Cloning of the cDNA encoding human skeletal-muscle fatty-acid-binding protein, its peptide sequence and chromosomal localization

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A cDNA clone for the human skeletal-muscle fatty-acid-binding protein (FABP) was isolated by screening of a human adult muscle λ gt11 expression library with an anti-(muscle FABP) serum. The identity of the clone was confirmed by transcription/translation *in vitro* in plasmid pSP6.5, followed by immunoprecipitation. The nucleotide sequence of the 551 bp cDNA insert showed an open reading frame of 399 nucleotides, coding for a protein of 133 amino acid residues with a calculated molecular mass of 14858 Da and a pI of 4.94. Only one cysteine residue was found, at position 125. Peptide sequence analyses of human skeletal-muscle and heart FABP, after carbamoylmethylation and lysyl endopeptidase digestion followed by automatic Edman degradation, showed that both proteins are identical. No evidence was found for the existence of isoproteins in muscle. The chromosomal localization of the human muscle FABP gene was determined by analysing 31 human $\times$ rodent somatic-cell hybrid lines. The human muscle FABP gene could be assigned to chromosome 1pter-q31.

INTRODUCTION

Fatty acid metabolism in mammalian cells depends on a flux of fatty acids, between the plasma membrane and mitochondria or peroxisomes for β -oxidation, and between other cellular organelles for lipid synthesis. Fatty-acid-binding proteins (FABPs) are believed to play a role as transport vehicles of these hydrophobic compounds throughout the cytoplasm. Experiments *in vitro* demonstrated the suggested transport function of FABPs [1,2]. *In vivo*, however, this phenomenon remains to be established. Until now, most investigations on FABPs concern the isolation and characterization of FABPs in several tissues of different species [3,4]. At least three different FABP types were characterized in liver, heart and intestine of man and rat [3,4]. The adipocyte lipid-binding protein [5,6] and the myelin P2 protein [7,8] are also considered to be members of the FABP family.

In 1983 the first primary sequence data appeared for rat liver FABP [9]. At the present time the primary sequence of liver FABP of man [10,11] and rat [9], heart FABP of rat [12], cattle [13] and mouse [14] and of intestinal FABP of rat [15,16] have been elucidated by cDNA sequencing. Also, primary sequence data have been obtained on human [17,18] and rat [19,20] heart FABP and bovine brain FABP [21] by peptide sequence analysis.

Recently, FABPs isolated from human skeletal muscle [22] and bovine brain [21] were found to show only slight differences from the heart FABPs in these species. At least three different chromosomal loci were found for the mouse heart FABP gene [23]. These findings suggest the existence of FABPs which are closely related to other FABPs, but diverge by functional and/or tissue-specific adaptation and have a different chromosomal localization. Another possibility is that skeletal-muscle and heart FABPs are identical proteins and that the different chromosomal loci found for the mouse heart FABP gene is due to the presence of pseudogenes. Therefore we decided to elucidate the primary

sequence of human skeletal-muscle FABP by cDNA and peptide analysis. We determined also the amino acid sequence similarity between skeletal-muscle FABP and other hydrophobic ligand-binding proteins and the chromosomal localization of the human skeletal-muscle FABP gene.

MATERIALS AND METHODS

Materials

The cDNA synthesis kit was obtained from Pharmacia, Uppsala, Sweden; the M13 Kilobase Sequencing System was from BRL, Gaithersburg, MD, U.S.A.; wheat-germ extract, L-[35 S]methionine (15 mCi/ml) and α -[35 S]thio-dATP (10 mCi/ml) were from Amersham International, Little Chalfont, Bucks., U.K.; alkaline phosphatase-conjugated swine anti-rabbit IgG was from Dakopatts A/S, Glostrup, Denmark. Acetonitrile, trifluoroacetic acid and lysyl endopeptidase were obtained from Wako Pure Chemical Industries, Japan; Nonidet P40 (NP40) was from Fluka AG, Buchs, Switzerland.

