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A 260-kDa Filamin/ABP-Related Protein in Chicken Gizzard Smooth Muscle Cells Is a New Component of the Dense Plaques and Dense Bodies of Smooth Muscle

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A 260-kDa protein, termed cgABP260, which localized in the dense plaques and dense bodies of smooth muscle cells, was found in a low-salt alkaline extract of chicken gizzard smooth muscle. An antibody against cgABP260 was used to screen a chicken gizzard cDNA library, and the nucleotide sequence of the partial cDNA encoding this protein was determined. Comparison of predicted amino acid sequences revealed that the protein had significant homology with human ABP-280 and chicken retina filamin [Barry, C.P. *et al.* (1993) *J. Biol. Chem.* 268, 25577-25586], but despite the high homology, cgABP260 was immunologically distinguishable from filamin. Immunoblot analysis showed that an anti-cgABP260 antibody reacted exclusively with the cgABP260 band of smooth, skeletal, and cardiac muscle tissues. By indirect immunofluorescence, the membrane-affinity-purified antibody against cgABP260 intensely stained the dense plaques of the isolated smooth muscle cells. Immunoelectron microscopy showed that immunogold particles representing cgABP260 were found abundantly on the dense plaques and less abundantly on the dense bodies. Its amino acid sequence, molecular size, immunological reactivity, and localization in smooth muscle thus indicated that cgABP260 is a new component of the dense plaques and dense bodies of smooth muscle cells.

Key words: dense body, dense plaque, filamin/ABP-related protein, immunogold, smooth muscle.

Dense plaques are specific structures of the smooth muscle cell membrane to which bundles of actin filaments attach themselves. In the cytoplasm, smooth muscle cells have dense bodies where actin filaments from opposite sides are bundled together *(2, 3).* They transmit force generated inside a smooth muscle cell by the contractile system, including myosin and actin, to the cell surface. As a result, the smooth muscle cell contracts. Many constituent proteins of the dense plaques and/or dense bodies have been found, although their physiological roles there have not been fully elucidated. The distributions of vinculin *(4-7),* talin (6, 7), paxillin *(8, 9),* and zyxin *(10)* are restricted to the dense plaques in smooth muscle cells, *ft-* Actin *(11)* is a common component of the dense plaques and dense bodies. or-Actinin, a major component of the dense bodies, also localizes in the dense plaques *(4, 12).* Antibodies against zeugmatin *(13),* which was recently found to be part of the Z-band region of titin, which is identical to connectin *(14),* stain the dense bodies intensely and the dense plaques less intensely *(15).* Filamin *(16)* is also found in the dense plaques, although it is mainly distributed in the cytoskeletal channel of smooth muscle cells *(17).* Filamin is closely related to ABP-280, which was first isolated from mammalian leukocytes *(18)* and has been found widely in nonmuscle tissues *(19-21).* Several cytoskeleton-associated proteins, such as a-actinin, filamin, vinculin, talin, and zyxin, have been isolated from the low-salt alkaline extract of avian gizzard smooth muscle. Recently we isolated fulcin and a 36-kDa protein from the low-salt alkaline extract of chicken gizzard. Fulcin, a 450-kDa filamin/ABP-related protein, localizes at the peripheral regions of dense bodies and dense plaques *(22).* The 36-kDa protein is a component of dense bodies and not dense plaques *(23).* In this study, we found a new filamin/ABP-related 260-kDa protein component of the dense plaques and dense bodies in a low-salt alkaline extract of chicken gizzard smooth muscle. Here, we refer to this protein as cgABP260 (chicken gizzard actin-binding protein of 260 kDa), from its apparent molecular mass on SDS-PAGE.

MATERIALS AND METHODS

Extraction of cgABP260—All procedures were performed in an ice box or a cold room. Two hundred grams of chicken gizzard smooth muscle was washed with 0.15 M NaCl and 20 mM sodium phosphate buffer, pH 7.2 (PBS), containing 10 mM EDTA and 0.1 mM phenylmethylsul-

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Abbreviation: MES, 2-(iV-morpholino)ethanesulfonic acid.

