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Identification of a Rat 30-kDa Protein Recognized by the Antibodies to a Recombinant Rat Cutaneous Fatty Acid–Binding Protein as a 14-3-3 Protein

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Immunoblot analysis with polyclonal antibodies raised against a recombinant rat cutaneous fatty acid–binding protein revealed a 30-kDa protein other than the 15-kDa fatty acid–binding protein in rat skin cytosol. This protein was present in a number of rat organs and in mouse 3T3 L1 cells. The amino acid sequences of the enzymatic peptides of the 30-kDa protein extracted from SDS-PAGE gels suggested that it was a mixture of the subunits of the eukaryotic signaling molecule, 14-3-3 protein. Glutathione *S*-transferase fusion proteins of 14-3-3 protein subunits were examined for cross-reaction by Western blotting, and the ε-subunit alone was found to be immunoreactive, so far as tested. It is likely that the 30-kDa protein detected in the rat tissues by the antibodies is the 14-3-3 protein ε-subunit. Although there is no apparent sequence similarity between the fatty acid–binding protein and the 14-3-3 protein subunit, they appear to share a common structural element recognized by the antibodies. Since 14-3-3 proteins and fatty acid–binding proteins are known to interact with a wide variety of cellular proteins, the presence of a common local structure might mutually modulate such interactions.

Key words: 14-3-3 protein, epidermis, fatty acid–binding protein, immunological cross-reaction, Western blot.

Fatty acid–binding proteins (FABPs) are *ca.* 15-kDa proteins whose principal functions are intracellular transport and storage of long-chain fatty acids (for recent reviews, see Refs. *1–3*). There is, however, convincing evidence of their roles in much more divergent biological processes such as cell growth and differentiation (*4–6*). A classical example of such a regulatory function is that of mammary-derived growth inhibitor from bovine mammary cells, which is now established to be heart FABP (*4*). The involvement of FABPs in the regulation of protein synthesis (*5*), spermatogenesis and germ cell apoptosis (*6*) has also been suggested. Cutaneous FABP (C-FABP, also known as epidermal FABP) was first identified as a highly up-regulated gene product in human psoriasis keratinocytes (*7*), and then as a normal constituent in rat skin (*8*) and many other tissues (*9–12*). C-FABP is remarkable for the fact that it is identical with protein DA11 involved in the repair of peripheral nerve injury (*13*), with an epidermal protein factor capable of inhibiting melanogenesis in pigmentary cells (*14*), which is an inhibitor of mammalian tyrosinase (*15*), and with a cancer metastasis–inducing protein (*16*). During an immunological study on the tissue distribution of C-FABP in rat, we found a protein of 30-kDa clearly recognized by polyclonal antibodies against a recombinant rat C-FABP. In this paper we describe the characterization and structural analysis of this cross-reactive protein.

MATERIALS AND METHODS

Materials—The chromatographic materials used were products of Amersham Pharmacia Biotech, Buckinghamshire (Sephadex, Sephacryl, Superdex, Mono S, and DEAE-Sephacel), Whatman, Maidstone (DEAE-cellulose, DE-32), and Shiseido, Tokyo (Capcell Pak C18 column). Polyvinylidene difluoride and nitrocellulose membranes were from Millipore Inc. (Bedford, MA), and kits for Western blotting were products of Bio-Rad Laboratories (Hercules) and Amersham Pharmacia Biotech. Lysyl endopeptidase from *Achromobacter lyticus* was purchased from Wako Pure Chemicals, Osaka. Glutathione *S*-transferase fusion proteins of subunits of the rat 14-3-3 protein were gifts from Drs. T. Ichimura and T. Isobe (Tokyo Metropolitan University), and Ms. C. Itagaki (Niigata University). Antibodies to mouse adipocyte FABP were a gift from Dr. J. Storch (Rutgers University).

Production of Anti–Recombinant Rat C-FABP Antibodies—Rat C-FABP was expressed in *Escherichia coli* and purified as described elsewhere (*17*). An emulsion of the recombinant C-FABP and Freund's complete adjuvant was injected intradermally into two rabbits. After 4 weeks the rabbits were boosted with recombinant C-FABP, and sera were prepared after an additional 4 weeks.

