6	ACCTATGCCCCATTAATTCA	GCTATAGGGACAATCCTCTG	199
7	TGTCCATGTACATACTGACA	CATGTGGGTCTGTGTAAAGC	257
8	ACCCCAATAGATGATAACAG	CACATGCATGCCACGCTCTC	318
9	ACTCCTCACGTGTTCTCCCT	TATCAAGCTTTTCTGGGCCT	172
10	ACTTTGGTCGAGCTTCCCCT	TATGCCATGGGGCACACATGT	303
11	CGAAGGAAGTTTGGATATAC	TTGAATCATTCAACTTACAC	268
12	ATCAGATGCTAGAGTGTATC	TCATCCTCTCATTTACACTT	224
13	ACACATACTAAATGTAGGTA	TGCCAAGAACAGAGCCACAC	220
14	GTGGATGGCATTCTATGTTT	CCAACTTGGGACCACACACT	425

Exon 5a amplifies polymorphic poly-T tract, whereas 5b does not include this region.

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-725 CTAATTTTCT--CAGACAATTTAGAACAGAATTGGGA-CAGTAGTAGCTTCCCTTCTTCTCCTTACTCTCCTAGCCTCTC H
-732 CTTATTTCCTTACAGG-A-TTT-GAA-ACAAT-GC-ATCAG--GTAGCTTCGCT-C--CTC-TG-CTC-CC-AGCCTCT- M

-648 TCTTTGAAAGACCTAA----GAG-CCT---T-GT-TCT-TC--ATAGCAGGTGCTGATGCCCAAAGGGCCATTATAAGAA H
-668 -----GAAAGATCAAAACACGAGTCCTAGATTGTGTCTGTTTTATAACAAATGCTGATACTCAA-GAACCATTATAAGAA M

-581 TCCTATAGCCTCCCCCCCTCCCCGCCACACACAT-G-AGATAATAAAGTAA-ATACAGAGAA-A-ATGGA---------T H
-594 TTCTAT--CATCACCAG------GC-ACATTCATAGGAGATA-TAAGGTAAGATAGAGAAAATAGATGGAAGGCGGGGGT M

-515 GG-A---AGGGAGGGA-----AA--GCACT-C--CCAGCCTTTG-ATTCTTGGTAGTTATGTTCAAGTGGGGCACTTCTC H
-524 GGGAGGTAGGGAGTGAGGGGTAATTGTTGTGCTTCCTGCCTTGGGAT-CTTGGTAGTTATATTCAAATGAGGCGGTTCTC M

-450 TTTGAA-AAGTGAAAATGTTTCTCACGG-TACAGAATTTATGGGCAGACAAGG-AGCAGA-CTTAACTGATCCCAGCAAA H
-445 TTTGAAGAA-TGGAGATGTTTCTCACAGCTACAGTATTTATG---A-ACA-GGCAGCAGAACTGACC-GATCCCA-C--- M

-374 CAGTTCCTT--CCTTTTCCTAATCCCTGGCTGCTTATTTTAGCCGCTTCTCCG-CCCGCCGGGAATACCATTCGGATCTT H
-376 CAGTTTCTTTTCCTTTTCTTAATCCTTCCCTGCCTATTTTAGTCACTTCTCCACCCCGCCGGGAATACCATCCAGTTCTC M
                                                       GC box
-297 AGTCCAGGTAGATCTGACGTCAAGAGATGGCTTTCGTCGATTTGACGTGTAAACACTCATTTCCATTCTGGCTGGGAAGG H
-296 AGTCCAGGTAGATCTGACGTCAAGAGATGGCTTTCGTCGATTTGACGTGTAAACACTCATTTCTATTCTGGTTGGTAAGG M
          * * *  CRE                    CRE
-217 GCTGGGGCTCCACTCAGCCTGGAGACCGAAGCGCTTCACTGAGCGCTCG-C-CGCCGCCCAGCCTCTCCTC--TCGCGCC H
-216 -CTGGGGCTCCACTCAGTCGGGAGACTGAAGCACTTTAGTGAGCGCTCGTCTCGCCGCCCAGCCTCTCC-CAGT-GAGCC M

-141 TCCTAGCTCTTCGCAGAGCAACC-AGGAGCCAGGAGTGGTCT-AGAGCCCGA-GGGTGGGAAGGGGGAGTCT-GTCTGGC H
-139 TC-TAGCTCTA-GTAGAGCAACCCAG-AGCCAGGAGAGGTTTTA-AGCT-GCTGGGTGGAAAGGTCGAGTCTAG-CTGG- M