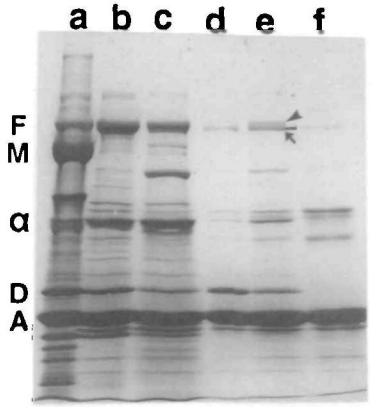

Fig. 1. **SDS-PAGE of each step in the extraction of cgABP260 from chicken gizzard.** (a) Myofibril-rich fraction; (b) soluble fraction in the low-salt alkaline solution (12.5 μg); (c) soluble fraction in 300 mM NaCl (12.5 μg); (d) residue insoluble in 300 mM NaCl; (e) soluble fraction in buffer A (12.5 μg); (f) flow-through fraction from a hydroxylapatite column (crude cgABP260, 12.5 μg). The arrow indicates the cgABP260 band, and the arrowhead indicates the filamin band. F, filamin; M, myosin; α, α-actinin; D, desmin; A, actin.

fonyl fluoride (PMSF). The myofibril-rich residue (Fig. 1a) was washed 3 times with 1 mM EDTA, pH 7.2, containing 0.1 mM PMSF. The resultant residue was collected by centrifugation at 7,000 × *g* for 10 min, washed with an equal volume of low-salt alkaline solution (2 mM Tris, 1 mM EDTA, and 1 mM PMSF, pH 9.2) developed by Feramisco and Burridge (*24*), and extracted twice with the same solution for 1 h. These extracts were combined and clarified by centrifugation at 7,000 × *g* for 20 min (Fig. 1b), solid ammonium sulfate was added to 10% saturation, and the mixture was left to stand overnight. The precipitate was collected by centrifugation at 20,000 × *g* for 10 min, washed sufficiently with 300 mM NaCl and 25 mM MES-NaOH buffer, pH 6.0, then washed 3 times with distilled water. Filamin was soluble in the supernatant (Fig. 1c). The residue (Fig. 1d) was collected by centrifugation at 20,000 × *g* for 10 min, suspended with buffer A (5% ethylene glycol and 50 mM sodium phosphate buffer, pH 8.0), and left to stand for 30 min. The supernatant was then collected by centrifugation at 20,000 × *g*. This process was repeated twice. The supernatant (Fig. 1e) was directly applied to a hydroxylapatite column (2.5 cm × 6 cm) which had been equilibrated with buffer A, and the flow-through fraction was collected. This fraction (Fig. 1f, crude cgABP260 fraction) contained actin, cgABP260, and other minor proteins. The yield of the crude cgABP260 was 11 mg.

Measurement of Protein Concentration—Protein concentration was determined by the biuret reaction.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot—SDS-PAGE was carried out by the method of Laemmli (*25*), using 3-15% gradient separation or 10% gels. Immunoblotting was performed essentially as described by Towbin *et al.* (*26*). For electrophoresis, chicken gizzard smooth muscle, breast muscle, and cardiac muscle were respectively homogenized in 5 volumes of an SDS solution (2% SDS, 65 mM Tris-HCl, pH 6.8, 2.5% 2-mercaptoethanol, and 10% glycerol), incubated at 100°C for 5 min, and centrifuged at 10,000 × *g* for 10 min. Aliquots of 2 μl of supernatant were applied to each well of the acrylamide gel.

Antibodies—Antiserum against cgABP260 was raised in a rabbit by our usual method (*27*). The crude cgABP260 fraction (Fig. 1f) was electrophoresed on SDS-PAGE gel. The cgABP260 band was cut out from the gel, emulsified in Freund's incomplete adjuvant, and injected 3 times into a rabbit at intervals of 2 wk. The antiserum was collected from the ear vein 10 d after the last injection. The antibody against cgABP260 was purified from the antiserum by membrane affinity adsorption (*28*). The affinity-purified antibody was stored at 4°C in PBS containing 1% bovine serum albumin (BSA) and 0.01% NaN_3. An anti-vinculin monoclonal antibody (clone VIN-11-5) was purchased from BioMakol. An anti-chicken gizzard filamin rabbit antiserum was purchased from TRI and affinity-purified in the same manner as cgABP260. An anti-β-galactosidase antibody was obtained from 5 Prime-3 Prime.