Western Blotting—Samples were electrophoresed with a Tris-Tricine SDS-PAGE system on 12% acrylamide gels (*18*), and electroblotted onto polyvinylidene difluoride or nitrocellulose transfer membranes (Millipore). Goat anti-

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Abbreviations: FABP, fatty acid–binding protein; C-FABP, cutaneous fatty acid–binding protein.

rabbit antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories and Amersham Pharmacia Biotech) were used as secondary antibodies, and the blots were developed with 4-chloro-1-naphthol or a chemiluminescence substrate (Amersham Pharmacia Biotech) according the manufacturers' instructions. Protein bands were stained with Coomassie Brilliant Blue.

Preparation of C-FABP from Rat Skin—This was performed according to the previously described method (*8*) with minor modifications. Briefly, dorsal skin from five male rats (*ca.* 150 cm^2) was minced and homogenized with a Polytron homogenizer in 0.1 M Tris-HCl, pH 8.0, 0.25 M sucrose. The homogenate was centrifuged for 2 h at 100,000 $\times g$. The supernatant was dialyzed against 30 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, and applied to a DEAE-cellulose column (2.5 × 20 cm, DE-32; Whatman) equilibrated with the dialysis buffer. The flow-through fraction was pooled and subjected to cation exchange chromatography on a Mono S column (0.5 × 5 cm; Pharmacia), with a linear NaCl gradient, from 0 to 0.5 M, in 50 mM sodium 2-morpholinoethanesulfonate buffer, pH 5.5. A Superdex 75 column (1.0 × 30 cm, Pharmacia) was used for the final purification of C-FABP (*8*).

Preparation of a 30-kDa Protein—A 30-kDa protein immunoreactive with recombinant rat C-FABP antibodies was purified from the fraction absorbed to DEAE-cellulose. The DE-32 column (2.5 × 20 cm) was washed with 500 ml of 0.5 M NaCl in 30 mM Tris-HCl, pH 8.0, and 1 mM EDTA. One hundred milliliters of the eluant was condensed to 5 ml by ultrafiltration and applied to a Sephadex G-100 column (2.5 × 90 cm) equilibrated with 50 mM Tris-HCl, pH 8.5. Fractions (3 ml) were collected at the flow rate of 20 ml/h, and individual fractions were examined for a 30-kDa protein by SDS-PAGE and Western blotting. Positive fractions were dialyzed against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and applied to a DEAE-Sephacel column (1.0 × 10 cm) equilibrated with the same buffer. The column was developed with a linear 80-min gradient of NaCl, from 0 to 0.5 M, at the flow rate of 1 ml/min. Fractions (1 ml) containing a 30-kDa protein detected on Western blotting were pooled and subjected to preparative SDS-PAGE. The band corresponding to the 30-kDa protein was excised and extracted by electroelution in 50 mM NH_4HCO_3 containing 0.1% SDS. The protein was freed from SDS and dye by repeated acetone precipitation at −20°C (*19*).

Enzymatic Digestion and Separation of Peptides of the 30-kDa Protein—The 30-kDa protein extracted from gel pieces was dissolved in 50 ml of 6 M guanidine-HCl, 30 mM Tris-HCl, pH 8.0, and heated for 30 min at 50°C. This was diluted with 100 ml of water and incubated overnight with 2 mg of *Achromobacter* lysyl endopeptidase at 25°C. The digest was separated on an octadecylsilane column (4.6 × 150 mm, Capcell Pak C18; Shiseido) equilibrated with 1% acetonitrile in 0.05% trifluoroacetic acid. A linear 120-min gradient of 1–75% acetonitrile in 0.05% trifluoroacetic acid was used for elution. The flow rate was 0.5 ml/min. Peptides were detected by measuring the absorbance at 215 nm.

