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# MOLECULAR CLONING OF A cDNA ENCODING A NOVEL FATTY ACID-BINDING PROTEIN FROM RAT SKIN

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**SUMMARY**: A novel skin-type fatty acid-binding protein, termed cutaneous(C)-FABP, has been purified from rat skin and a cDNA clone for this protein has been identified. The purified protein had the ability to bind long chain fatty acids like other rat FABPs. The deduced amino acid sequence of the cDNA clone comprises residues yielding a molecular mass for the polypeptide of 15.1 kDa and exhibits around 50% identity to myelin P2 protein, adipocyte FABP and heart FABP. Our results propose that C-FABP is a new member of the FABP family. 

Fatty acid-binding proteins (FABPs), found in the cytosol of various animal tissues, are low molecular mass (aroud 15 kDa) proteins that are capable of binding long chain fatty acids (1). Although they are thought to be involved in intracellular transport and metabolism of fatty acids (2), their precise functions in the cell remain unclear. These cytosolic proteins all have similar molecular weights and amino acid compositions, exhibit some sequence homology, and form a protein superfamily with other cytosolic proteins such as cellular retinoid-binding proteins and myelin P2 protein (3, 4).

As skin is one of the most active organs of lipid metabolism in mammals, it is possible that cutaneous tissues contain FABP-like proteins involved in lipid metabolism. Although there are some reports suggesting presense of FABP-like proteins in the skin (5, 6), no precise characterization of these proteins has been performed. Recently, Madsen et al. also described that FABP-like protein, designated PA-FABP (psoriasis-associated FABP), was highly expressed in psoriatic epidermis which is actively proliferative (7). Little, however, is known about the relationship between these FABP-

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like proteins present in the skin. Therefore it is of importance to isolate a FABP(s) from skin to determine the structure and its biological functions.

In the present study, we have first purified a skin-type FABP to homogeneity from normal rat skin and identified a cDNA clone encoding this protein. We here report that the skin-type FABP is abundantly expressed in normal rat skin cytosol and that this protein is a new member of the FABP family.

## MATERIALS AND METHODS

Preparation of Rat Skin Cytosol: Dorsal skin (5 cm x 5 cm, each) from 6 male Sprague-Dawley rats weighing 300 g, was shaved with electric clippers. The skin was dissected out, scraped with a sharp scalpel blade to remove subcutaneous fat and immediately rinsed with ice-cold saline. The skin was scraped in 250 ml of 30 mM Tris/HCl buffer, pH 7.3, containing 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol, and then homogenized with a Polytron tissue homogenizer. The homogenate was centrifuged at 10,000 x g for 30 min and the resulting supernatant was centrifuged at 125,000 x g for 90 min.

Purification of C-FABP and Heart FABP: The rat skin cytosol was dialyzed against 30 mM Tris/HCl buffer, pH 8.0, containing 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol, and then applied to a DE-52 column (2.5 x 15 cm), previously equilibrated with the above same buffer. The flow through was collected and dialyzed against 50 mM 2-morpholinoethane sulfonic acid buffer, pH 5.5, then applied to a Mono S column (bed volume 1 ml, Pharmacia), equilibrated with the same buffer. The column was washed with the same buffer and then developed with a linear gradient of 0-0.5 M NaCl in the same buffer (60 ml of each buffer). The 180-190 mM NaCl fractions were pooled and then applied to a Hiload 26/60 Superdex 75 column (2.6 x 62 cm, Pharmacia), equilibrated with 50 mM potassium phosphate buffer, pH 7.0. C-FABP was obtained as the purified form in the 15 kDa fraction, and gave a single band on SDS-polyacrylamide gel. Heart FABP was purified by the modified method as described previously (8).

Determination of Partial Amino Acid Sequence: C-FABP was digested with *Achromobacter lyticus* lysyl endopeptidase (Wako Pure Chemical Industries, Ltd.) at an enzyme/substrate ratio 1:20 (by mass) in 100 mM Tris/HCl, pH 8.5, for 8 h at 25 ℃. Lysyl endopeptidase peptides were separated by reversed-phase HPLC (Capcell pak, 0.46 x 15 cm, Shiseido). Column was developed with gradient of acetonitrile concentration in 0.1% (by vol.) trifluoroacetic acid. Elution was monitored by absorbance at 214 nm. Sequence analysis was performed using an Applied Biosystems 470A gas-phase sequencer, equipped with a 120 A phenylthiohydantoin analyzer.