Isolation of polyadenylated RNA [poly(A) $^{+}$]

Total cellular RNA was extracted from 5 g of frozen (-80°C) human adult muscle tissue with 70 ml of ice-cold 3 M-LiCl/6 M-urea. The solution was homogenized for 1 min with a Polytron homogenizer (PT10 tip) and incubated overnight on ice. After centrifugation for 30 min at 141 000 g the pellet was resuspended in 14 ml of 10 mM-Tris/HCl (pH 7.4)/0.5 % SDS. The suspension was vigorously shaken with 21 ml of phenol/chloroform/3-methylbutan-1-ol (25:24:1) for at least 5 min. After centrifugation for 10 min at 5000 g the phenol extraction was repeated once. Finally, total cellular RNA was precipitated at -20°C by the addition of 1.4 ml of 3 M-sodium acetate, pH 5.2, and 2 vol. of ethanol to the aqueous phase. Poly(A) $^{+}$ RNA was selected by oligo(dT)-cellulose chromatography as described by Maniatis *et al.* [24].

Abbreviation used: FABP, fatty-acid-binding protein; poly(A) $^{+}$, polyadenylated; NP40, Nonidet P40.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession no. X56549 (human CHMFA cDNA).

cDNA synthesis and library screening

To construct a human adult cDNA library in the λ gt11 vector, 5 μ g of poly(A)⁺ RNA was used according to the cDNA-synthesis-kit procedure. The human adult muscle cDNA library constructed contained 1.4×10^6 independent plaques. An anti-(human muscle FABP) serum, previously prepared [22], was diluted 1:400 to screen the cDNA library by the method of Young & Davis [25]. To detect specifically bound antibody, alkaline phosphatase-conjugated swine anti-rabbit IgG was used.

'In vitro' transcription/translation assay

The cDNA insert was ligated in pSP6.5 [26], grown in JM109, and afterwards linearized with *Bam*HI. RNA transcripts were synthesized *in vitro* from the *Bam*HI-digested clone in the presence of the dinucleotide primer G(5')ppp(5')G and SP6 RNA polymerase [27]. Translation *in vitro* in a nuclease-treated wheat-germ extract was performed for 60 min at 25 °C in 30 μ l of a reaction mixture containing 1 mM-amino acids, 1 mM-[³⁵S]methionine and 0.65 M-KCl.

Immunoprecipitation of newly synthesized FABP

A portion (200 μ l) of Protein A-Sepharose CL-4B in phosphate-buffered saline (10 %, v/v) was incubated with 75 μ l of anti-(human muscle FABP) serum for 2 h at room temperature under rotation. Non-specifically bound proteins were removed by washing the gel suspension four times with 500 μ l of buffer A (500 mM-NaCl/10 mM-Tris/HCl (pH 7.4)/0.05 % NP40). Subsequently, the gel suspension was incubated with 25 μ l of the translation assay mix in 175 μ l of 150 mM-NaCl/50 mM-Tris/HCl (pH 7.4)/0.05 % NP40 for 3 h at 4 °C, under rotation. Finally, the mixture was washed four times with buffer A and 50 μ l of SDS/gel-electrophoresis loading buffer was added to the gel suspension. SDS/PAGE was performed on a 12 % (w/v) polyacrylamide gel.

DNA sequence analysis

For sequence analysis, the 551 bp cDNA insert was ligated into M13 mp18 [26], grown in JM109, and subjected to dideoxy-chain-termination reactions [28], using the M13 Kilobase Sequencing System. cDNA inserts oriented in both directions in M13 mp18 were used for sequence analyses and subjected to gel electrophoresis (8 %, 60 cm) for 2 and 8 h on a LKB macrophor electrophoresis unit. Under these conditions DNA fragments up to 600 bp can be completely sequenced in both directions without making first restriction fragments.

Analytical h.p.l.c. of skeletal-muscle and heart FABPs

Skeletal-muscle and heart FABPs were isolated as described previously [22]. In addition, both preparations were further purified on a Toyo Soda ODS (octadecyl-silica) 120T column (0.46 cm $\times$ 25 cm). The FABPs were eluted with a linear gradient (1–42 %, v/v) of acetonitrile in 0.1 % (v/v) trifluoroacetic acid for the first 5 min and then with 42–48 % acetonitrile for the next 25 min, and, finally, with 48–75 % acetonitrile for 10 min. The flow rate was 1 ml/min.

Carbamoylmethylation and structural analysis of skeletal-muscle and heart FABPs

Carbamoylmethylation of both proteins after h.p.l.c. purification was performed with iodoacetamide by the method of Hirs [29]. After dialysis against 10 mM-Tris/HCl, pH 8.2, both FABPs were digested with lysyl endopeptidase at an enzyme/substrate ratio of 1:20 (w/w) in 100 mM-Tris/HCl, pH 8.2, for 6 h at 25 °C. The digests were applied to the Toyo Soda ODS 120T column. The peptides were eluted with linear gradient (1–60 %,

v/v) of acetonitrile in 0.1 % (v/v) trifluoroacetic acid in 60 min at a flow rate of 1 ml/min. Elution was monitored by A_{214} . Amino acid analyses and sequencing were described previously [20].