Amino Acid Sequence Analysis—The amino acid sequences of the peptides (50–200 pmol) were determined using protein sequencers, models 470A and 473A (Applied Biosystems).

Tissue Distribution of the 30-kDa Protein—Organs and tissues were excised from a female Sprague-Dawley rat (250 g), and homogenized in a double volume (v/w) of 0.05 M Tris-HCl, pH 8.0, 1 mM EDTA, and 0.25 M sucrose. The supernatants obtained on centrifugation at 100,000 $\times g$ (60 min) were analyzed by Western blotting.

Expression of C-FABP, Adipocyte FABP, and the 30-kDa Protein in Differentiating 3T3-L1 Cells—Mouse 3T3 L1 cells (provided by the Japanese Collection of Research Resources, Tokyo) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 50 units/ml penicillin essentially as described (*20*). Confluent cells (day 0) were converted to adipocytes by feeding them with the medium containing 0.5 mM 1-methyl-3-isobutylxanthine, 0.25 μM dexamethasone, and 10 μg/ml insulin (*20*). Cells were harvested every day, and proteins were extracted with a detergent solution (1% Nonidet P-40, 0.1% sodium deoxycholate, and 0.1% SDS in 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 5 mM EDTA), and analyzed by Western blotting using peroxidase-conjugated secondary antibodies and a chemiluminescence substrate.

RESULTS AND DISCUSSION

Detection of the 30-kDa Protein Recognized by Anti-Rat C-FABP Antiserum—When rat skin cytosol was separated by SDS-PAGE and then examined by Western blot using the antiserum to recombinant C-FABP, a band corresponding to 30-kDa was detected in addition to the expected 15-kDa one (Fig. 1, A and B). This high-molecular-mass protein was purified and analyzed as to the primary structure. The cytosolic fraction of rat skin was first applied to a DEAE-cellulose (DE-32) column, to which C-FABP did not bind but was eluted in the flow-through position (Fig. 1B). The 30-kDa protein band was detected for the DEAE-cellulose-bound fraction, which was eluted with 0.5 M NaCl (Fig. 1B). Since C-FABP contains five cysteine residues, this material could be a dimeric species of C-FABP formed through disulfide bonds. This is, however, unlikely because the 30-kDa protein was much more acidic than C-FABP, as judged from its chromatographic behavior, and the electrophoresis was conducted under reducing conditions. Immunological staining was performed with secondary anti-rabbit antibodies conjugated with peroxidase and, therefore, intrinsic peroxidases could be detected. However, without the secondary goat antibodies, no band was stained with the peroxidase reaction (data not shown). When rabbit antiserum raised against rat liver FABP was used, the reaction did not occur (data not shown). Contamination by some other rat proteins in the *E. coli*–derived antigen used for immunization is impossible. Western blotting of a cell lysate of the host bacterium, *E. coli* BL21 (DE3), also revealed no immunoreactivity to the anti–recombinant rat C-FABP antiserum (Fig. 1, C and D). Consequently, it was concluded that an acidic 30-kDa protein immunoreactive with recombinant rat C-FABP is present in the rat skin cytosol, which is different from C-FABP. A second, lightly stained band corresponding to a somewhat lower molecular mass was also detected for the skin cytosol and DEAE-bound fraction on Western blotting (Fig. 1B).

Purification of the 30-kDa Protein—Figure 2 shows Sephadex G-100 gel filtration of the 0.5 M NaCl-eluant from the DE-32 column. The fractions reactive to C-FABP antiserum comprised fractions 27 through 47, in which bovine