 -65 TTT-TCTCCT-ATCTTGCTTCTTTT-TCCTCTTCCCTTCCCACTCT-TGTTC-AAGCGAGTGTGT-GAGCT   ATG    H
 -66 TGTGTCTC-TGATCTTGCTTCTTTTCTCC-CAGCCCTTCCTACT-TGTGTGAGAA-CAAG-GTTTTGAGCC   ATG    M
                                                                           Met
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FIG. 2. Comparison of the sequences of the promoter regions of human and mouse PC3 genes. H and M represent human and mouse species, respectively. Nucleotide numbering is relative to the first nucleotide of the codon for the initiating methionine. Gaps in the alignment are indicated by dashes. Colons mean identical nucleotides. The putative transcriptional start sites (*) based on the mapping of the start site in the mouse gene is shown. One GC box and two CREs, which are conserved between human and mouse, are boxed. Another GC box-like sequence found only in the mouse (24,25) is underlined.

RESULTS

Isolation and characterization of the human PC3 gene. The human PC3 gene consists of 14 exons spanning more than 35 kb (Fig. 1 and Table 1). Sizes of the exons and introns are summarized in Table 1. The sequence has been deposited in the GenBank data base with accession numbers D73393–D73407. Boundaries of the exon-intron junctions conform to the GT-AG rule (21). Organization of the human PC3 gene is similar to that determined for the mouse except the mouse gene has an additional intron that interrupts the 3′-untranslated region of the mRNA; thus, the mouse PC3 gene has 15 exons (22).

The sequence of gene was in good agreement with that of the cDNA, which was determined independently by two groups (23,24), and we found only three differences: *1*) we could not locate nucleotides 1–13 of the sequence reported by Seidah et al. (24) in the sequence of the corresponding region of the gene; *2*) codon 330 is AGC (Ser) (24) and GGC (Gly) (23) in the cDNA and AGC in the genomic clone that we studied; and *3*) there is a C at nucleotide 3039 in the 3′-untranslated region of the gene not present in the cDNA sequence reported by Creemers et al. (23).

TABLE 3
Summary of polymorphic positions in the human PC3 gene

Nucleotide position	Change	Restriction enzyme site changed	NIDDM (wt:hetero: homo)	Nondiabetic control subjects (wt:hetero: homo)
Exon 2	Arg^{53} to Asn^{53} (CGA to CAA)	*Taq* I (−)	100:2:0	96:4:0
Exon 14	Gln^{638} to Glu^{638} (CAG to GAG)	*Scr*FI (−)	56:43:3	62:32:6
Intron 4	poly-T tract	ND	ND	ND

ND, not done.

The sequences of the putative promoter regions of the human and mouse PC3 genes (24,25) are shown in Fig. 2. There is 72% identity between the human and mouse promoter sequences including several regions of high homology, which presumably plays similar roles in regulation of expression of these genes in human and mouse tissues. The PC3 gene, like the PC2 gene, lacks TATA or CAAT boxes. However, there is a GC box and two cAMP response elements (CRE), the locations and sequences of which are conserved between human and mouse.

The genomic clones shown in Fig. 1 were screened for simple tandem repeats in the form (CA)*n* and (GA)*n*. Two interrupted (GA)*n* repeats were identified in intron 5, but they were not polymorphic and thus not suitable for genetic studies (data not shown).

Genetic variation in the human PC3 gene. One feature of NIDDM is an increased molar ratio of proinsulin to insulin. To determine if the defect in proinsulin processing may have a genetic basis, we scanned the PC3 gene of 102 Japanese NIDDM patients for mutations in each of the 14 exons using SSCP analysis. Three different SSCP patterns were detected (Table 3). There was a G-to-A substitution in codon 53 of exon 2 resulting in an Arg (CGA)-to-Gln (CAA) replacement. This nucleotide substitution abolished a *Taq* I restriction site, which facilitated its identification in 100 nondiabetic subjects. Arg^{53} and Gln^{53} alleles were found in both NIDDM and control subjects with the frequency of the uncommon Gln^{53} allele in NIDDM and control subjects being 1 and 2%, respectively. As expected for an uncommon variant, no individuals homozygous for the Gln^{53} allele were noted in the relatively small number of subjects tested. A common polymorphism was identified in codon 638 of exon 14 with a C-to-G substitution resulting in a Gln (CAG)-to-Glu (GAG) replacement; this substitution abolished a *Scr*FI restriction site. Frequency of the less common Glu^{638} allele in NIDDM and control groups was 24 and 22%, respectively.