Preparation of Rat Skin cDNA Library: Poly $(A)^+$ RNA was directly prepared from the skin of male Sprague-Dawley rats by absorption on oligo-dT cellulose tablet according to the instructions supplied by the manufacture (Invitrogen Corp.). Three μg of poly $(A)^+$ RNA was used to synthesize cDNA primed with an oligo-dT primer using a cDNA synthesis kit according to the instructions (Pharmacia). The cDNA inserts synthesized containing EcoRI linker were ligated into EcoRI site of λgt11 vector (CLONTECH). Approximately 1.5 x $10^6$ primary clones were recovered.

Cloning of a cDNA Encoding C-FABP: The polymerase chain reaction (PCR) was carried out to get a probe for screening cDNA library. For PCR, the sense and antisense primers were synthesized on the basis of sequencing of lysyl endopeptidase digested-

peptides (amino acids 34-42 and 116-125). The PCR was performed for 40 cycles (1 min. at 93℃, 12 min. at 55 ℃ and 3 min. at 72℃) in a reaction mixture (100 μl) containing the first strand cDNA synthesized from 1 μg of the rat skin poly $(A)^+$ RNA. A 276 bp fragment was isolated by PCR amplification. Sequencing of the PCR product revealed that it encoded the rat C-FABP. This fragment was used as a probe to screen $1.5 \times 10^5$ recombinants from the rat skin cDNA library. The positive clones were sequenced in both directions using an Applied Biosystems 373A DNA sequencer.

Fatty Acid-Binding Assay: Lipidex 1000 and [1-$^{14}$C] stearic acid (58 Ci/mol) were purchased from Sigma and Amersham, respectively. Delipidation of FABPs and the binding assay of fatty acid to C-FABP and heart FABP were performed according to Hitomi et al. (9). The radiolabeled fatty acids dissolved in ethanol and C-FABP or heart FABP were incubated in 10 mM potassium phosphate buffer, pH 7.4 at 37℃ for 1 h. The incubation volume was 500 μl and the final concentration of each component was 0.3 μM C-FABP or heart FABP, 1% (by vol.) ethanol and 2-20 μM fatty acid (specific activity, 12,000 dpm/nmol). After being chilled on ice for 10 min, incubation mixtures received 75 μl of ice-cold 50% (by vol.) Lipidex suspension in binding assay buffer, and then incubated at 4℃ for 10 min. Lipidex was removed by centrifugation at 15,000 x g for 5 min and 200 μl of the supernatant was removed for determination of radioactivity by liquid scintillation counting.

## RESULTS AND DISCUSSION

Tricine SDS-polyacrylamide gel electrophoresis (PAGE) pattern of rat C-FABP at various stages of purification is shown in Fig. 1. The mobility of the purified C-FABP on SDS-polyacrylamide gel was slightly slower compared to that of heart FABP. The amount of the purified protein recovered from 6 rats skin (5 cm x 5 cm, each) was about 1.0-1.2 mg, which is approximately 1% of the total cytosolic proteins. From this results, C-FABP appears to be abundantly present in normal rat skin cytosol.

Fig. 2 shows the binding assay of radiolabeled stearic acid with the purified rat C-FABP and heart FABP. Although both proteins binded stearic acid, the ability of C-FABP to bind stearic acid was slightly less than that of heart FABP. The binding assay with other fatty acids (palmitic, arachidonic, oleic, and linoleic acids, 10 μM each) were also performed under the same conditions and similar results were obtained. These results indicate that C-FABP is a new member of FABP family.

