Identification and Characterization of an Anterior Fat Body Protein in an Insect[1]

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We purified a novel protein with a molecular mass of 34 kDa from the *Sarcophaga* larval fat body. This protein, named AFP (anterior fat body protein), was restricted almost exclusively to the anterior fat body. The AFP content decreased after pupation on disintegration of the fat body tissue. cDNA analysis revealed that this protein consists of 306 amino acid residues and exhibits significant structural similarity with mammalian regucalcin (senescence marker protein-30), a calcium-binding liver protein. However, AFP did not seem to exhibit strong affinity with calcium. These results suggested that a seemingly uniform fat body tissue exhibits a regional difference in its function along the anterior-posterior axis.

Key words: AFP, anterior-posterior axis, fat body, regucalcin, *Sarcophaga peregrina*, senescence marker protein-30.

In the larvae of holometabolous insects such as *Sarcophaga peregrina* (flesh fly), fat body tissue is a conspicuous component of the internal anatomy. The fat body is an organ with multiple metabolic functions, including in the metabolism of carbohydrates, lipids and nitrogenous compounds, the storage of glycogen, fat and protein, the synthesis and regulation of blood sugar, and the synthesis of major hemolymph proteins (1, 2). The principal cell type found in the fat body is trophocytes, and there are two minor cell types, urocytes and mycetocytes. As tropocytes are morphologically uniform, they have been thought to be equivalent in terms of function. However, some findings have suggested a regional difference in fat body function (3).

In dipteran insects, extensive tissue remodeling takes place during metamorphosis and the larval fat body is replaced by the adult one. In *S. peregrina* (flesh fly), we found that the anterior part of the larval fat body is decomposed within 24 h after puparium formation, whereas the posterior part persists for at least for 44 h. Moreover, the fates of these two parts of the fat body during adult development seem to be different. Whereas the cells derived from the anterior part of the fat body undergo histolysis rapidly, those from the posterior part remain at eclosion

and degenerate during the first few days of adult life These facts strongly suggest that the larval fat body is not functionally uniform, but functionally differentiated along the anterior-posterior axis To gain more insight into this regional difference in the fat body, we precisely analyzed larval fat body proteins During the course of this study, we found a major protein with a molecular mass of 34 kDa that is almost exclusively expressed in the anterior part of the fat body.

This paper reports the purification and cDNA cloning of the 34-kDa protein, termed AFP (anterior fat body protein). The deduced amino acid sequence of this protein was found to exhibit significant similarity to that of mammalian regucalcin (senescence marker protein-30), a calcium-binding liver protein (4, 5) This is the first demonstration that an insect fat body contains a homologue of mammalian regucalcin.

MATERIALS AND METHODS

Animals—Flesh flies (*S. peregrina*) were reared according to the method of Ohtaki (6) The physiological age of pupae can be synchronized by dry-wet treatment; when third-instar larvae were transferred from wet conditions to dry conditions at 27°C, they started to pupate 16 h later (white pupae, puparium formation). Adults emerged 10 days after pupation under our rearing conditions.

Preparation of a Fat Body Extract—Third instar larvae or pupae were dissected and their fat bodies were excised under a binocular microscope. A larval fat body consists of bilaterally symmetrical monolayers of large cells in a basement membrane sac, which are spread throughout entire body cavity. In *Sarcophaga* larvae, clearly distinct fat body lobes are found along the anterior-posterior axis. The lobes that surround the salivary glands and brain in the thorax segment are separated from the rest of the posterior fat body. We removed three parts of the fat body separately, *i.e.* the pair of anterior lobes in the thorax segment (T1–T3),

[2] To whom correspondence should be addressed Tel +81-48-4679437, Fax +81-48-4624693, E-mail natori@postman riken go jp Abbreviations HPLC, high performance liquid chromatography, SDS, sodium dodecyl sulfate, FITC, fluorescein isothiocyanate, SSC, standard saline citrate; SSPE, standard saline phosphate containing EDTA.

the middle region (A1–A5), and the posterior region (A6–A8) of the fat body in the abdominal segment. Each fat body sample was homogenized in 10 mM Tris-HCl buffer (pH 7.9) containing 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 100 µg/ml leupeptin, and 0.1 µg/ml pepstatin The homogenate was centrifuged at 750 ×g for 5 min, and the supernatant was collected for use as the fat body extract. To obtain dissociated fat body cells from pupae, we carefully removed the cells with wide-end tips, rinsed them with the buffer carefully, and then homogenized them.

Electrophoresis and Immunoblotting—Electrophoresis on an SDS–polyacrylamide slab gel was carried out by the method of Laemmli (7). Samples were denatured by heating them for 20 min at 75°C in 1% SDS and 2% 2-mercaptoethanol. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. For immunoblotting, proteins separated by electrophoresis were transferred electrophoretically from the gel to a filter (Immobilon-P., Millipore). The filters were immersed in 20 mM Tris-HCl buffer (pH 7.9) containing 5% skim milk for 1 h. After washing with a rinsing solution [10 mM Tris-HCl buffer (pH 7.9) containing 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.01% sodium azide, and 0.25% skim milk], they were dipped in the rinsing solution containing affinity-purified antibodies against AFP and then kept overnight at 4°C. They were then washed well with the rinsing solution, transferred to 5 ml of the rinsing solution containing radioiodinated anti-rabbit IgG (3.7 kBq), and then kept at room temperature for 2 h. Finally, they were washed well with the rinsing solution, dried and then subjected to autoradiography with Kodak XAR film.

