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Identification of a rat liver dipeptidyl aminopeptidase IV with a liver plasma membrane glycoprotein (gp110)

A study using dipeptidyl aminopeptidase IV-deficient rats

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A rat liver plasma membrane glycoprotein, gp110, was compared with dipeptidyl aminopeptidase IV (DAP IV) by using Wistar rats (DAP IV-positive rats) and Fischer 344 rats (DAP IV-negative rats). Fischer rats also lacked gp110 and gp110 of Wistar rats had DAP IV activity. Furthermore, we showed that the C-terminal sequence of gp110 was Ser-Leu-Arg, which was the same as the C-terminal amino acid sequence deduced from the nucleotide sequence of the cDNA of DAP IV. According to these results, we concluded that gp110 was identical with DAP IV.

Dipeptidyl aminopeptidase IV; 110000 Da Glycoprotein; Fischer 344 rat; Wistar rat

1. INTRODUCTION

Dipeptidyl aminopeptidase IV (DAP IV: EC 3.4.14.5) is a plasma membrane-bound peptidase which is widely distributed in various organs, including liver, where it is expressed at a high level in bile canalicular membranes [1,2].

Many investigators have already reported that DAP IV may play an important role in the metabolism of peptides, blood clotting, extracellular matrix interactions and lymphocyte activation [3–7]. On the other hand, membrane-bound glycoproteins are thought to mediate various functions of plasma membranes such as cell-cell interactions, transport and enzymatic activity [8]. In particular, the 110 000 Da glycoprotein (gp110) isolated from the rat liver bile canaliculi has been studied, mainly as to turnover and biogenesis pathway [9–11].

In recent years, much attention has been paid to determining whether DAP IV and gp110 are the same protein or not, but no definitive conclusion has been drawn yet [12–16]. We have reported that Fischer 344 rats (F344 rats) specifically lack DAP IV [17]. In the present paper, we compare Wistar rats with F344 rats, and confirm that gp110 and DAP IV are identical.

2. EXPERIMENTAL

2.1. *Purification of gp110*

Plasma membranes were prepared according to [18]. Gp110 was purified according to [9] with some modifications. Briefly, plasma membranes were solubilized for 12 h at 4°C with 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $Cacl_2$, 0.02% (w/v) NaN_3 and 1.5% (v/v) Triton X-100 (buffer A), followed by centrifugation for 20 min at 30 000 × *g*. The supernatant was applied to a ConA-Sepharose 4B column (Sigma, St. Louis, MO). The column was washed with buffer B (the same as buffer A, but with only 0.5% (v/v) Triton-X-100) and eluted with 0.2 M methyl α-D-mannopyranoside in buffer B. The eluted fraction was dialyzed against buffer B and then applied to a wheat germ agglutinin (WGA)-agarose column (Seikagaku Kogyo, Tokyo). The retained glycoproteins were then eluted with 0.2 M *N*-acetyl-D-glucosamine in buffer B. The eluted fractions were dialyzed against 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl and 0.1% (v/v) Triton X-100, and then applied to a rabbit IgG anti-DAP IV-Sepharose 4B column equilibrated with the same buffer. The pure glycoprotein was eluted with 2 mM Tris-HCl (pH 8.0) at room temperature.

The purity of the plasma membrane fraction was checked by assaying the following marker enzymes according to the methods given in the references: 5'nucleotidase [19,20], β-glucuronidase [21], NADPH-cytochrome c reductase [22] and succinate dehydrogenase [23]. DAP IV was assayed as described previously [17]. Protein concentrations were determined by the BCA Protein Assay (Pierce, Rockford, IL) [24].

2.2. *Affinity chromatography of a chymotryptic digest of gp110*

Gp110 (320 μg) was denatured for 5 min at 100°C in 0.1 M sodium bicarbonate (pH 8.2) and then digested with TLCK-treated α-chymotrypsin (3.2 μg) in the same medium for 6 h at 37°C. The reaction was terminated by heating in boiling water for 5 min. Then the pH of the reaction mixture was adjusted to 5.5. The digest was applied to an anhydrotrypsin agarose column (0.8 × 2.0 cm; Takara, Kyoto) pre-equilibrated with 0.05 M sodium acetate (pH 5.5) containing 0.02 M $CaCl_2$ and then the column was washed with the same buffer. The adsorbed peptides were eluted with 5 mM HCl (pH 2.5).