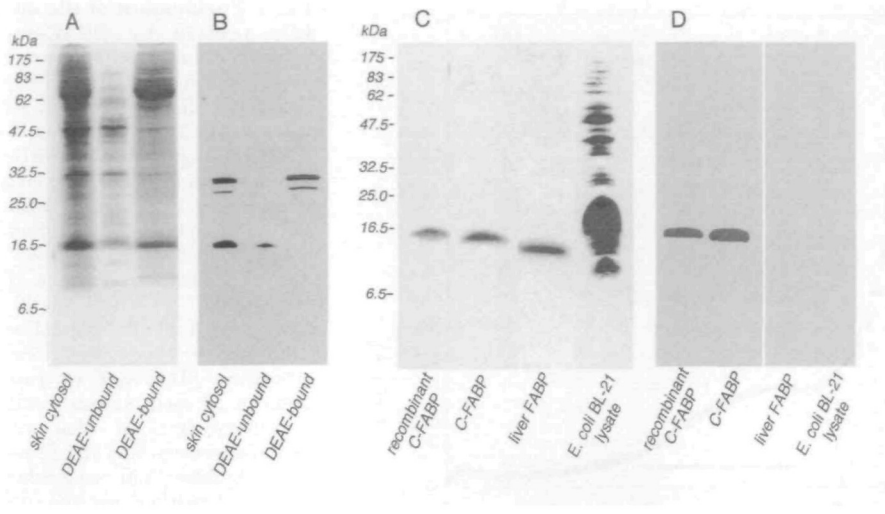

Fig. 1. **Western blot analysis of rat skin cytosol fractions.** Rat skin cytosol was applied to a DEAE-cellulose column (DE-32; Whatman) equilibrated with 30 mM Tris-HCl buffer, pH 8.0. The flow-through (unbound) fraction and the bound fraction eluted with 0.5 M NaCl were examined by SDS-PAGE and Western blotting (A, B). Recombinant and native rat C-FABP, rat liver FABP, and a lysate of the host cells, *E. coli* BL-21 (DE3), were also examined (C, D). A and C, Coomassie Brilliant Blue staining. B and D, Western blotting with rabbit antiserum to the recombinant rat C-FABP. The molecular masses of size marker proteins are shown.

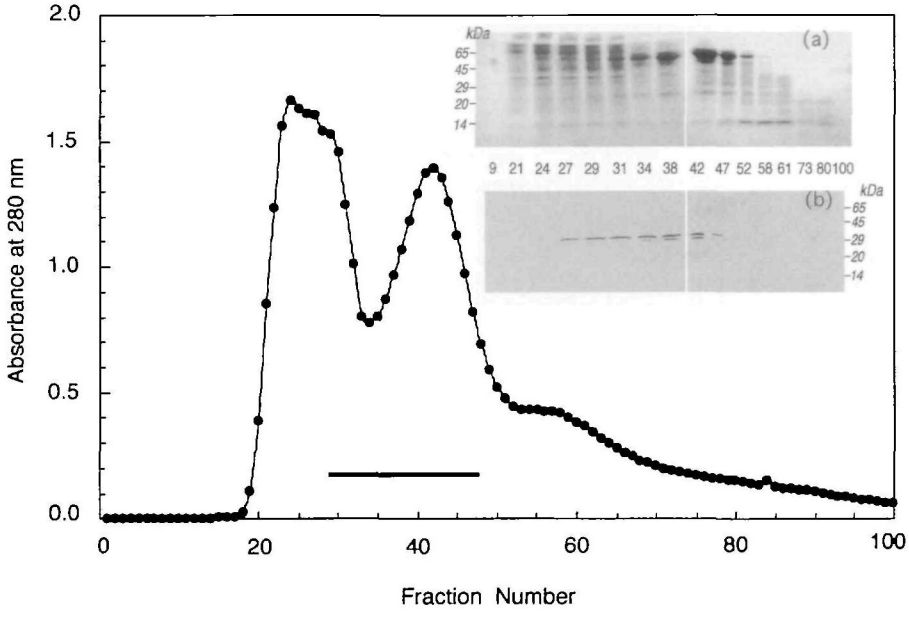

Fig. 2. **Gel filtration of the rat skin cytosol fraction bound to DEAE-cellulose.** The fraction bound to DEAE-cellulose was eluted with 0.5 M NaCl, condensed and then applied to a Sephadex G-100 column (2.5 × 90 cm) equilibrated with 50 mM Tris-HCl, pH 8.5. Fractions (3 ml) were collected at the flow rate of 20 ml/h. Insets show SDS-PAGE of 8 μl-aliquots of selected fractions stained with Coomassie Brilliant Blue (a), and Western blotting with anti–recombinant rat C-FABP antiserum (b). Fraction numbers and mass values of marker proteins are indicated. Fractions 29 through 47 were pooled and subjected to DEAE-Sephacel chromatography.