Purification of AFP—Purification of AFP was monitored by measuring the relative content of the 34-kDa band material on SDS–polyacrylamide gel electrophoresis. The anterior fat bodies (T1–T3) from 680 larvae were homogenized in 2.5 ml of 10 mM Tris-HCl buffer (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 100 µg/ml leupeptin, and 0.1 µg/ml pepstatin. The homogenate was centrifuged at 10,000 ×g for 20 min. The resulting supernatant was diluted 6 times with the same buffer, and then half of it was applied to a DEAE-cellulose column (1.1 × 6.5 cm) that had been equilibrated with 10 mM Tris-HCl buffer. The flow-through fraction was collected. Several proteins were in this fraction including AFP, but elution of AFP tended to be slow due to its weak interaction with the resin. The retarded fraction was combined and subjected to re-chromatography under the same conditions. AFP was almost pure at this stage and gave a single band on SDS–polyacrylamide gel electrophoresis. About 1 mg of pure AFP was obtained from 680 larvae.

Antibodies against AFP—Antibodies against AFP were raised by injecting 50 µg of purified protein into a male albino rabbit with complete Freund's adjuvant, followed by three booster injections of the same amount of protein at 14-day intervals. Affinity purification of the resulting antibodies was performed with the purified AFP. For this, the purified AFP (60 µg) was first separated by electrophoresis on a 10% polyacrylamide gel containing 0.2% SDS and then the protein was blotted onto a filter (Immobilon-P., Millipore). The small area of the filter in which AFP was concentrated was excised and treated with a skim milk solution (20 mM Tris-HCl buffer, pH 7.9, containing 5% skim milk).

Then the strip of filter paper was incubated in 6 ml of 1.5-fold diluted antiserum in the rinsing solution at 4°C overnight with gentle shaking. The strip was then cut into pieces and the antibodies specifically bound to AFP was extracted with 0.5 ml of 0.2 M glycine-HCl buffer, pH 2.8. The resulting extract was neutralized with 1 M KOH and then bovine serum albumin was added to a final concentration of 1%.

Cloning and Sequencing of AFP cDNA—The purified AFP (34 µg) was digested with lysyl endopeptidase and the resulting peptides were separated by HPLC on a C_{18} column (Gilson). The fraction containing each peptide was lyophilized and the sequences of the three peptides were determined. They were FYXIDTXDYEVK, XNVXISNX-LAXN, and EYDYDFETGK. Then we synthesized a degenerate oligodeoxyribonucleotide corresponding to EYDYDF-ETG, of which the sequence was 5'-GA(A/G)TA(T/C)GAIT-A(T/C)GA(T/C)TTIGA(A/G)ACIGG-3'. This oligodeoxyribonucleotide was labeled with [γ-^{32}P]ATP by the method of Sgaramella and Khorana (8), and used as a probe for screening a *Sarcophaga* fat body cDNA library (9) constructed by the method of Okayama and Berg (10). We screened $1.5 × 10^4$ colonies and found 10 hybridization-positive clones. The plasmid with the longest insert was sequenced by the dideoxy chain termination method of Sanger et al. (11) using an ABI 373A DNA sequencer and a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) The nucleotide sequences of both strands were determined.

Northern and Southern Blot Hybridization—Northern blot hybridization was performed in 50% formamide, 5 ×SSPE, 5 ×Denhardt's solution, 50 mM phosphate buffer, pH 6.5, 0.2 mg/ml single-stranded salmon sperm DNA overnight at 42°C. Then the filters were washed four times for 10 min each at room temperature with 2 ×SSC containing 0.1% SDS. For Southern blot hybridization, prehybridization was performed in 5 ×SSPE, 10 ×Denhardt's solution containing 2% SDS and 0.1 mg/ml single-stranded salmon sperm DNA at 65°C for 6 h. Then hybridization was performed under the same conditions for 16 h. After hy-
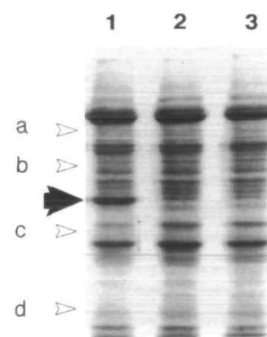

Fig. 1 **SDS–polyacrylamide gel electrophoresis of various regions of the fat body.** Three regions of the fat body were excised from *Sarcophaga* third instar larvae. Their extracts were subjected to SDS–polyacrylamide gel electrophoresis. Each lane contained 5 µg protein Parts of the fat body: Lane 1, anterior part (T1–T3); lane 2, middle part (A1–A5); lane 3, posterior part (A6–A8). The gel was calibrated with the following molecular markers: a, bovine serum albumin (66 kDa); b, ovalbumin (43 kDa), c, α-chymotrypsinogen (26 kDa), d, cytochrome c (12 kDa). The arrow indicates the position of 34 kDa

bridization the filters were washed twice for 10 min each time at room temperature with 2 ×SSC containing 0.05% SDS and once for 30 min at 60°C. The DNA used as a probe was a part of the AFP cDNA (+569 to +952) amplified by PCR.