2.3. *Synthesis of Ser-Leu-Arg*

Ser-Leu-Arg was synthesized by means of the solid phase procedure. In this study, we used a semi-manual peptide synthesizer (RaMPS; Dupont/NEN Research Products, Boston, MA).

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Table I

Intracellular distribution of marker enzymes, DAP IV and protein in liver of Wistar and F344 rats

		Fractions			
		Homogenate	1000 × g sup.	1000 × gppt.	Plasma membrane fr.
Plasma membrane[a]	Wistar	0.050 ± 0.013	0.052 ± 0.018	0.173 ± 0.066	0.352 ± 0.229
	F344	0.046 ± 0.006	0.062 ± 0.001	0.115 ± 0.021	0.224 ± 0.037
Lysosomes[b]	Wistar	3.09 ± 1.21	3.34 ± 1.85	1.38 ± 0.63	2.01 ± 0.63
	F344	3.41 ± 0.33	3.03 ± 0.55	0.945 ± 0.177	2.35 ± 0.49
Microsomes[c]	Wistar	0.046 ± 0.003	0.153 ± 0.111	0.065 ± 0.034	0.039 ± 0.005
	F344	0.044 ± 0.005	0.108 ± 0.100	0.060 ± 0.004	0.065 ± 0.009
Mitochondria[d]	Wistar	0.011 ± 0.003	0.009 ± 0.003	0.039 ± 0.005	0.003 ± 0.001
	F344	0.014 ± 0.004	0.009 ± 0.003	0.036 ± 0.004	0.004 ± 0.002
DAP IV[e]	Wistar	0.038 ± 0.013	0.017 ± 0.012	0.197 ± 0.04	2.43 ± 0.76
	F344	0.002 ± 0.001	0.002 ± 0.002	0.003 ± 0.003	0.001 ± 0.001
Protein recovery[f]	Wistar	100	79.7 ± 13.9	2.31 ± 0.56	0.130 ± 0.014
(%)	F344	100	77.8 ± 23.8	5.26 ± 1.92	0.145 ± 0.007

The data represent the means ± SD for 4 separate experiments. One unit of enzyme activity equals 1 μmol of substrate changed per min.
[a]5'-Nucleotidase, U/mg protein
[b]β-Glucuronidase, mU/mg protein
[c]NADPH-cytochrome c reductase, U/mg protein
[d]Succinate dehydrogenase, U/mg protein
[e]U/mg protein
[f]%, yield based on the homogenate

3. RESULTS AND DISCUSSION

First we purified gp110. The liver homogenate was centrifuged at 1000 × g, the pellet was rehomogenized in a sucrose solution, and then fractionated by discontinuous sucrose density gradient centrifugation, and finally the plasma membrane fraction was collected [18].

As shown in Table I, the intracellular distribution patterns of the activities of marker enzymes and protein recovery were almost the same in Wistar and F344 rats, except for that of DAP IV activity. Also, solubilized