To assess whether these missense mutations were associated with an elevated proinsulin to insulin ratio, fasting serum proinsulin and insulin levels were measured in 95 NIDDM subjects and 20 nondiabetic control subjects. Fasting serum proinsulin level was significantly ($P < 0.01$) higher in NIDDM subjects (6.3 ± 0.4 pmol/l) compared with control subjects (3.7 ± 1.0 pmol/l). Two of the NIDDM subjects were heterozygous for the codon 53 mutation (i.e., Arg^{53}/Asn^{53}). One subject was a 55-year-old woman who also had the A-to-G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene. This woman is currently being treated with insulin and hemodialysis because of diabetic renal failure. The other patient was a 59-year-old woman being treated with oral hypoglycemic agents, and her fasting serum proinsulin level (24.7 pmol/l) and proinsulin to insulin ratio (26.2%) were relatively high.

NIDDM patients were divided into three subgroups based on the presence or absence of codon 638 polymorphism; i.e., Gln^{638}/Gln^{638}, Gln^{638}/Glu^{638}, and Glu^{638}/Glu^{638}. Serum proinsulin levels in these three groups were 5.97 ± 0.38, 6.77 ± 0.66, and 3.69 ± 0.58 pmol/l, respectively, and the proinsulin to insulin molar ratios were 19.2 ± 1.1, 18.8 ± 1.8, 15.3 ± 2.5%, respectively. There were no significant differences in serum proinsulin levels or proinsulin to insulin molar ratios among these three genetically distinct groups.

In addition to the two missense mutations described above, we found a simple sequence repeat polymorphism in

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cagccaggatgggaaggcatagggtaagaggcttgggaagtgatgc
                    forward primer 1
ttttttttttttttttttaatgctgccacagtgttatatttgttg
                    forward primer 2
tctctttagGATCCAGAGGCTAGCTATGATTTTAATGATAATGACC
                                   Exon5
ATGATCCATTTCCCCGATATGTACCCACAAACGAGAACAA gtgagt

aaatatgcatttgtgtgaaataaagtactttacaat
        reverse primer
```

FIG. 3. Sequence of polymorphic poly-T tract in intron 4. The sequence of exon 5 and adjacent intron segments are shown. The primers used for the SSCP analysis are indicated. Exons sequences are capitalized and intron sequences are lowercased. The polymorphic poly-T tract is boxed. Forward primer 1 amplifies the polymorphic poly-T tract, whereas forward primer 2 does not include this region.

intron 4 resulting from variation in the length of a poly-T tract upstream of the splice acceptor site (Fig. 3). DNA sequence analysis of this region in 10 unrelated subjects revealed eight alleles varying in length from 18 to 25 repeats.

DISCUSSION

Proinsulin is converted to insulin by the concerted action of two sequence-specific subtilisin-like proteases termed PC2 and PC3 (3,26,27). PC2 cleaves predominately at Lys^{64}-Arg^{65}, and PC3 cleaves at Arg^{31}-Arg^{32}. Elevated proinsulin levels and/or proinsulin to insulin molar ratios are often observed in NIDDM subjects. The molecular basis for this hyperproinsulinemia is uncertain, but PC2 and/or PC3 mutations might be responsible by contributing to inefficient processing of proinsulin leading to hyperproinsulinemia, insulin deficiency, and NIDDM.

To examine this hypothesis, we identified the human PC3 gene that will facilitate molecular genetic studies of NIDDM and other metabolic disorders (28). Using the sequences adjacent to exon-intron junctions, we screened NIDDM patients for mutations of each of the 14 exons of human PC3 gene by SSCP analysis. We identified two amino acid substitutions in patients with NIDDM and in normal healthy control subjects: Arg/Gln^{53} and Gln/Glu^{638}. Arg^{53} is located in the prosegment of PC3, and it is conserved in mouse (29), rat (30), and amphioxus PC3 (31). Gln^{638} is located in the COOH-terminal region, and the corresponding amino acid in mouse and rat is Lys. Neither polymorphism appears to be associated with NIDDM or to affect proinsulin levels or proinsulin to insulin ratio. Thus, genetic variation in the PC3 gene does not appear to play a major role in the pathogenesis of NIDDM. However, the promoter region was not screened for mutations, and it is possible that analysis of the promoter region may reveal variation that affects the expression of the PC3 gene in NIDDM patients.

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