Immunofluorescence Staining of Fat Bodies with Antibodies against AFP—The anterior or posterior part of the fat body was removed from third instar larvae and then subjected to immunofluorescence staining to locate AFP. For this, each fat body was frozen in Tissue Tek and 10 μm sections of the frozen fat body were prepared on gelatin-coated, fluorescence-free glass slides. Samples were fixed in 50 mM phosphate buffer, pH 6.0, containing 1.5% formaldehyde for 15 min and then washed in buffered insect saline. Then they were incubated with 30 μl of the affinity-purified antibody solution for 1 h at room temperature. As negative controls, other sections were treated with normal IgG. The preparations were then washed well with buffered insect

saline and incubated with 30 μl of FITC-conjugated goat anti–rabbit IgG for 1 h. Finally they were immersed in 90% fluorescence-free glycerol and mounted with cover-slips for examination.

RESULTS

Detection and Purification of a Protein Specifically Expressed in the Anterior Part of the Fat Body—To examine regional differences in fat body tissue, we isolated three parts of the larval fat body of *Sarcophaga* along the anterior-posterior axis and prepared extracts of them. The electrophoretic profiles of these extracts are shown in Fig 1. Overall the protein patterns were very similar, except that the intensity of the band of a 34-kDa protein in the anterior fat body extract was very high, indicating that the content
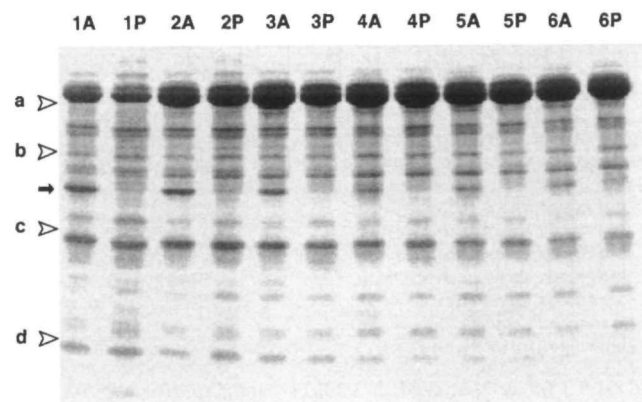

Fig 2. **SDS–polyacrylamide gel electrophoresis of the anterior and posterior parts of the fat body during the initial stage of metamorphosis.** Two parts of the fat body from third instar larvae or pupae harvested at various times after puparium formation were homogenized and subjected to SDS–polyacrylamide gel electrophoresis. Each lane contained 5 μg protein A indicates anterior fat body (T1–T3) and P posterior fat body (A6–A8) Lane 1, third instar larvae, lane 2, white pupae (0 h after puparium formation), lanes 3 to 6 pupae harvested at 7, 24, 31, and 50 h after puparium formation The arrow indicates AFP The gel was calibrated as described in the legend to Fig 1
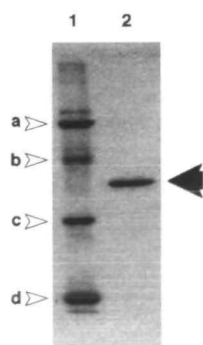

Fig. 3 **Electrophoretic profile of the purified AFP.** The purified AFP (2 μg, lane 2) was subjected to SDS–polyacrylamide gel electrophoresis with the molecular mass markers (lane 1) described in the legend to Fig. 1