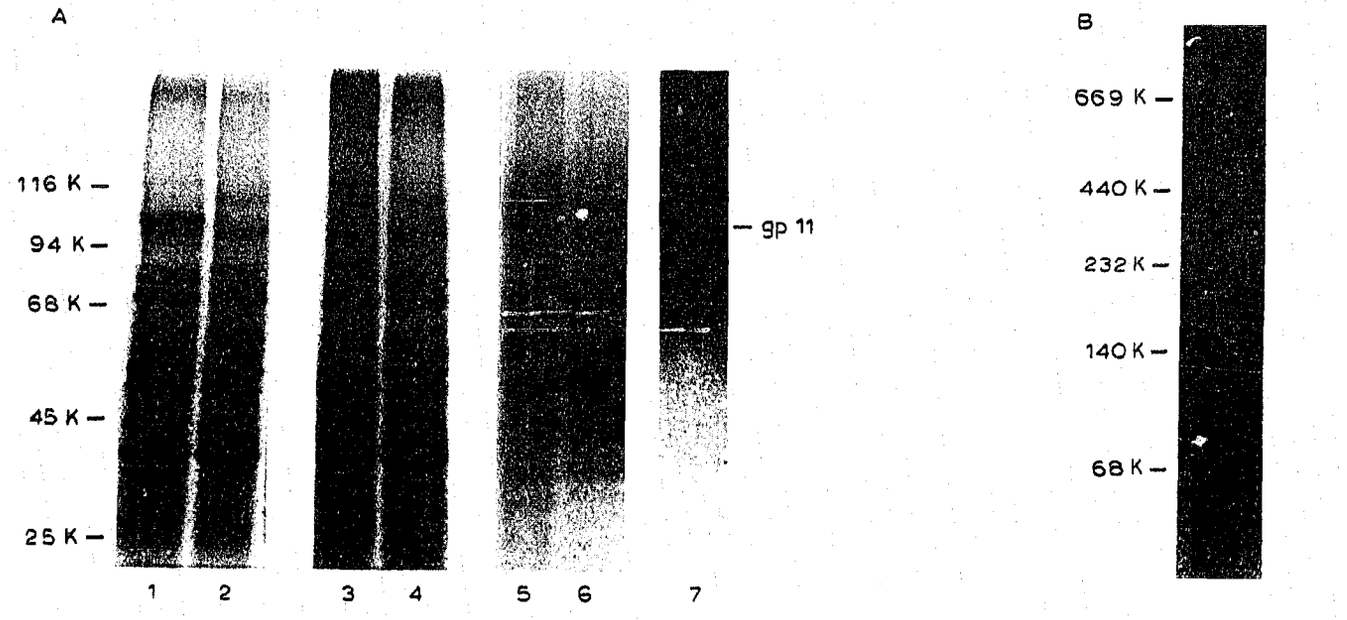

Fig. 1 (A). SDS-PAGE analysis of the fractions obtained during the purification of gp110. The electrophoresis was carried out on 7.5% SDS-polyacrylamide gels, and subsequently stained with Coomassie brilliant blue. Lanes 1 and 2: total plasma membrane proteins extracted with 1% Triton X-100. Lanes 3 and 4: ConA-Sepharose column flow-through fraction. Lanes 5 and 6: ConA-Sepharose column eluted fraction. Lane 7: anti-DAP IV-Sepharose column eluted fraction. Lanes 1, 3, 5 and 7 were samples from Wistar rats. Lanes 2, 4 and 6 were samples from F344 rats. (B) Activity staining of gp110 after non SDS-PAGE (6% gel). Specific demonstration of DAP IV activity in the gel was carried out using the simultaneous azo-coupling method. The gel was incubated with Gly-Pro-pNA at 37°C until colouring became visible and then with a coupling salt [26] to yield a purple azo dye. Bars (left) indicate the positions of protein standards: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), β-galactosidase (116 kDa), phosphorylase *a* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa) and chymotrypsinogen (25 kDa).

plasma membrane proteins from the two strains showed similar SDS-polyacrylamide gel electrophoresis (SDS-PAGE) patterns, a difference being one protein band, which corresponded to a molecular weight of about 110,000 (Fig. 1A, lanes 1 and 2). The SDS-PAGE patterns were similar to those that we had already reported for the membranes of Wistar and F344 rat kidneys [17].

Following the purification of gp110, the solubilized plasma membranes were applied to a ConA-Sepharose column. The 110 000 Da protein of Wistar rats was adsorbed on ConA-Sepharose (Fig. 1A), lane 5), but the other proteins of F344 rats showed a similar pattern to that of Wistar rats (Fig. 1A, lanes 3–6). The SDS-PAGE patterns of WGA-agarose-adsorbed fractions, after ConA-Sepharose column chromatography, were the same as those seen in lanes 5 and 6 of Fig. 1A (data not shown). Though other investigators have purified gp110 in a denatured form from gels after SDS-PAGE of a WGA-agarose adsorbed sample, we used another approach to get an undenatured form of gp110. The WGA-agarose-adsorbed fraction was applied to a rabbit IgG anti-DAP IV-Sepharose column. The effluent only showed the 110 000 Da protein band (Fig. 1A, lane 7). The purified gp110 was run on non-SDS-PAGE. In the range of 200 000 ~ 300 000 Da, which corresponded to the molecular weight of native DAP IV [25], Gly-Pro-pNA was hydrolyzed (Fig. 1B). From these results, we deduced that F344 rats also lack gp110 and that gp110 is identical with DAP IV.

