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ANGIOTENSIN CONVERTING ENZYME AND GENETIC HYPERTENSION: CLONING OF RAT cDNAs AND CHARACTERIZATION OF THE ENZYME

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SUMMARY: Using genetic mapping approaches, a gene on chromosome 10, *Bp1*, has been identified in the stroke-prone spontaneously hypertensive rat (SHRSP) in the same region that contains the gene for angiotensin converting enzyme (ACE). Since ACE plays an important role in blood pressure regulation, the ACE gene is a leading candidate for *Bp1*. To examine the possibility that a structural abnormality of ACE exists in the SHRSP, we cloned and characterized the cDNAs for the Wistar-Kyoto rat (WKY) and SHRSP ACE. Both cDNAs encode a single polypeptide of 1,313 amino acid residues with an estimated molecular weight of 150.9 KDa. Five nucleotide differences were identified between the WKY and the SHRSP ACE cDNAs. One of these differences resulted in an amino acid substitution (Lys-207 in the WKY to Arg-207 in the SHRSP). But the enzymatic properties of partially purified ACE from the two strains were similar. Thus the data suggest that an alteration in the primary structure of rat ACE does not contribute to the hypertension in the SHRSP.

Hypertension is a major risk factor for cardiovascular diseases such as stroke, heart failure, and renal failure (1, 2). Epidemiological data suggests that human essential or primary hypertension is a heterogeneous, polygenic disease. The primary causes of hypertension remain to be elucidated despite intensive physiological and pathophysiological studies in man and experimental animal models (2-8). Several of these models are genetic, including the spontaneously hypertensive rat (SHR) and the stroke-prone spontaneously hypertensive rat (SHRSP), and these models have been well characterized physiologically. Recently the SHRSP and the John Rapp Salt Susceptible (SS/JR) have been investigated by several laboratories using molecular genetic approaches (9-12). These studies revealed that a gene, designated *Bp1*, near the gene that encodes angiotensin converting enzyme (ACE), on chromosome 10 is responsible for a significant portion of the blood pressure increase observed after sodium loading. The important question to be answered is whether ACE is *Bp1*?

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ACE (peptidyl-dipeptidase A, kininase II; EC 3.4.15.1) is a zinc-containing dicarboxypeptidase that cleaves angiotensin I to form the vasoconstrictor angiotensin II and degrades the vasodilator bradykinin (13). ACE plays a critical role in blood pressure homeostasis (14) and is the target of an important class of anti-hypertensive agents -- ACE inhibitors. To determine if the ACE was a viable candidate gene for *Bp1* as suggested by the linkage analysis, we set out to study the ACE gene in the SHRSP and the normotensive control Wistar Kyoto rat (WKY). The cDNAs that encode ACE of mouse, human, and bovine have been cloned and reported (15-18); however, the rat ACE cDNA cloning has not been reported. Here we report the cloning and the comparison of the cDNAs for ACE gene from the WKY and SHRSP, as well as the initial characterization of the enzymatic properties.

MATERIALS AND METHODS

Preparation of Probes - The probes for screening cDNA libraries were prepared by reverse transcription of total RNA of the SHRSP using an oligo(dT) primer followed by amplification using polymerase chain reaction (RT-PCR) (Perkin Elmer Cetus, Norwalk, CT). PCR primers were selected based on the mouse and human ACE cDNA sequences (15-17). Two RT-PCR fragments corresponding to the 5'-end (Probe-1; forward primer p1: ATATCTAGACTG(T/C)CGCTGC(T/C)(G/C)GTGCTGTTG, reverse primer p2: TATAGAGCTCCAGGGAGGTGAAGAACTCCTCTG) and to the 3'-end (Probe-2; forward primer p3: GCCACATCCAGTATTTCATGCAGT, reverse primer p4: AACTGGAACTGGATGATGAAGCTGA) (Fig. 1) were used to insure the entire cDNA, estimated to be 4 kbp based on mouse and human cDNA. The two fragments were partially sequenced to confirm proper fragment amplification (data not shown). The probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol, Du Pont-New England Nuclear, Boston, MA) by a random primer labeling kit (Life Technologies, Inc., Gaithersburg, MD), and purified with NICK column (Pharmacia LKB Biotechnology, Uppsala).