Immunofluorescence and Immunoelectron Microscopy—Gizzard smooth muscle cells were isolated without using protease, as described previously (*22, 23*). Isolated smooth muscle cells were mounted on a glass slide and covered with a glass slip. The cells were washed with PBS containing 10 mM EDTA and 0.1 mM PMSF, fixed with 3% formaldehyde in PBS, washed with PBS, and incubated with 1% BSA for 30 min. The cells were then incubated with affinity-purified antibody against cgABP260 or filamin for 2 h at room temperature, washed well with PBS, incubated with the FITC-labeled goat anti-rabbit IgG antibody (Cappel) for 1 h, and washed well with PBS. For double immunostaining, the cells were successively incubated in the same way with the mouse anti-vinculin first antibody and rhodamine-labeled goat anti-mouse IgG second antibody (Cappel). The specimen was washed with PBS, fixed with 3% formaldehyde in PBS, washed again with PBS, and embedded in the glycerol/PBS solution of Citifluor (Agar). For immunoelectron microscopy, small blocks (1 mm) of chicken gizzard smooth muscle were dissected and fixed with 3% formaldehyde in 150 mM NaCl, 10 mM EDTA, 0.1 mM PMSF, and 20 mM sodium phosphate buffer, pH 7.2, for 30 min. Blocks were washed 3 times with PBS for 20 min, dehydrated, and embedded in LR white (London Resin). Samples were ultrathin-sectioned, blocked with 1% BSA in PBS, reacted with the affinity-purified antibody against cgABP260 for 2 h, and rinsed 5 times with PBS. Sections were all labeled in the same manner with colloidal gold particle-conjugated goat anti-rabbit IgG antibody (E-Y Laboratories). Immunostained sections were fixed with 2.5% glutaraldehyde in PBS, stained with 2% uranyl acetate and 0.1% lead citrate, and observed under an electron microscope (JEOL 100CX).

Screening of the cDNA Library—A chicken gizzard cDNA library (CLONETECH Laboratories) prepared in λgt11 was screened with antiserum against cgABP260. For preparation of antigen-bound nitrocellulose membrane filters and antibody screening, the method of Huynh *et al.* (*29*) was used with slight modifications. Nonspecific binding sites on the filters were saturated by preincubation for 30 min with 500 mM NaCl and 20 mM Tris-HCl buffer, pH 8.0 (TBS) containing 3% gelatin. Filters were then washed with 0.05% Tween 20 contained TBS (TTBS), incubated with antiserum diluted in TTBS (1:25,000) overnight, washed with TTBS to remove unbound antibodies, and

incubated with horseradish peroxidase conjugated goat anti-rabbit IgG antibody (Bio-Rad) for 1 h. They were washed well with TTBS, then with TBS, and the color reaction was using a Konica immunostaining HRP kit. Color development proceeded for a maximum of 20 min and was stopped by washing with distilled water.

Cloning and Sequencing—The *Eco*RI inserts were excised from the immuno-positive λgt11 recombinant and subcloned into plasmid vector pBluescript KS+. Recombinant plasmid vectors were sequenced by the chain termination method, as described by Sanger *et al.* (*30*). Nucleotide sequences were determined using the program GENETIX-MAX 6.2.0 (Software Development). Homologies were analyzed by computer sequence search of the GenomeNet database (http://www.tokyo-center.genome.ad.jp/SIT/BLAST.html). It was confirmed that determined sequences of both strands of the cDNA were consistent.

Production of λgt11 Fusion Protein—To obtain the cgABP260 and β-galactosidase fusion protein, crude lysate of λgt11 recombinant lysogen in *Escherichia coli* Y1089

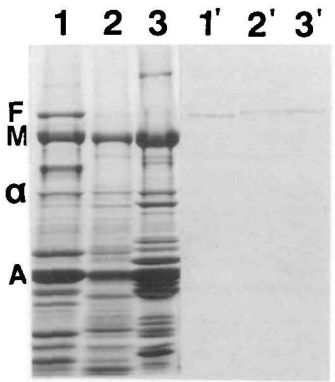

Fig. 2. **Immunoblot analysis of muscle tissues with the anti-cgABP260 antiserum.** Samples were run on a 3-15% gradient gel. The left half of the gel was stained with Coomassie Brilliant Blue, and the right half, containing the same samples as the left half, was analyzed by immunoblotting. Lane 1, gizzard; lane 2, heart; lane 3, breast muscle; lanes 1′-3′, immunostaining patterns of the corresponding samples. F, filamin; M, myosin; α, α-actinin; A, actin.