serum albumin (M_r = 65,000 kDa) was eluted during calibration of the column. Therefore, the 30-kDa protein appears to be in a dimeric form under nondenaturing conditions. These fractions were further purified on a DEAE-Sephacel column with NaCl gradient elution (Fig. 3). Fractions were tested for immunoreaction by Western blotting after SDS-PAGE, and the positive fractions (fractions 43 through 60) were pooled and concentrated to a small volume with Centricon (Amicon). The 30-kDa protein was further purified by preparative SDS-PAGE.

Sequence Analysis of the 30-kDa Protein—The 30-kDa protein extracted from the SDS-PAGE gels was digested with lysyl endopeptidase. Peptides were separated on an octadecylsilane column (4.6 × 150 mm, Capcell Pak C18), as shown in Fig. 4. Selected peaks were analyzed as to their N-terminal sequences with a protein sequencer and partial sequences were used to search for homologous proteins in protein databases. These are summarized in Table I. The sequences obtained so far are homologous to parts of 14-3-3 protein subunits β, γ, σ, and ζ, suggesting that the 30-kDa protein isolated from rat skin cytosol is a mixture of 14-3-3 protein subunits. It has been suggested that 14-3-3 proteins exist as complex dimers comprising different subunits (*21–24*). The elution position of the 30-kDa protein on Sephadex G-100 gel filtration confirmed its dimeric nature (Fig. 2).

Cross-Reaction between Anti–Rat C-FABP Antibodies and 14-3-3 Protein Subunits—Next we examined the cross-reactivity between anti–rat C-FABP antibodies and recombinant rat 14-3-3 protein subunits available. These were glutathione *S*-transferase fusion proteins of the ε-, γ-, σ-, and ζ-subunits with molecular masses around 50 kDa. From Fig. 5 it can be seen that among the recombinant 14-3-3 protein subunits examined, only the ε-subunit was reactive. It was concluded that the immunoreactive 30-kDa protein present in rat skin was the ε-subunit of a 14-3-3 protein. Although partial sequences of the selected peptides did not match parts of this subunit, there will be sequences of the ε-subunit that do when more peptides are sequenced, for epidermal keratinocytes and other tissues are known to