Preparation and Screening of cDNA Libraries - Total RNA was isolated as previously described (19) from lung of the WKY and the SHRSP obtained from colonies maintained at the University of Heidelberg, Germany. These colonies, derived from the original Aoki-Okamoto stock (20), have been maintained under brother-sister breeding conditions for over 25 generations. Using these RNAs, cDNA libraries, primed with oligo(dT) and random primers, were constructed in λZap II (Clontech Laboratories, Inc. Palo Alto, CA) (WKY cDNA library; independent plaques: 1.74 x 10^6, SHRSP cDNA library; independent plaques: 2.1 x 10^6). The cDNA libraries were plated on *Escherichia coli* strain BB4 and screened by plaque hybridization (21) using Colony/Plaque Screen (Du Pont-New England Nuclear) or Magna nylon membrane (Micron Separations Inc., Westboro, MA). Hybridization was carried out at 42 °C in 50 % formamide, 5 x SSPE (1 x SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5 x Denhardt's solution, 0.1 % SDS and 100 µg/ml denatured salmon sperm DNA. Filters were washed at 68 °C in 0.1 x SSC (1 x SSC is 150 mM NaCl and 15 mM sodium citrate), 0.1 % SDS for Colony/Plaque Screen or 0.2 x SSC, 0.1 % SDS for Magna. Positive clones, localized following autoradiography at -70 °C with an intensifying screen, were isolated by repeated phage purification. The pBluescript SK(-) plasmids with cDNA insert were excised from phage DNA with R408 helper phage by the *in vivo* excision method (22).

DNA Sequencing - Double-stranded DNA, prepared with Qiagen columns (Diagen GmbH, Dusseldorf) was sequenced by the dideoxy chain termination method (23) using Sequenase (United States Biochemical, Cleveland, OH). Sequencing reactions were primed either with T7 and T3 primer (Stratagene, San Diego, CA) or with internal primers complementary to a confirmed cDNA sequence.

ACE Extraction - ACE was extracted from lungs of the WKY (1.2 g) and the SHRSP (1.8 g) using a published procedure (24) with minor modification. After collecting

the membrane fraction, membrane bound ACE was solublized in 10 mM Tris-HCl (pH 7.8) containing 0.5 % Nonidet-P40. The supernatant containing the extracted ACE was aliquoted and stored frozen until analysis. All operations were performed at 4 °C.

Assay of ACE Activity - ACE was assayed by the spectrofluorometric procedure of Cushman and Cheung (25) using Hip-His-Leu as substrate. A unit of enzyme activity is the amount required to catalyze formation of 1.0 mmol of hippuric acid per min at 37 °C using standard conditions. ACE activity was normalized based on the concentration of protein. The concentration of protein was determined using BSA as a standard as described by Bradford (26).

RESULTS

Screening of cDNA Libraries - The WKY cDNA library was screened with Probe-1 (2.9 x 10^6 clones screened) and Probe-2 (2.7 x 10^6 clones screened) yielding three clones, which were isolated and then mapped using restriction enzymes. The largest of these clones, pGKW154, represented the 5' region of the cDNA and contained three EcoRI fragments (Fig. 1). The 3' fragment (pGKW154-3) of this clone was an artifact of library preparation since 1) it contained EcoRI linker sequences on both ends; 2) its sequence exhibited no homology with the overlapping clone, pGKW102; 3) its sequence exhibited