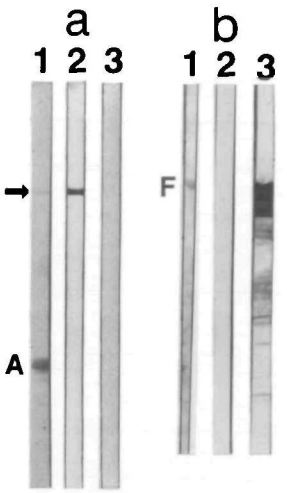

Fig. 3. **Immunological difference between cgABP260 and filamin.** Five micrograms of crude cgABP260 (a) and 1 μg of chicken gizzard filamin (b) were each run on 3-15% gradient gels and blotted onto PVDF membrane sheets. Lane 1, staining patterns with Amido Black 10B; lane 2, immunostaining patterns with anti-cgABP260; lane 3, immunostaining patterns with affinity purified anti-filamin. The arrow indicates the position of cgABP260 band. F, filamin; A, actin.

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                    30                  60                  90                 120
GGGCGAGACTGGAACGACGGGCGCGCGCTGGGGGCGCTGGTGGACAACTGCGCGCCTGGGCTGTGCCCGGACTGGCAGAGCTGGGACCCCGCGCAGTCGGTGCAGAACGCGCGCGATGTG
G  R  D  W  N  D  G  R  A  L  G  A  L  V  D  N  C  A  P  G  L  C  P  D  W  Q  S  W  D  P  A  Q  S  V  Q  N  A  R  D  V   40
                   150                 180                 210                 240
ATGCAGCAGGCCGATGATTGGCTCGGAGTGCCGCAGGTGATCGCCCCGGAGGAGATCGCGGACCCCAACGTGGACGAGCACTCCGTGATGACGTATCTGTCGCAGTTCCCCAAAGCCAAA
M  Q  Q  A  D  D  W  L  G  V  P  Q  V  I  A  P  E  E  I  A  D  P  N  V  D  E  H  S  V  M  T  Y  L  S  Q  F  P  K  A  K   80
                   270                 300                 330                 360
CTGAAGCCCGGCGCCCCTCTGCGGCCGCGCGCGGTGCGGCCCGAACGCGTCCGCGCATATGGGCCCGGGCTGGAGCCGCAGGGGAACGTGGTGCTGCAGCCGGCGCGCTTCACGGTGGAG
L  K  P  G  A  P  L  R  P  R  A  V  R  P  E  R  V  R  A  Y  G  P  G  L  E  P  Q  G  N  V  V  L  Q  P  A  R  F  T  V  E  120
                   390                 420                 450                 480
ACGTGGACGCGGGCACGGCAGGTGTTGGAGAACGCCAGAGACCACCGAGCACACCACGAGGAGGCCCAGGTGGTGGCCAACAACGATGAGAAGAGGACCTTCTCTGTCACCTACGTCCCC
T  W  T  R  A  R  Q  V  L  E  N  A  R  D  H  R  A  H  H  E  E  A  Q  V  V  A  N  N  D  E  K  R  T  F  S  V  T  Y  V  P  160
                   510                 540                 570                 600
AAAGTGGCGGGGGCTGCACAAGGTGACGGTGCTGTTTGGGGGGCAGAACATCCCCGGGAGCCCCTTTGTGGGGGTGGCGATGGCTCACGGCGATGCCAGCAAAGTGTCTGCGCGCGGCCCC
K  V  A  G  L  H  K  V  T  V  L  F  G  G  Q  N  I  P  G  S  P  F  V  G  V  A  M  A  H  G  D  A  S  K  V  S  A  R  G  P  200
                   630                 660                 690                 720
GGCATCGAACCCAGCGGCAACGTGGCCAACAAACCCACCTACTTCGACATCTACACCGCAGGTGCGGGTTCGGGTGACGTCGGGGTGGTGTTGGAGGACCCCGCGGGACCGCGTGACACC
G  I  E  P  S  G  N  V  A  N  K  P  T  Y  F  D  I  Y  T  A  G  A  G  S  G  D  V  G  V  V  L  E  D  P  A  G  P  R  D  T  240
                   750                 780                 810                 840
GTGGAGGGTGACATGGAGGACCGCGGGGACAGCACCTTCCGCTGCTCCTATCGGCCCACCCTGCCCGGCCCCCACCGCGTCGCCGTCACCTTCGCCGGAGCCCACATCCCAAACAGCACC
V  E  G  D  M  E  D  R  G  D  S  T  F  R  C  S  Y  R  P  T  L  P  G  P  H  R  V  A  V  T  F  A  G  A  H  I  P  N  S  T  280