Fig 4 **Nucleotide sequence of the cDNA encoding AFP and its predicted amino acid sequence.** The deduced amino acid sequence of AFP is shown below the nucleotide sequence. The numbers of the nucleotides are shown to the left of each line Chemically determined partial amino acid sequences are boxed. The probe used for cDNA cloning is represented by shaded areas The asterisk and double underlining denote the termination codon and poly (A) addition signal, respectively
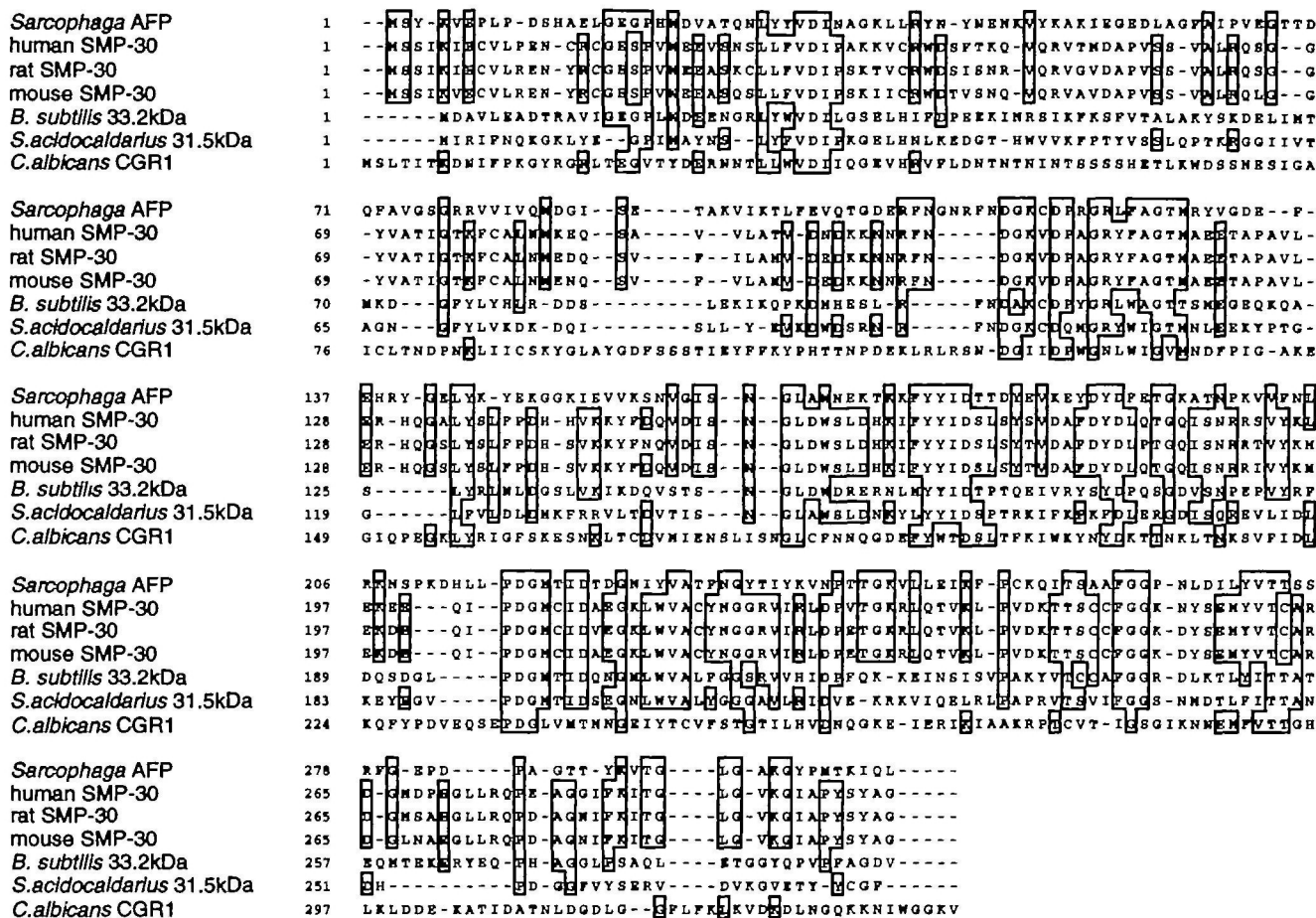
Sarcophaga AFP	1	
human SMP-30	1	
rat SMP-30	1	
mouse SMP-30	1	
B. subtilis 33.2kDa	1	
S.acidocaldarius 31.5kDa	1	
C.albicans CGR1	1	

Sarcophaga AFP	71
human SMP-30	69
rat SMP-30	69
mouse SMP-30	69
B. subtilis 33.2kDa	70
S.acidocaldarius 31.5kDa	65
C.albicans CGR1	76

Sarcophaga AFP	137
human SMP-30	128
rat SMP-30	128
mouse SMP-30	128
B. subtilis 33.2kDa	125
S.acidocaldarius 31.5kDa	119
C.albicans CGR1	149

Sarcophaga AFP	206
human SMP-30	197
rat SMP-30	197
mouse SMP-30	197
B. subtilis 33.2kDa	189
S.acidocaldarius 31.5kDa	183
C.albicans CGR1	224

Sarcophaga AFP	278
human SMP-30	265
rat SMP-30	265
mouse SMP-30	265
B. subtilis 33.2kDa	257
S.acidocaldarius 31.5kDa	251
C.albicans CGR1	297

Fig 5. **Comparison of the amino acid sequences of AFP and other SMP-30 family proteins.** The deduced amino acid sequences of human SMP-30 (EMBL Q15493), rat SMP-30 (EMBL Q03336), mouse SMP-30 (EMBL Q64374), *Bacillus subtilis* hypothetical 33.2 kDa protein (EMBL O34940), *Sulfolobus acidocaldarius* hypothetical 31 5 kDa protein (EMBL P46218), and *Candida albicans* cell growth protein CGR1 (EMBL P56553) were aligned Gaps were introduced to obtain maximum matching The numbering is based on the sequence of each protein The residues conserved among them (but not in all of them) are boxed
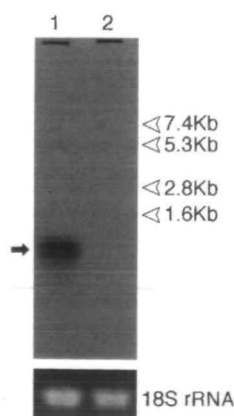
Fig. 6 **Northern blot analysis of AFP mRNA.** Total RNA extracted from the anterior or posterior part of the fat body was subjected to Northern blot hybridization The probe used was a PCR product of positions +569 to +952 of AFP cDNA. The recovery and integrity of each RNA were assessed on the basis of the intensity of the 18S ribosomal RNA band Lane 1, RNA from the anterior part of the fat body (25 μg); lane 2, RNA from the posterior part of the fat body (25 μg) The arrow indicates the position of AFP mRNA.