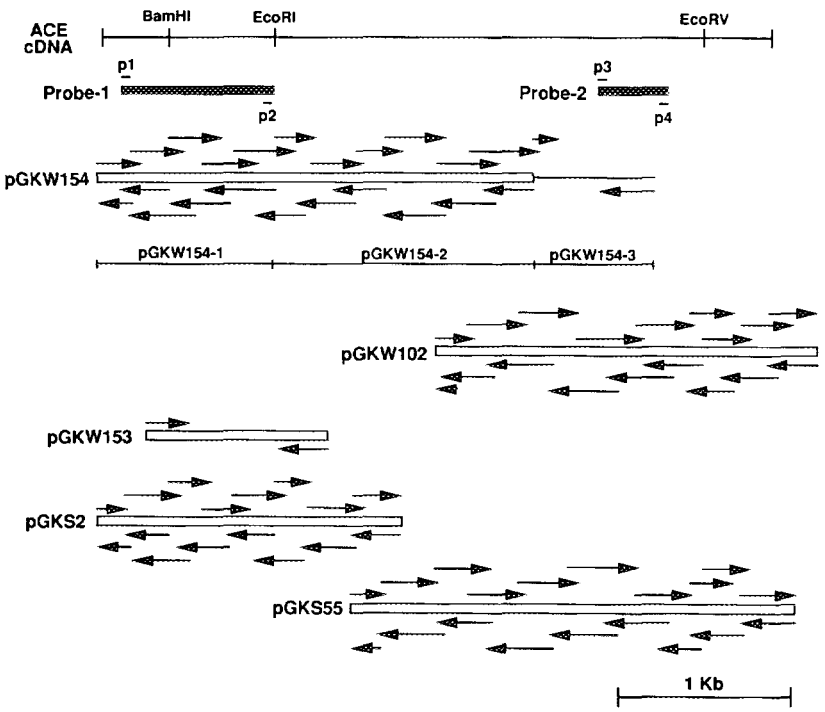

Fig. 1. **The schematic diagram of the WKY and the SHRSP ACE cDNA and strategy for DNA sequencing.** The top line with restriction enzyme sites that used in sequencing indicates ACE cDNA coding region. Open box (□) indicates the cDNA clones. Line (-) in pGKW154 indicates the region of pGKW154-3. The line under pGKW154 indicates the organization of the pGKW154. Horizontal arrows show the directions and the regions of the sequence analyzed. Black boxes (■) indicate probes prepared by RT-PCR.

no homology to mouse, human or bovine ACE; and 4) its sequence exhibited no homology to the SHRSP ACE. Several clones pGK154 (pGKW154-1 and pGKW154-2) and pGKW102 were sequenced in both directions (Fig. 1). A third independent clone pGKW153 was partially sequenced to confirm the position of pGKW154-1 and 2. Similarly, the cDNA library from the SHRSP was screened with Probe-1 (2.2×10^6 clones screened) and Probe-2 (1.1×10^6 clones screened). Two overlapping clones (pGKS2, pGKS55) were identified and then sequenced in both directions. The structure of ACE cDNA, the organization of these clones and the sequencing strategy are shown in Fig. 1.

Primary Structure of the ACE cDNAs - A similar open reading frame corresponding to a protein composed of 1,313 amino acid residues were identified from the sequences of WKY and SHRSP ACE cDNA (Fig.2A). There were five nucleotide differences in the coding region of ACE between the WKY sequence and the SHRSP sequence (A-620, C-871, C-2514, T-2649, G-2940 in WKY to G-620, T-871, T-2514, C-2649, A-2940 in SHRSP). Only one of these differences resulted in an amino acid substitution, Lys-207 in WKY and Arg-207 in SHRSP. The 3' untranslated regions were identical for the first 38 nucleotides past the stop codon (Fig. 2B). Differences further 3' are presumably due to different priming of the cDNA during library construction.

Characterization of ACE Extract - We prepared partially purified (9-fold purification; data not shown) membrane-bound ACE protein from lungs of WKY and SHRSP. As summarized in Table I, the specific activity of the ACE protein was 0.26 units/mg for the WKY vs. 0.27 units/mg for the SHRSP. The *Km* values were 3.03 mM (WKY) and 3.17 mM (SHRSP). The *Vmax* values were 0.34 mmole/min/mg (WKY) vs. 0.34 mmole/min/mg (SHRSP). There were no significant differences in any of these values that estimate ACE enzyme activity, between the WKY and the SHRSP.

DISCUSSION

Recently we and others performed genetic cosegregation studies using a total genomic search strategy and discovered a gene, *Bp1*, on rat chromosome 10 which cosegregates with salt loaded hypertension in the SHRSP and accounts for more than 20% of genetic variance (9, 10). Interestingly, the ACE gene is localized in this region of *Bp1*. Since ACE plays a significant role in blood pressure homeostasis (14), and previous physiological studies had demonstrated that the tissue ACE activity in the SHR was altered (27, 28), we hypothesize that the ACE gene is a candidate gene for *Bp1*. There are two possible mechanisms for altering tissue ACE activity, a difference of primary structure resulting in an alteration of enzyme characteristics or alternatively, the aberrancy of ACE gene regulation. In order to examine the first hypothesis, we set out to clone and compare the cDNAs for ACE of both the WKY and the SHRSP.