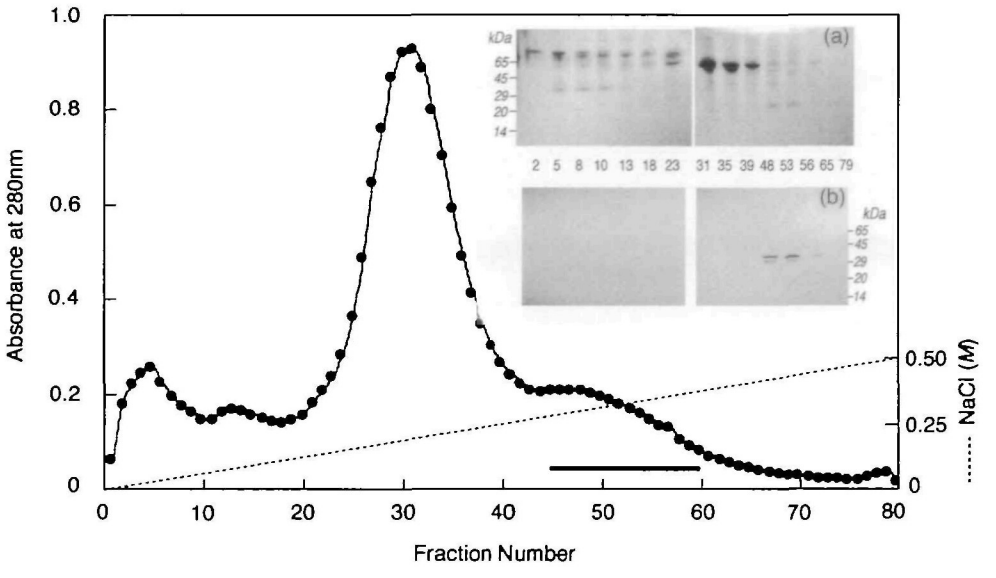

Fig. 3. **Purification of the 30-kDa protein by DEAE-Sephacel column chromatography.** Sephadex G-100 fractions were dialyzed against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and applied to a DEAE-Sephacel column (1.0 × 10 cm; Pharmacia) equilibrated with the same buffer. The column was developed with a linear 80-min concentration gradient of NaCl, from 0 to 0.5 M, at the flow rate of 1 ml/min. One-milliliter fractions were collected. Insets show SDS-PAGE of fractions (8 μl each) stained with Coomassie Brilliant Blue (a), and Western blotting (b). Fraction numbers and molecular masses of marker proteins are indicated. Fractions 43 through 60 were pooled and further purified by preparative SDS-PAGE.

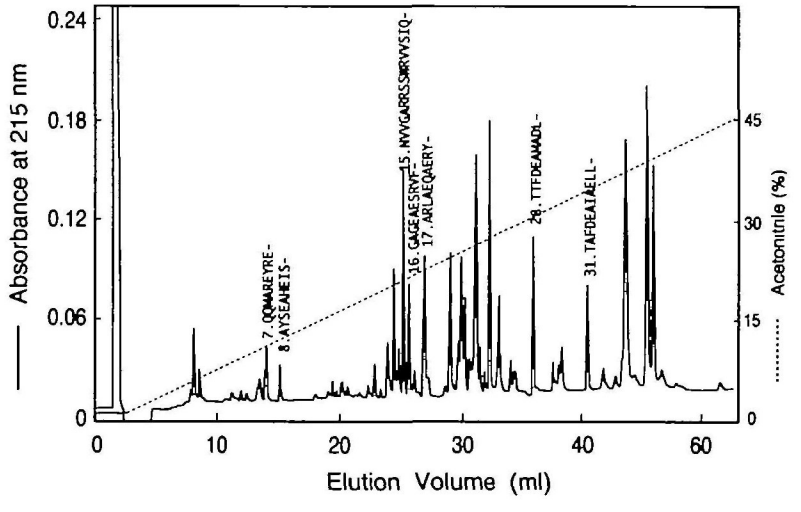

Fig. 4. **Separation of lysyl endopeptidase peptides of the rat 30-kDa protein by reversed-phase HPLC.** The digest was separated on an octadecylsilane column (4.6 × 150 mm, Capcell Pak C18; Shiseido) equilibrated with 0.05% trifluoroacetic acid and eluted with a linear concentration gradient of acetonitrile, as shown by the dotted lines. The flow rate was 0.5 ml/min. The amino acid sequences of selected peptides are also shown.

express many 14-3-3 proteins including this subunit (*25, 26*). There is also possible that other subunit(s) not examined here may also be recognized by the same antibodies, as a faint band corresponding to a slightly lower molecular mass was seen on Western blotting (Figs. 1B, 2, and 3), and the ε-subunit is the largest of 14-3-3 protein subunits (*21*).

Tissue Distribution of the 30-kDa Protein—Figure 6 shows the results of SDS-PAGE and Western blotting of cytosolic fractions of rat skin, liver, kidney, rectum, heart, skeletal muscle, and brain. The 30-kDa protein was detected in all these tissues except for skeletal muscle. For the skin cytosol, a 15-kDa band corresponding to C-FABP was observed. It is of interest that the antibodies also recognized high-molecular-mass (50–60 kDa) proteins in the brain and rectum cytosol. These proteins are now under investigation.