                   870                 900                 930                 960
TTCTGCGTCAACGTGGCCGAAGCGTGCAACCCTTCGGCGTGCCGTGCGTGGGGGCGGGGGCTGCAGCCCAAAGGGCTGCGCGTGCACGAGACGGCCGACTTCCGCGTGCACACCAACGCG
F  C  V  N  V  A  E  A  C  N  P  S  A  C  R  A  W  G  R  G  L  Q  P  K  G  L  R  V  H  E  T  A  D  F  R  V  H  T  N  A  320
                   990                1020                1050                1080
CCGGCAGAACTGCGCGTCACCGTGCCGGACCGAGGCACAGAGGTTCCGGTGTCGGTGCGCCCCACGGCTGATGGCGTTTATGAGTGCGAATACCGCCCCACTGTGGCCGGGACCCACAGC
P  A  E  L  R  V  T  V  P  D  R  G  T  E  V  P  V  S  V  R  P  T  A  D  G  V  Y  E  C  E  Y  R  P  T  V  A  G  T  H  S  360
                  1110                1140                1170                1200
GTCTCCATCACGTGGGGGGGATACAGCATCCCGAGGAGCCCCCTGGAGGTGGAGGTGTCTCCGGCCGCGGGCGCTCAGAAGGTCCGTGCGTGGGGGCCGGGGCTGCACGGCGGCGTCGTG
V  S  I  T  W  G  G  Y  S  I  P  R  S  P  L  E  V  E  V  S  P  A  A  G  A  Q  K  V  R  A  W  G  P  G  L  H  G  G  V  V  400
                  1230                1260                1290                1320
GGCGACTCGGCCGACTTCGTGGTGGAGGCTATCGGGGACGACGTACGGACTCTGGGGTTCTCCATTGAGGGTCCGTCTCAGCCAAAGATCGAATCCGACGACCCGGGCGACGGTTCGTGC
G  D  S  A  D  F  V  V  E  A  I  G  D  D  V  R  T  L  G  F  S  I  E  G  P  S  Q  P  K  I  E  S  D  D  P  G  D  G  S  C  440
                  1350                1380                1410                1440
GACGTTCGCTATTGGCCCACGGAGGCGGGGCCGTACCCCGTGCACGTCGTGTGCGACGATGAGGACATCGCCCGCAGCCCCTTCATGGCCCACATCCGCCCCGCCGCCCCCGACTGCAGC
D  V  R  Y  W  P  T  E  A  G  P  Y  P  V  H  V  V  C  D  D  E  D  I  A  R  S  P  F  M  A  H  I  R  P  A  A  P  D  C  S  480
                  1470                1500                1530       1545
CCCGACAAGGTGAAGGTTTGGGGTCCCGGCCTGGAGCCCACGGGGGTCATCGTCAACCGCCCCACTGAGTTCTGTGTGGACGCCCGCGCTGCCGGAAAGGGGCCC
P  D  K  V  K  V  W  G  P  G  L  E  P  T  G  V  I  V  N  R  P  T  E  F  C  V  D  A  R  A  A  G  K  G  P   515
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Fig. 4. **Nucleotide sequence and predicted amino acid sequence of partial cDNA encoding cgABP260.** The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB000832.

was prepared as described by Huynh *et al.* (*29*). Recombinant lysogen was cultured in 50 ml of LB medium containing ampicillin (50 μg/ml) at 32°C to $A_{600}=0.5$. After incubation of cells at 42°C for 20 min, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 10 mM. Incubation was continued at 37°C for 2 h, then cells were pelleted, resuspended in sample buffer (4 M urea, 50 mM Tris-HCl buffer, pH 6.8, 1.5% SDS, and 50 mM dithiothreitol) at 3% original volume, and mixed well by passage several times through a 21-gauge needle. The β-galactosidase fusion protein was subjected to immunoblot analysis.