Screening of cDNA libraries of WKY and SHRSP rat yielded two clones from each cDNA library. Both cDNA sequences encoded a single open reading frame, corresponding to a protein composed of 1,313 amino acid residues. The sizes of these

Fig. 2. **The nucleotide sequence and deduced amino acid sequence of ACE cDNA.** A, The nucleotide sequence of the cDNA for the WKY ACE. The nucleotide sequence is numbered on the left. The deduced amino acid sequence, presented below the nucleotide sequence is numbered on the right. The termination codon TGA is denoted by asterisks (***). The bold characters above the nucleotide sequence indicate the different nucleic acid in the cDNA for the SHRSP ACE. The bold characters below the amino acid sequence indicate the different amino acid in the amino acid sequence in the SHRSP ACE. The italic characters boxed with shade indicate the active site. The putative transmembrane region near the carboxyl terminus is boxed. B, The 3' untranslated region of the cDNA for the SHRSP ACE.

open reading frames are consistent with the sizes that were detected by northern blot analysis (data not shown). ACE gene from the rat is 80-90 % homologous to ACE gene reported for other species (15-18) at both the nucleic acid and amino acid level. The

Table I. Comparison of ACE extract from WKY and SHRSP

	Specific Activity (units / mg)	*Km* (mM)	*Vmax* (μmol / min / mg)
WKY	0.26	3.03 ± 0.23	0.34 ± 0.26
SHRSP	0.27	3.17 ± 0.23	0.34 ± 0.25

Km : the Michaelis constant (mean ± S.D.)
Vmax : the maximum velocity (mean ± S.D.)

molecular weight of the protein, estimated from the predicted amino acid sequence is approximately 150.9 kDa, which is also similar to other species (15-18). Further analysis revealed that the deduced amino acid sequence contains two homologous domains and hydrophobic regions are similar to those reported for ACE from various species suggesting that the enzyme has been highly conserved during evolution (data not shown). Previously it has been shown that ACE (15-18) contains two active sites, which contain a consensus sequence, His-Glu-X-X-His. This consensus sequence is conserved in ACE from the WKY and the SHRSP (Fig. 2A).

We compared the primary structure of the ACE between the WKY and the SHRSP. 5 nucleotide differences were observed between the coding regions of the WKY and the SHRSP cDNA, one of which result in an amino acid substitution of Lys-207 in the WKY by Arg-207 in the SHRSP. While it is possible that single amino acid substitutions can alter significantly the function of a protein resulting in genetic diseases, such as sickle-cell anemia (29) and familial hypertrophic cardiomyopathy (30), these substitutions have tended to be major substitutions and/or substitutions within an active site. Conservative amino acid substitutions, such as that observed in the comparison of the WKY and the SHRSP ACE, do not generally have dramatic consequences for the function of the protein. Consistent with this, our data of enzymatic analysis revealed similar *Km* and *Vmax* for ACE extracted from the lungs of the WKY and the SHRSP (Table I).

We conclude that a mutation in the primary structure of the SHRSP ACE is an unlikely cause for the genetic basis for salt-loaded hypertension in this strain of rat. Our data in conjunction with the reported differences ACE activity between the SHRSP and the WKY, suggests that the regulatory region of the ACE gene must be different between these two rat strains. We are currently investigating this hypothesis.