Expression of C-FABP, Adipocyte FABP, and the 30-kDa Protein in Differentiating 3T3-L1 Cells—It has been well established that adipocytes express two FABPs, adipocyte FABP and C-FABP. Adipocyte FABP (adipocyte P2 protein) was first recognized as a 15-kDa protein that was induced during adipocyte differentiation from fibroblasts (*27, 28*). In order to evaluate the potential physiological role of the 30-kDa protein in this process, the modes of expression of these proteins were analyzed during the differentiation of adipocytes from mouse 3T3-L1 cells. As shown in Fig. 6, the

TABLE I. **Amino acid sequences of the lysyl endopeptidase peptides of the 30-kDa protein in Fig. 4.** Amino acid sequences were compared with those of the 14-3-3 protein subunits of rat, except for the σ-subunit (mouse), of which D 113 is replaced by E in the rat protein (the 4th residue of peptide 16). The corresponding positions in the 14-3-3 protein subunit are indicated.

Peak No.	Sequence	Position in 14-3-3 subunits
7	QQMAREYRE	ζ: 76 to 84
8	AYSEAHEIS	γ: 152 to 160
15	NVVGARRSSWRVVSSIEQ	ζ: 50 to 67
16	GAGEAESRVF	σ: 110 to 119
17	ARLAEQAERY	γ: 10 to 19
28	TTFDEAMADL	σ: 196 to 205
31	TAFDEAIAEL	β: 195 to 204

30-kDa protein is constitutively expressed from day 0 to day 4, while the two FABPs are induced exclusively after conversion to adipocytes (day 4).

Significance of the Immunological Cross-Reactivity of C-FABP and 14-3-3 Protein—At present no functional link between C-FABP and the signaling molecule, 14-3-3 protein, is seemingly known. 14-3-3 proteins comprise a well-conserved protein family that modulates a wide variety of eukaryotic cellular signaling processes (*22–24*, *29*, *30*). Alignment of the sequences of C-FABP and the 14-3-3 protein ε-subunit by means of Clustal W software (*31*) or eye-inspection failed to reveal any convincing partial similarity between them. The three-dimensional structures of these proteins are quite different; that is, 14-3-3 proteins are almost exclusively composed of α-helices (*22*), while C-FABP is a variation of a β-barrel protein with two short α-helices (*32*). Despite this apparent lack of relationship between the primary and tertiary structures, they must share a common local structure recognized by anti–C-FABP antibodies. Individual 14-3-3 protein subunits bind a variety of cellular proteins in a phosphorylation-dependent manner and, therefore, the dimers of 14-3-3 protein subunits are regarded as "clamps" or "coordinators" that facilitate the association between two target molecules in a signaling process (*22–24*, *27*). Our results indicate the coexistence of the two proteins (at least) in the epidermis and adipocyte cytosol (Figs. 1 and 6). Consequently, it may be possible that C-FABP, having a similar structural element, interferes with the interaction to modulate the function of 14-3-3 proteins, if the common epitope region is involved in the interaction. Although there is no direct evidence of a functional linkage between C-FABP and the 14-3-3 protein family, one recent observation that expression of C-FABP and 14-3-3σ protein is markedly increased in multidrug-resistant cell lines of human pancreatic adenocarcinoma (*33*) might allow some speculation about it. Complex formation between C-FABP and an S100 protein species (S100A7) was shown with biochemical methods, and their co-localization in the cytoplasm of differentiating keratinocytes from lesional psoriatic skin was established on confocal microscopy (*34*, *35*). In this instance, 14-3-3 protein might modulate the interaction between C-FABP and the S100 protein. Direct interaction between other FABPs and cellular proteins, such as hormone-sensitive lipase (*36*), glycoprotein CD36 (*37*), and a 33-kDa nuclear protein (*38*), is also known.

Interestingly, Schurer *et al.* (*39*) recognized the presence of a 32-kDa protein strongly stained by anti–rat heart FABP antibodies in the mouse epidermal cytosol. Although no further characterization of the 32-kDa protein has been reported, its molecular mass suggests that it might be a 14-3-3-protein subunit. Other reports have described the immunological cross-reaction between a lipid-binding protein and a cellular protein. In human kidney glomeruli, a 110-kDa protein is stained by antibodies to human heart FABP (*40*). Anti–sterol carrier protein-2 antibodies react with rat liver 26-kDa glutathione *S*-transferase and 30-kDa carbonic anhydrase (*41*). Although the physiological significance cannot be readily assessed, these cross-reactions indicate the regional similarity in structure among them, which might affect their interaction with other cellular proteins in certain instances.