RESULTS

Immunoblot Analysis—The whole SDS extracts of adult chicken muscle tissues were electrophoresed on a polyacrylamide gel and transferred to a polyvinylidenedifluoride (PVDF) membrane sheet. In the chicken gizzard, the

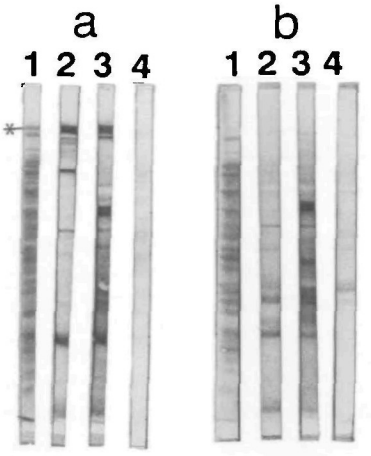

Fig. 6. **Immunoblot analysis of the cgABP260 and β-galactosidase fusion protein.** Lysates of λgt11-260a lysogenic Y1089 bacteria (a) and control Y1089 bacteria (b) were subjected to 10% SDS-PAGE and blotted onto PVDF membranes. Lane 1, Amido Black 10B staining patterns of the PVDF membranes; lanes 2, 3, and 4, immunostaining patterns of the PVDF membranes with anti-β-galactosidase (lane 2), anti-cgABP260 (lane 3), and anti-filamin (lane 4) antisera.

TABLE I. **Identity (%) of the amino acid sequence.**

	ABP-280		Retina filamin	
	ABD[a]	ROD	ABD	ROD
cgABP260	83.1	63.3	75.0	56.1

[a]ABD, actin-binding domain.

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260k   1  GRDWNDGRAL GALVDNCAPG LCPDWQSWDP AQSVQNARDV MQQADDWLGV  50
HABP 189  SRDWQSGRAL GALVDSCAPG LCPDWDSWDA SKPVTNAREA MQQADDWLGI 238
RFIL 181  NQNWQDGKAL GALVDSCAPG LCPDWETWDP SKPVDNAREA MQQADDWLGV 230

260k  51  PQVIAPEEIA DPNVDEHSVM TYLSQFPKAK LKPGAPLRPR AVRPERVRAY 100
HABP 239  PQVITPEEIV DPNVDEHSVM TYLSQFPKAK LKPGAPLRPK -LNPKKARAY 287
RFIL 231  PQLLPPEEII HPDVDEHSVM TYLYTFPKAK LKPGAPLKPK -LNPKKARAY 279

260k 101  GPGLEPQGNV VLQPARFTVE TWTRAR-QVL ENARDHRAHH EEAQVVANND 149
HABP 288  GPGLEPTGNM VKKRAEFTVE TRSAGQGEVL VYVEDPAGHQ EEAKVTANND 337
RFIL 280  GPGLEPHGNM VKQPAIFTVD TISAGQGDLM VFVEDPEGNR EEAKIMPSSD 329

260k 150  EKRTFSVTYV PKVAGLHKVT VLFGGQNIPG SPF-VGVAMA HGDASKVSAR 198
HABP 338  KNRTFSVWYV PEVTGTHKVT VLFAGQHIAK SPFEVYVDKS QGDASKVTAQ 387
RFIL 330  KNKTYSVQYV PKVTGPHKVS VLFAGQHISK SPFEVNVDKA QGDASKVTAk 379

260k 199  GPGIEPSGNV ANKPTYFDIY TAGAGSGDVG VVLEDPAGPR DTVEGDMEDR 248
HABP 388  GPGLEPSGNI ANKTTYFEIF TAGAGTGEVE VVIQDPMGQK GTVEPQLEAR 437
RFIL 380  GPGLEATGNI ANKPTYFDLY TAGAGVGDVI VEVEDPQG-R CLAEVAVEDK 428

260k 249  GDSTFRCSYR PTLPGPHRVA VTFAGAHIPN STFCVNVAEA CNPSACRAWG 298
HABP 438  GDSTYRCSYQ PTMEGVHTVH VTFAGVPIPR SPYTVTVGQA CNPSACRAVG 487
RFIL 429  GNQVYRCVYK PVQAGPHVVK VTFAGEAIPK TPCSVLIGEA CNPNACRATG 478

260k 299  RGLQPKGLRV HETADFRVHT N--APAELRV TVPDRGTEVP VSVRPTADGV 346
HABP 488  RGLQPKGVRV KETADFKVYT KGAGSGELKV TVKGPKGEER VKQKDLGDGV 537
RFIL 479  RGLQPKGVRI RETATQLIRE Q--QAVETLG TIKTRLEEL- VKQKGFMDGV 525