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REFERENCES

1. Dollery, C.T. (1985) In Cecil Textbook of Medicine (Wyngaarden, J.B. and Smith, L. Eds.), pp. 266-280. W. B. Saunders Company, Philadelphia, PA.
2. Haber, E. and Slater, E.E. (1986) In Scientific American Medicine: Cardiology (Rubinstein, E. and Federman, D. Eds.). Scientific American Press, New York, NY.
3. Havlik, R.J., Garrison, R.J., Feinleib, M., Kannel, W.B., Castelli, W.P., and McNamara, P.M. (1979) Am. J. Epidemiol. 110, 304-312.
4. Higgins, M., Keller, J., Moore, F., Ostrander, L., Metzner, H., and Stock, L. (1980) Am. J. Epidemiol. 111, 142-155.
5. Levine, R.S., Hennekens, C.H., Perry, A., Cassady, J., Gelbrand, H., and Jesse, M.J. (1982) Am. J. Epidemiol. 116, 759-764.
6. Rapp, J.P. (1982) In Hypertention (Genest, J., Kuchel, O., Hamet, P. and Cantin, M., Eds.), pp. 582-598. McGraw-Hill, New York, NY.
7. Yamori, Y. (1982) In Hypertention (Genest, J., Kuchel, O., Hamet, P. and Cantin, M., Eds.), pp. 556-581. McGraw-Hill, New York, NY.
8. Bianch, G., Ferrari, P., and Barber, B.R. (1990) In Hypertension: Pathophysiology, Diagnosis, and Management (Laragh, J.H. and Brenner, B.M., Eds.), Chapter 78. Raven Press, Ltd., New York, NY.
9. Jacob, H.J., Lindpaintner, K., Lincoln, S.E., Kusumi, K., Bunker, R.K., Mao, Y-P., Ganten, D., Dzau, V.J., and Lander, E.S. (1991) Cell 67, 213-224.
10. Hilbert, P., Lindpaintner, K., Beckmann, J.S., Serikawa, T., Soubrier, F., Dubay, C., Cartwright, P., De Gouyon, B., Julier, C., Takahashi, S., Vincent, M., Ganten, D., Georges, M., and Lathrop, G.M. (1991) Nature 353, 521-529.
11. Nara, Y., Nabika, T., Ikeda, K., Sawamura, M., Endo, J., and Yamori, Y. (1991) Biochem. Biophys. Res. Comm. 181, 941-946.
12. Deng, Y. and Rapp, J.P. (1992) Nature Genet. 1, 267-272.
13. Soffer, R.L. (1976) Annu. Rev. Biochem. 45, 73-94.
14. Soffer, R.L. (1981) Biochemical Regulation of Blood Pressure, pp. 123-164. Wiley Interscience, New York, NY.
15. Bernstein, K.E., Martin, B.M., Bernstein, E.A., Linton, J., Striker, L., and Striker G. (1988) J. Biol. Chem. 263, 11021-11024.
16. Bernstein, K.E., Martin, B.M., Edwards, A.S., and Bernstein, E.A. (1989) J. Biol. Chem. 264, 11945-11951.
17. Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, G., and Corvol, P. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 9386-9390.
18. Shai, S-Y., Fishel, R.S., Martin, B.M., Berk, B.C., and Bernstein, K.E. (1992) Circ. Res. 70, 1274-1281.
19. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Biochemistry 18, 5294-5299.
20. Okamoto, K. and Aoki, K. (1963) Jpn. Circ. J. 27, 282-293.
21. Benton, W. D. and David, R. W. (1977) Science 196, 180-182.
22. Short, J.M., Fernandez, J.M., Sorge, J.A., and Huse, W.D. (1988) Nucleic Acids Res. 16, 7583-7600.
23. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467.
24. Das, M. and Soffer, R.L. (1975). J. Biol. Chem. 250, 6762-6768.
25. Cheung, H.S. and Cushman, D.W. (1973) Biochim. Biophys. Acta 293, 451-463.
26. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
27. Grima, M., Welsch, C., Giesen-Crouse, E-M., Coquard, C., Barthelmebs, M., and Imbs, J-L. (1990) J. Hypertens. 8, 697-702.
28. Okunishi, H., Kawamoto, T., Kurobe, Y., Oka, Y., Ishii, K., Tanaka, T., and Miyazaki, M. (1991) Clin. Exp. Phamacol. Physiol. 18, 649-659.
29. Ingram, V.M. (1957) Nature 180, 326-328.
30. Geisterfer-Lowrance, A.A.T., Kass, S., Tanigawa, G., Vosberg, H-P., McKenna, W., Seidman, C.E., and Seidman, J.G. (1990) Cell 62, 999-1006.