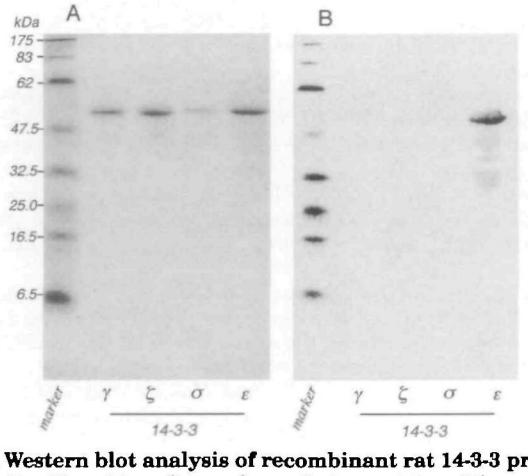

Fig. 5. **Western blot analysis of recombinant rat 14-3-3 protein subunits.** Glutathione *S*-transferase fusion proteins of the γ-, ζ-, σ, and ε-subunits of 14-3-3 proteins (*ca.* 5 μg each) were analyzed by Western blotting with anti–recombinant rat C-FABP antiserum. A, Coomassie Brilliant Blue staining. B, Western blot analysis. Marker, size marker proteins.

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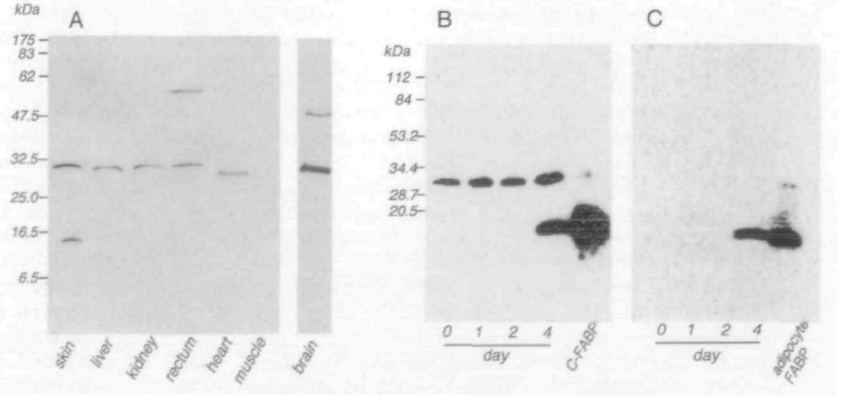

Fig. 6. **Detection of the 30-kDa protein in some rat organs and in differentiating mouse adipocytes.** A: cytosolic fractions of rat organs prepared as described under "MATERIALS AND METHODS" were examined for the presence of the 30-kDa protein by SDS-PAGE followed by Western blot analysis with the anti–recombinant rat C-FABP antiserum. Muscle, skeletal muscle. Brain was analyzed in a separate experiment. High-molecular-mass immunoreactive bands can be seen for rectum and brain. B and C: Western blotting of differentiating mouse adipocytes with the anti–recombinant rat C-FABP and anti–rat adipocyte FABP antisera. Confluent mouse 3T3 L1 cells (day 0) were converted to adipocytes by feeding them with the medium containing 0.5 mM 1-methyl-3-isobutylxanthine, 0.25 μM dexamethasone, and 10 μg/ml insulin (*20*). Cells were harvested every day and proteins were extracted with a detergent solution (1% Nonidet P-40, 0.1% sodium deoxycholate, and 0.1% SDS). The broad spot of C-FABP (C) is due to sample overloading. Anti–recombinant rat C-FABP (B) and anti–adipocyte FABP (C) antisera were used for Western blotting.

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