Fig 7. **Immunofluorescence study of AFP in the fat body.** Thin sections (10 μm) of the fat body from third instar larvae were treated with an affinity-purified antibody followed by fluorescein isothiocyanate–conjugated secondary IgG, and then the nuclei were localized by 4′,6-diamidino-2-phenylindole staining. a, immunofluorescence of APF of the anterior part of the fat body; b, localization of the nuclei (the same field as in a), c, magnification of the immunofluorescence of APF of the anterior part of the fat body; d, localization of the nuclei (the same field as in c), e, immunofluorescence of APF of the posterior part of the fat body; f, localization of the nuclei (the same field as in e), g, background fluorescence of the anterior part of the fat body obtained with the same amount of normal antibodies, h, localization of the nuclei (the same field as in g)

of this protein, named AFP (anterior fat body protein), in the anterior fat body is much greater than those in other regions The anterior part of the fat body disintegrates within 24 h after puparium formation to yield fat body cells, but the other parts remain intact for more than 40 h. Therefore, we compared the protein patterns of the anterior (T1–T3) and posterior (A6–A8) parts of the fat body with time after puparium formation. As shown in Fig. 2, the AFP content in the anterior part of the fat body clearly decreased with disintegration of the fat body tissue, but other
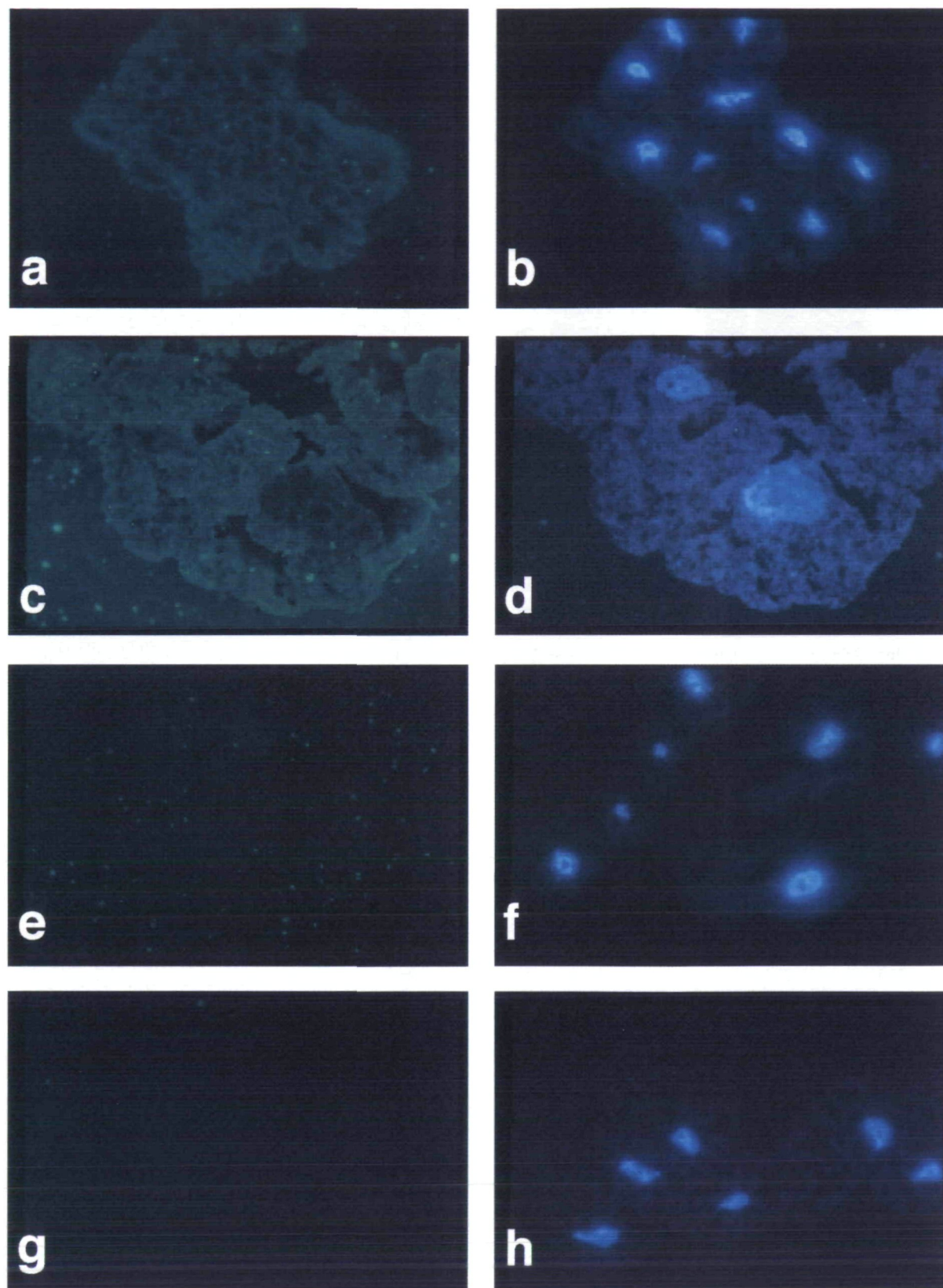
Fig. 7

