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MOLECULAR CLONING OF cDNA FOR RAT LIVER GENERAL ACYL CoA DEHYDROGENASE AND HOMOLOGY BETWEEN THE RAT LIVER AND PIG KIDNEY ENZYMES

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Summary. cDNA clone for general acyl CoA dehydrogenase (GAD) was isolated from a rat liver cDNA expression library in λgtll using anti-pig kidney GAD antibody. Size of the isolated cDNA was estimated to be 1.5-1.6 kb. By immunological analysis of fusion protein and epitope selection, the cDNA clone was identified as that containing the GAD gene. Partial amino acid sequence deduced from nucleotide sequence of the cDNA coincided with that of the pig kidney enzyme. The antibody cross-reacted with rat liver enzyme and molecular weights of these enzyme proteins were shown to be almost the same. All these results indicate that rat liver GAD shares a common structure with pig kidney enzyme.

Introduction

General acyl CoA dehydrogenase (EC. 1.3.99.3) (GAD), one of the mitochondrial flavin enzymes, has been purified to homogeneity from pig kidney (1), bovine liver (2), and rat liver (3). However, the structure of the enzyme and the extent of homology among tissues and species have remained unclear. Recently it has been shown that the enzyme is synthesized as a precursor having a larger molecular weight than that of the mature enzyme in rat liver (4) and pig kidney (5). In order to clarify the gene structure of the enzyme, we attempted cDNA cloning of the enzyme. In this paper, the isolation and identification of a cDNA clone for rat liver GAD are presented and homology between rat liver and pig kidney enzymes is also described.

Materials and Methods

Purification of rat liver GAD. GAD was purified from rat liver homogenate according to Ikeda et al. (3) with a slight modification. In brief, the enzyme was purified by a series of chromatographic steps, using columns of DEAE-Sepharose, hydroxyapatite, and anti-pig kidney GAD IgG coupled Sepharose

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4B. A solution containing the enzyme was subjected to SDS-PAGE according to Laemmli (6), the segment of gel containing the enzyme was cut out, and the enzyme was extracted with 50 mM Tris-HCl (pH 7.5)-150 mM NaCl (TBS) containing 0.1% SDS. The enzyme was examined by its activity using n-octanoyl CoA as substrate (1) or by immunoblotting (5). The eluate containing the enzyme was stored at -20°C until used.

Anti-GAD antiserum. Antibodies against pig kidney enzyme were raised in a rabbit as described previously (5) and shown to be specific for pig kidney GAD (5).

Isolation of cDNA clone for rat liver GAD. cDNA clones for GAD were isolated by immunoscreening a rat liver cDNA library constructed in λgtll (Clontech Laboratories, Inc., Palo Alto, CA), in which antiserum against the pig kidney enzyme was used according to the method of Young and Davis (7). Escherichia coli Y1090 infected with recombinant phages (approx. 5×10^5) was plated on LB agar plates. The plates were incubated at 42°C for 3.5 h and were overlaid with nitrocellulose membranes (BA85, Schleicher and Schüll, Dassel, FRG) impregnated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After the induction of fusion protein at 37°C for 2.5 h, the membranes were washed with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.05% Tween 20 (TBST), and incubated with TBST containing anti-GAD antiserum and then TBST containing goat anti-rabbit IgG-peroxidase (Bio-Rad Laboratories, Richmond, CA) at 25°C for 1 h. The filters were washed with TBS, and then stained with TBS containing 0.5 mg/ml of 4-chloro-1-naphthol (Wako Pure Chemicals, Osaka) and 0.15% of H_2O_2. Positive clones isolated were suspended with 1 ml of TBS containing 10 mM $MgSO_4$ and 0.01% gelatin.

Preparation and analysis of fusion protein. E. coli Y1090 was infected with the isolated cDNA clone-bearing phages, incubated at 42°C for 3.5 h, and further incubated at 37°C for 2.5 h in the presence of 10 mM IPTG. Fusion protein was directly recovered from plates by washing with 0.125 M Tris-HCl (pH 6.8) containing 20% glycerol, 1 mM EDTA, 1.5 mM β-mercaptoethanol, 0.005% Bromophenol Blue, and 0.1% SDS, and subjected to SDS-PAGE and then to immunoblotting as described previously (5).