To isolate a cDNA clone encoding C-FABP, partial amino acid sequences of rat C-FABP was determined. Lysyl endopeptidase digestion of C-FABP and the subsequent fractionation by HPLC yielded a peptide map in which eleven peaks gave sequence information (data not shown). With primers based on these sequences, the PCR

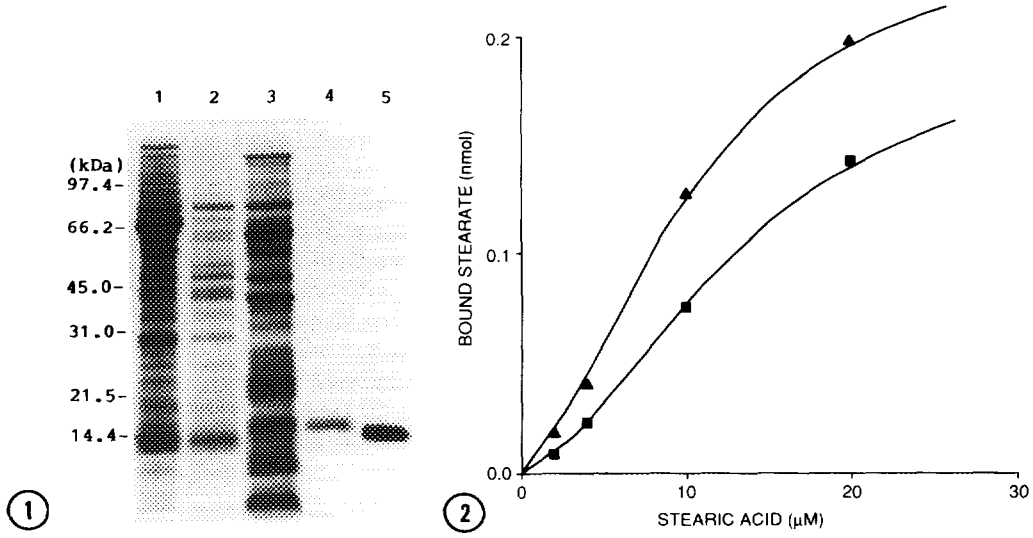


Fig. 1. Tricine/SDS/PAGE of rat cutaneous FABP at various stages of purification. The gel was stained with Coomassie brilliant blue. Lane 1, rat skin high speed supernatant (25 μg of protein); lane 2, DE52 flow through (10 μg of protein); lane 3, Mono S fraction (18 μg of protein); lane 4, purified cutaneous FABP (0.6 μg of protein); lane 5, purified heart FABP (2 μg of protein).

Fig. 2. Binding assay of radiolabeled stearic acid with the purified rat cutaneous FABP (■) and heart FABP (▲). [1-$^{14}$C] Stearic acid was incubated with 150 pmol FABP at 37 ℃ for 1 h. Unbound fatty acid was separated from bound fatty acid by Lipidex 1000.

technique was used to isolate partial C-FABP cDNA clones as described under "Materials and Methods". One was found to contain a 276 bp fragment with the partial peptide sequences derived from the purified C-FABP. This fragment was used to screen a rat skin cDNA library. Fig. 3 shows nucleotide sequence of the full length of rat C-FABP cDNA and the deduced amino acid sequence. The isolated cDNA clone contained all of the peptide sequences derived from the purified C-FABP and one open reading frame encoding a 135-amino acid polypeptide with a calculated relative molecular mass of 15,061. This molecular weight is consistent with the molecular mass of 15 kDa on SDS-polyacrylamide gel electrophoresis of C-FABP purified from rat skin. The predicted amino acid sequence of C-FABP contains the highly conserved N-terminal regions among the FABP family (10), which has been suggested to be involved in forming the fatty acid-binding structure (11, 12). Other part of the polypeptide of C-FABP also has a sequence similarity to other rat FABPs. Particularly, 54.1, 52.6 and

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   1                CGCCACCGCGTCTCCTTGCTGCTTTTGTGCTCTCCCGCTCGCC  43

  44 ATGGCCAGCCTTAAGGACCTGGAAGGGAAGTGGCGTCTGGTGGAAAGCCACGGG  97
   1 M  A  S  L  K  D  L  E  G  K  W  R  L  V  E  S  H  G    18

  98 TTTGAGGACTACATGAAGGAACTAGGAGTAGGGCTGGCTCTTAGGAAGATGGGT 151
  19 F  E  D  Y  M  K  E  L  G  V  G  L  A  L  R  K  M  G    36