proteins did not change appreciably regardless of the tissue disintegration. The thick band at the top of each gel represents a storage protein named arylphorin. This protein is known to be taken up by the fat body from the hemolymph
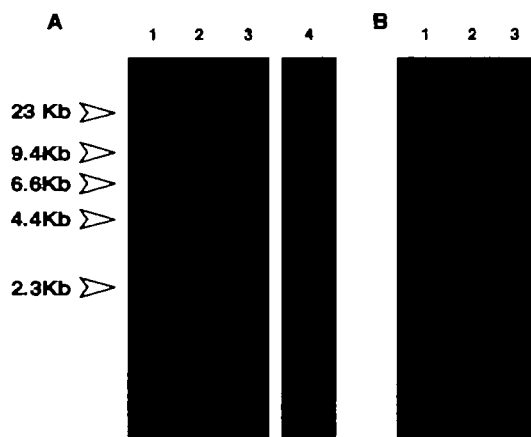
Fig. 8 **Southern blot hybridization of the AFP gene.** Digests of *Sarcophaga* genomic DNA were subjected to Southern blot analysis. The probes used were a PCR product of the +569 to +952 fragment of AFP cDNA for A and a PCR product of the +314 to +760 fragment, containing an *Eco*RI site, for B. The restriction enzymes used were Lane 1, *Eco*RI, lane 2, *Xho*I, lane 3, *Sal*I, lane 4, *Hin*dIII. The positions of the λ/*Hin*dIII fragments used as molecular mass markers are indicated by arrowheads to the left.

and accumulates in fat body cells with time after puparium formation (*12*). These facts suggest an intimate relationship between the AFP content and the function of the anterior part of fat body. Therefore, we purified AFP from the anterior part of the fat body to homogeneity (Fig. 3). About 1 mg of pure protein was obtained from 680 larvae and its content was estimated to be about 0.8% of total fat body protein.

cDNA Cloning of AFP—To determine the amino acid sequence of AFP, we isolated its cDNA. For this, we first determined the amino acid sequences of the three peptides derived from AFP. The amino-terminal residue of AFP could not be determined probably due to blocking. With this sequence information, we synthesized a DNA probe to screen the cDNA library of *Sarcophaga* larval fat body. We selected 10 hybridization positive clones out of about 15,000 colonies. The nucleotide sequence of the longest insert and the deduced amino acid sequence encodes by its ORF are shown in Fig. 4.

This protein consists of 306 amino acid residues and contains the sequences of the three peptides derived from the purified AFP, indicating that this cDNA is that of AFP. As shown in Fig. 5, AFP was found to exhibit significant sequence similarity to mammalian senescence marker protein-30 (SMP-30) (*5*), which is the same protein as a novel Ca^{2+}-binding protein, regucalcin (RC) (*4, 13, 14*), and unidentified proteins in bacteria or yeast (*15*). Nothing is known about the functions of bacterial proteins, but mammalian SMP-30 is a hepatic protein having Ca^{2+}-binding activity and is known to decrease with aging. The maximal sequence identity between AFP and rat SMP-30 was about 33%. Thus, it is likely that AFP is an insect homologue of mammalian SMP-30. In mammalian SMP-30, eight Cys residues are conserved, but none of these Cys residues is present in AFP. These structural features suggest that AFP is a SMP-30–like protein, but that its function is different from that of mammalian SMP-30.

We examined whether or not AFP has calcium-binding activity. After dot-blotting of AFP onto a nitrocellulose membrane, the membrane was washed with DDW and then soaked in a solution of 10 mM imidazole-HCl buffer (pH 6.8) containing 60 mM KCl and 5 mM $MgCl_2$. Then the membrane was incubated in the same buffer containing ^{45}Ca (1 μCi/ml) for 10 min, washed well, and then subjected to autoradiography (*16*). Although 5 μg of bovine brain calmodulin (SIGMA) clearly bound calcium under these conditions, no significant calcium binding was detected with 20 μg of AFP, suggesting that AFP is not a calcium-binding protein (data not shown).

Localization of AFP in the Larval Fat Body and Analysis of the AFP Gene—Northern blotting revealed that mRNA for AFP was exclusively expressed in the anterior part of fat body (Fig. 6). To identify the cells synthesizing AFP, we located AFP by means of the indirect immunofluorescence method using affinity purified antibodies. As shown in Fig. 7, AFP was detected in the cytoplasm of the large cells in the anterior part of the fat body, but not in the posterior part. These results indicate that the major cells of the fat body, trophocytes, synthesize AFP in the anterior part of the fat body.