Epitope selection. Epitope selection was carried out according to Weinberger et al. (8). E. coli Y1090 was infected with cDNA clone-bearing phages and fusion protein produced as described above was transferred to a nitrocellulose membrane. The membrane was blocked with TBS containing 10% bovine serum albumin (BSA), incubated with anti-GAD antiserum at 25°C for 2 h, and washed with TBS containing 0.5% Triton X-100. Antibodies immobilized on the membrane were recovered by washing for 1 min with 1 ml of 5 mM glycine-HCl (pH 2.3) containing 150 mM NaCl, 0.5% Triton X-100, and 100 μg/ml of BSA. This washing procedure was repeated. The washings were adjusted to pH 7.5 with 1 M Tris. The epitope-selected antibodies were used to probe the purified enzyme on the immunoblot.

DNA sequencing. Restriction fragments of 200-300 base pairs obtained by agarose (Low Gel Temperature, Bio-Rad Laboratories) gel electrophoresis were subcloned directly in the phage M13 mp18 (9). DNA's were sequenced by the dideoxynucleotide chain termination method (10) using an M13 sequencing kit (TAKARA Shuzo Co., Ltd., Kyoto).

Amino acid sequencing of pig kidney GAD. Pig kidney GAD purified according to Thorpe et al. (1) was digested with trypsin and cyanogen bromide. The digested peptides were separated by reverse-phase HPLC and sequenced by automated Edman degradation using an automated sequencer.

Results and Discussion

Immunoreactivity of anti-pig kidney GAD antiserum against rat liver GAD.

Prior to carrying out cDNA cloning for rat liver GAD, we examined the immunoreactivity of the anti-pig kidney GAD antiserum against rat liver GAD. Figure 1 shows the results of immunoblotting analysis of mitochondrial extracts from rat liver (lane a) and pig liver (lane b) and purified pig kidney enzyme (lane c), indicating that the antibody can cross-react with rat liver and pig liver enzymes. This result also indicates that the molecular weights of rat liver and pig liver enzymes are almost the same as that of pig kidney (42 K).

Isolation of cDNA for rat liver GAD.

Three positive clones for GAD were obtained among 5×10^5 recombinant phages after a third immunoscreening and designated λ-GAD-1, 2, and 3. In order to determine insert size of the cDNA clones, the phage DNA's were digested with EcoRI and subjected to 1% agarose gel electrophoresis. As shown in Fig. 2, the sizes of the inserts were estimated to be 1.5-1.6 kb. λ-GAD-1 was examined with respect to cDNA for GAD.

Identification of cDNA clone.

Figure 3A (lane a) shows that a fusion protein of approximately 155-160 K produced in the bacteria was detected on the immunoblots, whereas no band was detected when the fusion protein of a negative clone was examined (Fig. 3A, lane b).

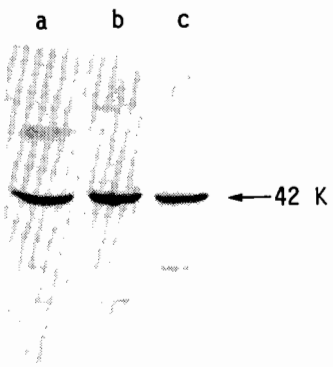

Fig. 1. Immunoblotting analysis of general acyl CoA dehydrogenase from rat liver and pig liver mitochondria. Mitochondrial extracts were subjected to immunoblotting. Lane a, rat liver mitochondrial extract (30 μg); lane b, pig liver mitochondrial extract (30 μg); lane c, purified pig kidney GAD (0.5 μg).