Recently, the sequences of cDNAs for rat DAP IV and gp110 were published [16,27]. The predicted amino acid sequences of DAP IV and gp110 showed striking homology, but there were differences in 5 amino acid residues at corresponding positions when they were compared up to position 767. DAP IV has the entire sequence of 767 residues, while gp110 has 792 residues. The C-terminal sequences of DAP IV and gp110 are shown in Fig. 2. As DAP IV and gp110 are the same protein, we analyzed the C-terminal sequence of gp110, which we obtained from Wistar rat liver, to resolve this inconsistency.

The C-termini of DAP IV and gp110, predicted from their cDNA sequences, contain an Arg residue, so we used an efficient method that is useful for the selective isolation of C-terminal peptide from a digestion mixture of a protein, taking advantage of a unique property of immobilized anhydrotrypsin [28]. The chymotryptic digest of Wistar rat liver gp110 was applied to an anhydrotrypsin agarose column. Most of peptides passed through the column. The adsorbed fraction was subjected to reversed-phase high-performance liquid chromatography (HPLC). The HPLC pattern of the fraction is shown in Fig. 3A. The adsorbed fraction gave a few peaks, but there was only one peak which contained a peptide that could be determined by means of the fluorescamine reaction (indicated by a shaded bar). The peptide in this peak was concentrated and its amino acid composition was determined with an amino acid analysis system (Shimadzu, Kyoto). Three amino acids, that is, Ser, Leu and Arg, were detected. As anhydrotrypsin shows strong affinity for peptides containing Arg or Lys residues at their C-termini, the C-terminal sequence of gp110 is thought to be (Ser, Leu)-Arg. We then synthesized a peptide, Ser-Leu-Arg, using a semi-manual peptide synthesizer, and subjected to reversed-phase HPLC (Fig. 3B). The synthetic Ser-Leu-Arg showed the same retention time as that of the peptide peak. From this, the C-terminal sequence of gp110 was found to be Ser-Leu-Arg, which is the same sequence as the predicted sequences from the cDNA of DAP IV [16]. The inconsistency of the C-terminal sequence of DAP IV and gp110 might be caused by a frame shift of reading nucleotides.

DAP IV (81): · · · C F S L R (767) (STOP)

gp110 (91): · · · C F S L T L A W · · · · L K T A L L R (792) (STOP)

Fig. 2. Comparison of the C-terminal amino acid sequences of DAP IV and gp110. a) reference [16], b) reference [27]. A single letter code is used for the amino acids.

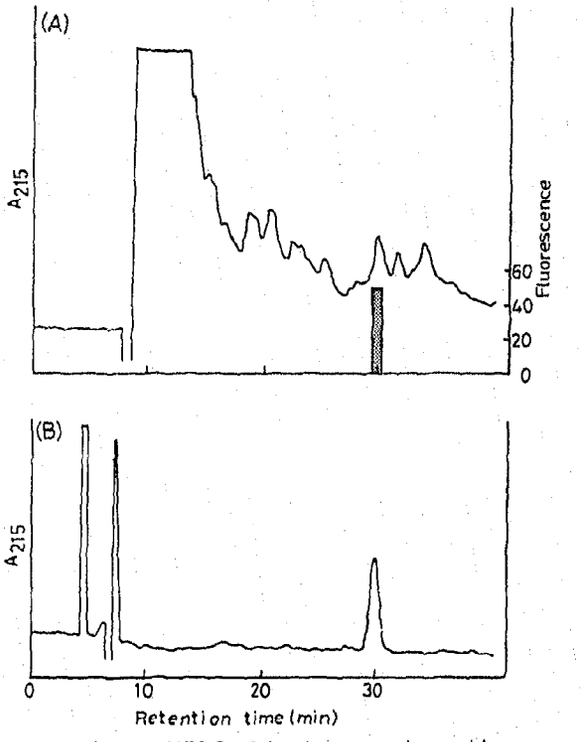

Fig. 3. Reversed-phase HPLC of the chymotryptic peptides recovered in the adsorbed fraction on anhydrotrypsin agarose chromatography (A) and the synthetic Ser-Leu-Arg (B). (A) Chymotryptic peptides prepared as described in section 2 were subjected to reversed-phase HPLC on a column of TSK-gel ODS-120 (0.46 × 25 cm). Elution was performed with 6% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min and the 215 nm absorbance was measured. Fractions of 0.5 ml were collected. The peptide concentration of each fraction was measured by fluorometry with fluorescamine (shaded bar). (B) 2 nmol synthetic Ser-Leu-Arg.

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