To determine the copy number of the AFP gene, we performed Southern blot hybridization with *Sarcophaga* DNA digested with various restriction enzymes. One major band was detected on probing with a DNA fragment of 383 bp corresponding to the C-terminal region (Fig. 8A) or 447 bp corresponding to the central region of AFP (Fig. 8B). As the latter probe contained an *Eco*RI site, two major bands were detected when DNA was digested with *Eco*RI (Fig. 8B, lane 1). These results suggested that the AFP gene is a single copy gene. Thus, it is clear that there is a regional difference in the function of the *Sarcophaga* larval fat body and that the AFP gene is active only in its anterior part.

cDNA Cloning of Drosophila AFP—To investigate the biological roles of AFP, it would be useful to find a *Drosophila* counterpart of *Sarcophaga* AFP, because genetical techniques are available for *Drosophila*. Therefore, we tried to isolate cDNA of *Drosophila* AFP. We performed RT-PCR using a *Drosophila* adult cDNA library with primers specific for *Sarcophaga* AFP and isolated a 345-bp fragment. This fragment encoded a peptide consisting of 115 amino acid residues that exhibited 80% similarity to a partial sequence of *Sarcophaga* AFP. This fragment was used as a probe for screening the *Drosophila* adult cDNA library. We isolated 10 hybridization positive clones on the screening of about 400,000 plaques. These clones were found to be derived from the same cDNA, and we finally determined the complete nucleotide sequence and the amino acid sequence of the protein encoded in this cDNA (Fig. 9A). This protein consists of 303 amino acid residues and 75% of its sequences are identical with those of *Sarcophaga* AFP (Fig. 9B), suggesting that this protein is *Drosophila* AFP.

DISCUSSION

The distinct lobes of the fat body along the anterior-posterior axis of larval dipteran insects have long been known, but in most cases protein expression has been studied using the entire fat body, not its different parts. Few obvious regional differences in the fat body have been reported so far. In *Drosophila* larvae, the enzymes needed to synthesize ommochromes are produced predominantly by the

A

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  1  AATTCGGCACGAGCGGCACGAGGATCTCCGTGACCAACATCTACAACTACTACAAGAAGTTCGGCTACAAGACCC
 76  TGGTCATGGGCGCCTCCTTCCGGAAACGTGGGCGAAATCAAGGCCCTGGCTGGCTGCGATCTGCTCACCATCAGCC
151  CAGCTCTGCTCAAGCTTAAGCTATCTCCAACATGTCGTACAAGGTGGAACCATTGCCCGATTCCTACGCCGGCCT
                                             M  S  Y  K  V  E  P  L  P  D  S  Y  A  G  L

226  GGGCGAGGGTCCCCATTGGGATGTGGCCAGGCAAAGCCTGTACTACGTGGATTTGGAGGCGGGCAGCCTGCTCCG
      G  E  G  P  H  W  D  V  A  R  Q  S  L  Y  Y  V  D  L  E  A  G  S  L  L  R

301  CTACGACTATGCCCAGAACAAGGTCTACAAGACGAAGATCGAGGGCGAAACCTTGGCCGGATTCGTGCTGCCGGT
      Y  D  Y  A  Q  N  K  V  Y  K  T  K  I  E  G  E  T  L  A  G  F  V  L  P  V

376  GGAGGGACGTCCGCAGGAGTTCGCCGTCGGCTGCGGTCGACGCGTGGTGATCGTCAACTGGGATGGCGTCTCGCC
      E  G  R  P  Q  E  F  A  V  G  C  G  R  R  V  V  I  V  N  W  D  G  V  S  P

451  CAGCGCCAAGGTGGTGCGCACACTGTTCGAGGTGCAGCCACTGATGGAGAAGAATCGTTTGAACGACGCCAAGGT
      S  A  K  V  V  R  T  L  F  E  V  Q  P  L  M  E  K  N  R  L  N  D  A  K  V

526  TGATCCCCGTGGTCGCTTCTTTGGCGGCACCATGCGCTACATTGGCGATGAGTTCGAGTTCCGTCACGGCGAGCT
      D  P  R  G  R  F  F  G  G  T  M  R  Y  I  G  D  E  F  E  F  R  H  G  E  L

601  GTACCGCTGGGAGGCCGGTGGCCAGGTGTCGGTGATCAAGGGCGATGTGGGCATCTCCAATGGACTGGCATGGGA
      Y  R  W  E  A  G  G  Q  V  S  V  I  K  G  D  V  G  I  S  N  G  L  A  W  D

676  CGAGAAGGCCAAGAAGTTCTACTACATCGATACCACCGACTACGAGGTGAAGTCGTATGACTATGATTTCGAGAC
      E  K  A  K  K  F  Y  Y  I  D  T  T  D  Y  E  V  K  S  Y  D  Y  D  F  E  T

751  CGGCGTGGCTAGCAATCCCAAGGTTATATTCAATCTGCGCAAGAACAGTCCCAAGGATCATCTGCTGCCCGATGG
      G  V  A  S  N  P  K  V  I  F  N  L  R  K  N  S  P  K  D  H  L  L  P  D  G

826  CCTGACCATCGATACCGAGGGCAACCTGTATGTGGCCACCTTCAATGGCGCCACCATCTACAAGGTTAATCCCAA
      L  T  I  D  T  E  G  N  L  Y  V  A  T  F  N  G  A  T  I  Y  K  V  N  P  N