Chromosomal localization of the muscle FABP gene

For the chromosomal localization of the human muscle FABP gene, a panel of 31 well-defined human $\times$ rodent somatic-cell hybrids was used [31]. These hybrid lines were isolated after fusion of human cells, obtained from different donors, with hypoxanthine phosphoribosyltransferase- or thymidine kinase-deficient rodent cell lines. Parental and hybrid cells were grown in F10 or RPMI-1640 medium supplemented with 2 mM-glutamine, penicillin (100 units/ml), streptomycin (100 μ g/ml) and 10 % (v/v) fetal-calf serum. For the chromosomal analysis of the hybrid cell lines, air-dried chromosome spreads were made according to standard procedures [31]. Of each cell line, at least 16 metaphases were examined, using R-banding after heat denaturation. The cells, used for chromosome analysis and DNA extraction, were always derived from the same culture batch.

RESULTS AND DISCUSSION

Isolation and identification of a full-length cDNA clone for the human skeletal-muscle FABP

Initial screening of 200 000 independent plaques of a human adult skeletal-muscle cDNA library, constructed in the λ gt11 expression vector with a high-titre anti-(muscle FABP) serum, yielded two individual clones. Only one of these two clones, named λ -hmF, remained antigen-producing after several screening procedures. After plaque purification, the identity of this clone was confirmed by an immunoprecipitation assay. The cDNA insert of λ -hmF was isolated by an *Eco*RI digestion and had a size of about 550 bp, estimated on agarose-gel electrophoresis. Subsequently, the insert was ligated in pSP6.5 and named pSP-hmF. After linearization with *Bam*HI, pSP-hmF was used in an 'in vitro' transcription assay. The RNA product obtained yielded, in an 'in vitro' translation assay, only one product (Fig. 1, lane 1). Its molecular mass was in good agreement with that of muscle FABP [22], which is somewhat higher than

Fig. 1. SDS/PAGE and subsequent autoradiography of the products obtained in 'in vitro' transcription of pSP-hmF and translation of its RNA, followed by immunoprecipitation

Lane M, molecular-mass markers; lane 1, 1 μ l of the total translation mixture; lane 2, 4 μ l of the immunoprecipitation assay; lane 3, [¹⁴C]formaldehyde-labelled pig liver FABP [2], with a molecular mass of about 14.2 kDa (based upon the molecular masses of liver FABP of rat [9] and man [11], which are 14 178 and 14 184 Da respectively).

that of pig liver FABP (Fig. 1, lane 3). The primary translocation product could also be immunoprecipitated from the translation mixture (Fig. 1, lane 2).

Nucleotide sequence and deduced amino acid sequence of the skeletal-muscle FABP

The nucleotide sequence of the cDNA insert was determined by the dideoxy method [28]. The complete nucleotide sequence comprises 551 bp (Fig. 2). A single long open reading frame of 399 nucleotides is present. The designated ATG translation initiation codon at position 46 is the first ATG codon occurring. It exists within a sequence which only diverges in position -1 (one nucleotide upstream of the ATG codon) from the optimal context for initiation, ACCATGG [32]. The TGA translational stop signal at position 444 is followed by a non-coding region of 107 nucleotides.

The predicted amino acid sequence for skeletal-muscle FABP (Fig. 2) indicates that the molecular mass of the protein is 14858 Da and the pI is 4.94. One cysteine residue is present, at position 125, and two tryptophan residues are present at positions 9 and 98. The molecular mass is in good agreement with that estimated from SDS/polyacrylamide gels [22]; the pI, however, is somewhat lower than that obtained from isoelectric-focusing assays [22]. Others [17,33,34] found comparable data for M_r and pI of human heart FABP, but the presence of cysteine was established only by Spener and his co-workers [18,33]. The presence of the cysteine residue can cause the self-aggregation of muscle FABP, which was observed during storage of pure muscle FABP and explained as denaturation [22].

Amino acid sequence of both skeletal-muscle and heart FABP

H.p.l.c. analysis of the purified proteins revealed two peaks for both skeletal-muscle and heart FABP. The amino acid compositions of these four peaks were identical with each other (results not shown).