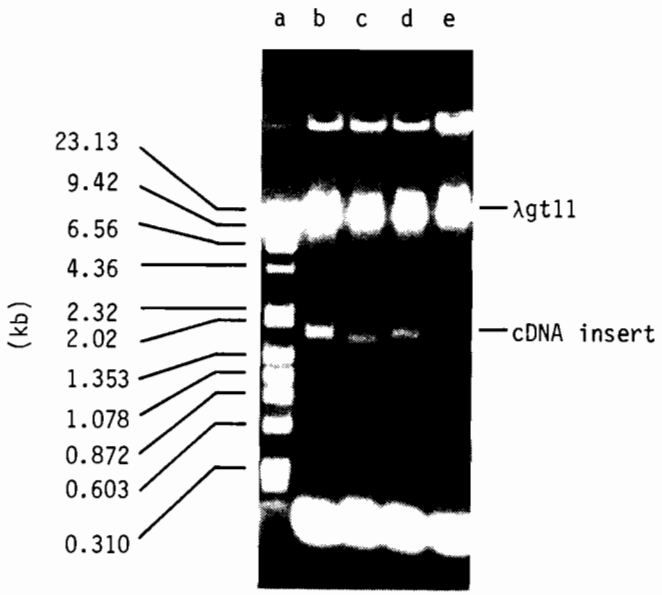

Fig. 2. Agarose gel electrophoresis of λ-GAD-1, 2, and 3 after digestion with EcoRI. Lane a, marker DNA; b, λ-GAD-1; c, λ-GAD-2; d, λ-GAD-3; e, λgt11 alone.

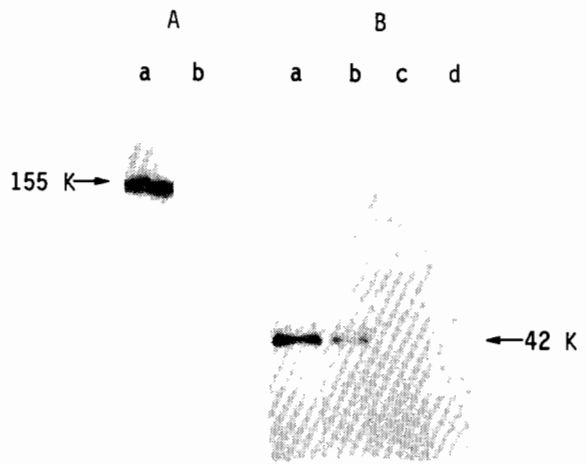

Fig. 3. Immunoblotting analysis of fusion protein prepared using λ-GAD-1 (A) and epitope selection (B). In A, lane a contained fusion protein from λ-GAD-1; lane b, fusion protein from a negative clone (insert size; 1.7 kb). In B, antibodies epitope-selected by using λ-GAD-1 were used in lanes a and b and those by using negative clone in lanes c and d. Lanes a and c, purified pig kidney GAD, lanes b and d, rat liver GAD.

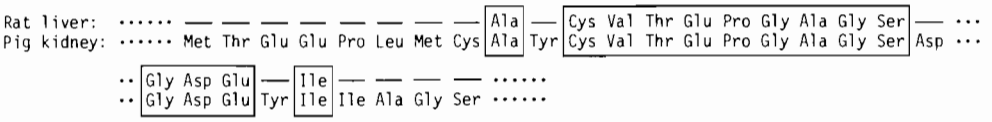

Fig. 4. Homologous amino acid sequences between rat liver and pig kidney GAD's.

In order to confirm the identity of the clone as a true GAD clone, we attempted epitope selection. The specific IgG epitope-selected by using the clone λ-GAD-1 was used to recognize GAD by immunoblotting analysis. As shown in Fig. 3B, lanes a and b, the epitope-selected IgG clearly recognized pig kidney and rat liver GAD's, whereas no band was detected when IgG obtained from a negative clone was used (lanes c and d). From these results, the cDNA clone isolated was identified to be that containing the GAD gene.

Partial amino acid sequence of rat liver and pig kidney GAD.

After digestion of the cDNA with restriction enzymes, DNA sequencing of smaller subfragments was carried out. As shown in Fig. 4, the amino acid sequence deduced from the DNA sequence of such subfragment was found to be identical with that of a portion of the pig kidney enzyme. This indicates that the isolated cDNA clone contains the GAD gene. N-Terminal sequencing of pig kidney GAD was not successful, suggesting that the terminus is blocked.

From the present results of immunoreactivity, molecular weight, and partial amino acid sequence of the enzyme, rat liver GAD is considered to be homologous to some extent with pig kidney enzyme. Matsubara et al. (11) have reported that rat liver GAD is homologous with human GAD. All these results indicate that GAD has homology among mammalian tissues and species.

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