 152 GCCATGGCCAAACCAGACTGCATCATTACCCTCGACGGCAACAACCTCACCGTC 205
  37 A  M  A  K  P  D  C  I  I  T  L  D  G  N  N  L  T  V    54

 206 AAAACTGAGAGCACGGTGAAGACGACCGTGTTTTCTTGCACCTTGGGAGAGAAG 259
  55 K  T  E  S  T  V  K  T  T  V  F  S  C  T  L  G  E  K    72

 260 TTTGATGAAACCACAGCTGATGGCAGGAAAACTGAGACGGTCTGCACCTTCACA 313
  73 F  D  E  T  T  A  D  G  R  K  T  E  T  V  C  T  F  T    90

 314 GACGGTGCCCTGGTCCAGCACCAGAAGTGGGAAGGGAAAGAAAGCACGATAACG 367
  91 D  G  A  L  V  Q  H  Q  K  W  E  G  K  E  S  T  I  T   108

 368 AGAAAACTGAAGGACGGGAAGATGGTCGTCGAGTGCGTCATGAACAATGCCATC 421
 109 R* K  L  K  D  G  K  M  V  V  E  C  V  M  N  N  A  I   126

 422 TGTACTCGGGTCTATGAGAAGGTACAATGAGGACTGGCTAACTCGTCATCCTGG 475
 127 C  T  R  V  Y  E  K  V  Q  end                         135

 476 ACAGCAGTCAGCTGGCTGAGGGAATAAGCTCAATTCAATGAGCAGGTCGTACAG 529

 530 AACCACACTGCTTCACTTCTTTGGTTTTATTTTTCATGACTTTTCATCATAGAC 583

 584 ACTTTACCCGAAACCCATGTCAGACCGTTGGTTTACCCAGGATCATTCCTTTGG 637

 638 TTAGTAAATAAATGCGTTTGTGCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 691

 692 AAAAAAAAAAAAA                                          704
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Fig. 3. Nucleotide and deduced amino acid sequences of C-FABP cDNA. The sequence of the C-FABP cDNA clone and the translated amino acid sequence are shown. Amino acid sequences for the sense and antisense primers are underlined. A potential polyadenylation signal is double underlined. The conserved arginine residue (R) which may electrostatically interact with carboxylate group of fatty acids is indicated by asterisk.

48.1% of C-FABP amino acid residues were identical with those of rat myelin P2 protein, mouse adipocyte FABP (aP2) and rat heart FABP, respectively. And C-FABP appears to contain the conserved arginine(residue 109) among FABP family which has been believed to be involved in the ionic interaction with the carboxylate group of fatty acids(13). Interestingly, C-FABP contains a tyrosine at a position where mouse aP2 is phosphorylated by insulin receptor (residue 19 of the aP2)(14). Although the significance of the tyrosine phsphorylation remains unclear, it would be interesting to examine whether C-FABP can be phosphorylated on tyrosine (residue 22) by the insulin receptor tyrosine kinase. C-FABP also contains cystein residues which are rarely found in other FABPs so far isolated. To date, there is few examples that five cystein residues (residues, 43, 67, 87, 120 and 127) are found in the polypeptide of FABP. It is

possible that these cystein residues might be involved in intra- and/or intermolecular disulfide bond formation. It would be of interest to examine whether site-directed mutagenesis of a specific cystein residue of C-FABP affects the flexibility of protein conformation and the binding specificity for lipophilic ligands.

More interestingly, C-FABP has 81.5% sequence homology to human PA-FABP derived from psoriasis epidermis. The level of PA-FABP mRNA was found to be very low in normal human keratinocytes, compared to that of the psoriatic keratinocytes (7). On the other hand, rat C-FABP was abundantly present even in normal rat skin. The precise relationship between these two FABPs remains to be determined. It would be of interest to examine whether PA-FABP is a human homologue of C-FABP, because PA-FABP may be related to the pathogenesis of psoriatic disorder.

In this report, we demonstrate that a skin-type FABP is a structurally novel protein and a new member of FABP family on the basis of cDNA cloning of this protein.

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