H.p.l.c. analysis of the lysyl endopeptidase digest of carbamoyl-methylated skeletal-muscle FABP gave 12 peaks (Fig. 3), representing the purified peptides, which were necessary for the determination of the complete amino acid sequence of muscle FABP. For heart FABP a similar elution profile was obtained (results not shown). Two peaks contained no amino acids (Fig. 3, peaks a and c) and only one peak (Fig. 3, peak b) contained a mixture of peptides, which were later derived from peak 10 by secondary enzymic hydrolysis. No amino acid phenylthiohydantoin derivatives were released, indicating that the *N*-termini of both proteins were blocked, like those of other FABPs [7,10,11]. On the basis of the amino acid compositions and the partial sequences of the peptides obtained, only one amino acid sequence of skeletal-muscle FABP could be established (Fig. 4). For heart FABP a similar amino acid composition of the peptides was found as for muscle FABP. Sequence analyses of the peptide products 2, 9 and 11 gave similar data to those found for peptides derived from skeletal-muscle FABP (Fig. 4). FABPs from human skeletal muscle and heart are thus identical proteins and exist in only one form.

The amino acid sequence of skeletal-muscle and heart FABP (Fig. 4) perfectly matches with the predicted amino acid sequence obtained from our muscle cDNA (Fig. 2). The amino acid sequence of human heart FABP was reported by Offner *et al.* [17] and recently revised by Borchers *et al.* [18]. Now our cDNA data confirm the latter results and show also the presence of leucine at position 105 and cysteine at position 125. No isoforms for human [18] and rat [20] heart FABP were detected, in contrast with bovine heart FABP [35].

FABPs from bovine [13], rat [19,20] and mouse [14] heart show a high amino acid sequence similarity to human muscle FABP

THE cDNA SEQUENCE ENCODING HUMAN SKELETAL MUSCLE FABP

20																				40																				ATG		GTG		
gaattc ggcacgaggtagcttctctcagcctagccagcatcact																																								met		val		2
60										80																																		
GAC	GCT	TTC	CTG	GGC	ACC	TGG	AAG	CTA	GTG	GAC	AGC	AAG	AAT	TTC																														
asp	ala	phe	leu	gly	thr	trp	lys	leu	val	asp	ser	lys	asn	phe															17															
100										120																																		
GAT	GAC	TAC	ATG	AAG	TCA	CTC	GGT	GTG	GGT	TTT	GCT	ACC	AGG	CAG																														
asp	asp	tyr	met	lys	ser	leu	gly	val	gly	phe	ala	thr	arg	gln															32															
160										180																																		
GTG	CCC	AGC	ATG	ACC	AAG	CCT	ACC	ACA	ATC	ATC	GAA	AAG	AAT	GGG																														
val	ala	ser	met	thr	lys	pro	thr	thr	ile	ile	glu	lys	asn	gly															47															
200										220																																		
GAC	ATT	CTC	ACC	CTA	AAA	ACA	CAC	AGC	ACC	TTT	AAG	AAC	ACA	GAG																														
asp	ile	leu	thr	leu	lys	thr	his	ser	thr	phe	lys	asn	thr	glu															62															
240										260																																		
ATC	AGC	TTT	AAG	TTG	GGG	GTG	GAG	TTT	GAT	GAG	ACA	ACA	GCA	GAT																														
ile	ser	phe	lys	leu	gly	val	glu	phe	asp	glu	thr	thr	ala	asp															77															
280										300																																		
GAC	AGG	AAG	GTC	AAG	TCC	ATT	GTG	ACA	CTG	GAT	GGA	GGG	AAA	CTT																														
asp	arg	lys	val	lys	ser	ile	val	thr	leu	asp	gly	gly	lys	leu															92															
340										360																																		
GTT	CAC	CTG	CAG	AAA	TGG	GAC	GGG	CAA	GAG	ACC	ACA	CTT	GTG	CGG																														
val	his	leu	gln	lys	trp	asp	gly	gln	glu	thr	thr	leu	val	arg															107															
380										400																																		
GAG	CTA	ATT	GAT	GGA	AAA	CTC	ATC	CTG	ACA	CTC	ACC	CAC	GGC	ACT																														
glu	leu	ile	asp	gly	lys	leu	ile	leu	thr	leu	thr	his	gly	thr															122															
420										440																																		
GCA	GTT	TGC	ACT	CGC	ACT	TAC	GAG	AAA	GAG	GCA	TGA	cctgactgca																																
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ttttctctctgattttgtacaaatccac gaattc

Fig. 4. Primary structure of human skeletal-muscle FABP derived from amino acid composition and sequence analyses

Arrows indicate the residues identified by automated Edman degradation of peptides derived from lysyl endopeptidase digestion. K1–K12 correspond to peaks 1–12 of Fig. 3. Lys⁵² was not determined.