901  CACTGGCAAGATTCTGCTTGAGATCAAGTTCCCAACCAAACAGATTACCTCCGCCGCCTTCGGTGGCCCCAACTT
      T  G  K  I  L  L  E  I  K  F  P  T  K  Q  I  T  S  A  A  F  G  G  P  N  L

976  GGACATCCTGTACGTGACCACTGCCGCCAAGTTCGATCAGCCCGCTCCAGCTGGCACCACCTACAAGGTGACCGG
      D  I  L  Y  V  T  T  A  A  K  F  D  Q  P  A  P  A  G  T  T  Y  K  V  T  G

1051 ACTGAACGCCACCGGCTATCCCGGCGTCAACCTGAAGGTCTAGGAGCTGCAATCTCTATCTATCCTTAGCCCGCA
      L  N  A  T  G  Y  P  G  V  N  L  K  V  *

1126 GTAAAATGTACCAATCGAATCCAAGTGCAATTATATGTTATGCTCTTTAACGTTTTAAAAAAAAAAAAAAAAAAC
1201 TCGTGCCG  1208
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B

Fig. 9. **Analysis of *Drosophila* AFP cDNA.** A, nucleotide sequence of the cloned cDNA. The numbers of the nucleotides are shown to the left of each line. The deduced amino acid sequence is shown below the nucleotide sequence. B, comparison of the amino acid sequences of *Sarcophaga* and *Drosophila* AFPs. Gaps were introduced to obtain maximal sequence matching Identical residues are boxed


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Sarcophaga    1   MSYKVEPLPDSHAELGEGPHWDVATDNLYYVDINAGKLLRYNTNENKVYKAKIEGEDLAGPAISVEGTTDQFAVG
Drosophila    1   MSYKVEPLPDSYAGLGEGPHWDVARQSLYYVDLEAGSLLRYDMAQNKVYKTKIEGETLAGFVLPVEGRPQEFAVG

Sarcophaga   76   SGRRVVIVQWDGISETAKVIKTLFEVQTGDERPNGNRFNDGKCDPRGRLFAGTMRYVGDEFEHRYGELYKYSKGG
Drosophila   76   CGRRVVIVNWDGVSPSAKVVRTLFEVQPLMEKN---RLNDAKVDPRGRFFGGTMRYIGDEFEFRHGELYRWEAGG

Sarcophaga  151   KIEMVRSNVGISNGLAWNEKTKKFYYIDTTDYEVKEYDYDFETGKNTNPKVVFNLRKNSPKDHLLPDGMFIDTDE
Drosophila  148   QVSMIKGDVGISNGLAWDEKAKKPYYIDTTDYEVKSYDYDFETGVNSNPKVIFNLRKNSPKDHLLPDGLFIDTEG

Sarcophaga  226   NIYVATPNGYTIYKVNFTTGRVLLEIKFPCKQITSAAFGGPNLDILYVTTSSRFGEDPAGTTYKVTGLGNKGYP
Drosophila  223   NLYVATFNGATIYKVNFNTGKILLEIKPHTKQITSAAFGGPNLDILYVTTAAKFDQPAPAGTTYKVTGLNNTGYP

Sarcophaga  301   MTKIQL
Drosophila  298   GVNLKV
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anterior segment of the fat body (*17*). A regional difference in the fat body has also been detected in the ability to take up storage protein from the hemolymph when *Drosophila* larvae pupate (*18*).

This paper reported a clear regional difference in the

Sarcophaga larval fat body in the synthesis of AFP. AFP was found to be a novel SMP-30-like protein of insect. Mammalian SMP-30 (regucalcin) is known to be distributed in the hepatic cytosol and is supposed to play a physiological role in the regulation of liver cell function through

its Ca²⁺ binding activity (13, 14). SMP-30 has been reported to decrease with aging in rat liver (5), and to prevent Ca²⁺-induced cell death in mammalian cell lines when a large amount of SMP-30 is expressed (19). AFP is located in the cytoplasm of trophocytes in the anterior part of the fat body, and its content reaches about 1% of soluble fat body protein. Nothing is known about the function of AFP at the moment, but contrary to mammalian SMP-30, AFP does not seem to have calcium-binding activity. As the anterior part of fat body is in contact with the brain hemispheres and ring gland, its function might be related to endocrine control in *Sarcophaga*. On a search of genomic databases, we found that bacteria and fungi possibly produce SMP-30–like proteins, but their function also remains to be elucidated. The sequence identity among vertebrate SMP-30 (regucalcin) family proteins is more than 70%, but that between AFP and vertebrate proteins is 30% at most. Thus, we suppose that insect SMP-30–like proteins form a novel protein family different from vertebrate SMP-30 family, although they are derived from a common ancestral gene.

It is noteworthy that the anterior part of the fat body rapidly decomposes during metamorphosis, and the content of AFP decreases before the fat body tissue decomposition. This is the only major change in fat body protein detected on SDS–polyacrylamide gel electrophoresis. The decrease in AFP might be related to the rapid decomposition of the anterior fat body. We think that the homologue of AFP of *Drosophila* has a similar function to that of *Sarcophaga*. Analysis of mutant and/or transgenic *Drosophila* will provide more information on the function of this protein.

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