Table 1. Amino acid sequence similarity of human muscle FABP to other members of the family of hydrophobic ligand-binding proteins

Abbreviation used: BP, binding protein.

Protein	Species	Similarity (%)	Reference
Heart FABP	Cattle	99	[13]
	Rat	89	[19,20]
	Mouse	84	[14]
Brain FABP	Cattle	76	[21]
Adipocyte FABP	Man	65	[5]
Myelin FABP	Man	61	[7]
Retinol BP II	Rat	41	[36]
Retinoic acid BP	Cattle	40	[36]
	Mouse	40	[38,39]
Retinol BP I	Man	34	[37]
Liver FABP	Man	27	[10]
Intestinal FABP	Man	25	[16]

and intestine [16] FABP types. The high extent of conservation of amino acid sequence between (heart) muscle FABPs of different species throughout evolution and the occurrence of this FABP type in many other tissues [4,12,39] demonstrate the importance of this protein in fatty acid metabolism.

Chromosomal localization of the muscle FABP gene

To determine the chromosomal localization of the muscle FABP gene, the FABP cDNA insert was hybridized to a panel of 31 human × mouse or human × hamster cell lines, containing various sets of human chromosomes (Table 2). From the hybridization results it is obvious that the muscle FABP gene must be present on human chromosome 1. The optimal score of 0% discordancy means that the muscle FABP gene was detected in all cell lines retaining human chromosome 1 and not in any of the lines lacking this chromosome. All other chromosomes showed at least 13% discordance (Table 1). Two cell lines contained only a part of chromosome 1 (one chromosome 1pter-q31 and the other 1q42-qter). Of these two cell lines only the former showed a positive reaction with our cDNA probe, whereas

Table 2. Relationship between the human-muscle-FABP gene and the presence or absence of human chromosomes in the 31 somatic cell hybrids analysed

Chromosome no.	Chromosome/FABP; no. of clones				Discordance (%)
	+/+	+/-	-/+	-/-	
1*	11	0	0	18	0
2	5	2	7	17	29
3	5	3	7	16	32
4	6	5	6	14	35
5	11	5	1	14	19
6	7	4	5	15	29
7	8	6	4	13	32
8	6	5	6	14	35
9	6	2	6	17	26
10	6	4	6	15	32
11	7	4	5	15	29
12	6	6	6	13	39
13	5	3	7	16	32
14	5	6	7	13	42
15	6	4	6	15	32
16	10	4	2	15	19
17	9	4	3	15	23
18	8	2	4	17	19
19	6	9	6	10	55
20	9	1	3	18	13
21	9	5	3	14	26
22	8	8	4	11	39
X	8	9	4	10	42

* The two cell lines that only contained a part of chromosome 1 are not included.

the other cell line did not. Therefore, the human muscle FABP gene must reside on human chromosome 1 in the pter-q31 region.

The presence of one chromosomal locus for the human muscle FABP gene contrasts with the three chromosomal loci found for the mouse heart FABP gene [40] and disproves the presence of pseudogenes, as has been suggested for the mouse heart FABP gene [14,40]. These data also support the notion that skeletal-

muscle and heart FABPs are identical proteins. Although the FABP types are closely related and possibly originate from a common ancestral gene, their genes are dispersed throughout the genome in both men and mice [16,40,41]. The genes for the liver and intestinal FABP types of man are located on chromosome 2 and 4 respectively. For myelin and adipocyte FABP types of man, the chromosomal localization is not yet known. The genes encoding the retinoid-binding proteins appear to reside on the same chromosome in some species [23,39,42,43], but on different chromosomes in man [44,45].

In conclusion, human skeletal-muscle and heart FABPs are identical proteins, encoded by one gene located on chromosome 1. No evidence was found for the existence of isoproteins or closely related proteins in muscle. At the present time several important biological questions are unsettled: e.g., why are the FABP types dispersed throughout the genome? and which roles do these proteins play in the intracellular metabolism of fatty acids and other hydrophobic ligands? Future studies with the obtained cDNA may help us to unravel some of these questions.

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