260k 347  YECEYRPTVA GTHSVSITWG GYSIPRSPLE VEVSPAAGAQ KVRAWGPGLH 396
HABP 538  YGFEYYPMVP GTYIVTITWG GQNIGRSPFE VKVGTECGNQ KVRAWGPGLE 587
RFIL 526  YAFEYYPATP GKYVVTITWG GHNIPKSPFE VHIGHEAGPQ KVRAWGPGLH 575

260k 397  GGVVGDSADF VVEAIGDDVR TLGFSIEGPS QPKIESDDPG DGSCDVRYWP 446
HABP 588  GGVVGKSADF VVEAIGDDVG TLGFSVEGPS QAKIECDDKG DGSCDVRYWP 637
RFIL 576  EGIVGRSADF VVESIGTEVG SLGFAIEGPS QAKIECDDKN DGSCDVKYWP 625

260k 447  TEAGPYPVHV VCDDEDIARS PFMAHIRPAA PDCSPDKVKV WGPGLEPTGV 496
HABP 638  QEAGEYAVHV LCNSEDIRLS PFMADIRDAP QDFHPDRVKA RGPGLEKTGV 687
RFIL 626  KEPGEYAVHI MCDDEDIKDS PYMAFIRPAS GDFNPDKVRA YGPGLERSGC 675

260k 497  IVNRPTEFCV DARAAGKGP  515
HABP 688  AVNKPAEFTV DAKHGGKAP  706
RFIL 676  IVNNPAEFTV ETKDAGKAP  694
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Fig. 5. **Comparison of amino acid sequence of cgABP260, filamin (*1*) and ABP-280 (*31*) over their homologous region.** A part of the actin-binding domain is included in this region. Bold characters indicate the position of the identical residues. Underlines indicate the partial potential actin-binding region.

antiserum against cgABP260 reacted exclusively with the cgABP260 band (Fig. 2, lanes 1 and 1′) just beneath the filamin band. This antiserum did not react with filamin, although the primary structure of cgABP260 is similar to that of filamin (Fig. 5). In the cardiac muscle (Fig. 2, lanes 2 and 2′) and breast muscle (Fig. 2, lanes 3 and 3′), an immunoreactive band appeared with similar mobility on SDS-PAGE to that of cgABP260. The affinity-purified anti-cgABP260 antibody also reacted exclusively with a single band in the whole peptides of chicken gizzard. The affinity-purified anti-filamin antibody did not react with the cgABP260 band of the crude cgABP260 fraction, while the affinity-purified anti-cgABP260 antibody did react with it (Fig. 3a). Conversely, the affinity-purified anti-cgABP260 antibody did not react with the chicken gizzard filamin band, while the anti-filamin antibody reacted with it intensely (Fig. 3b). The concentration of anti-filamin antibody used for these two reactions was the same.

cDNA Clones Encoding cgABP260—A cDNA clone encoding cgABP260 was isolated from a λgt11 gizzard cell cDNA library using the antiserum against cgABP260. The immunopositive λgt11-260a clone contained a cDNA insert of 1,545 bp (Fig. 4). The nucleotide sequence and the predicted amino acid sequence of cgABP260 were compared with sequences in the GenomeNet database. Chicken retina filamin (*1*) and human ABP-280 (*31*) were shown to have significant homology with cgABP260. Based on the alignment shown in Fig. 5, the calculated percentage identities of the amino acid sequence between cgABP260 and these two proteins are shown in Table I. The identity in partial potential actin-binding region (Fig. 5, underlined) was higher than that in the rod region.

Immunoblot Analysis of cDNA-Encoded Protein—The λgt11-260a clone was used to generate a λ lysogen after infection of *E. coli* Y1089. As Fig. 6 shows, cgABP260 and β-galactosidase fusion protein (asterisk) reacted with the anti-cgABP260 antiserum (Fig. 6a, lane 3), while the fusion protein did not react with the anti-filamin antiserum (Fig. 6a, lane 4). These results confirmed that the isolated clone is a partial cDNA of cgABP260.

Immunofluorescence Localization of cgABP260 in Smooth Muscle Cells—Isolated chicken gizzard smooth muscle cells were examined by indirect immunofluorescence microscopy using the membrane-affinity-purified

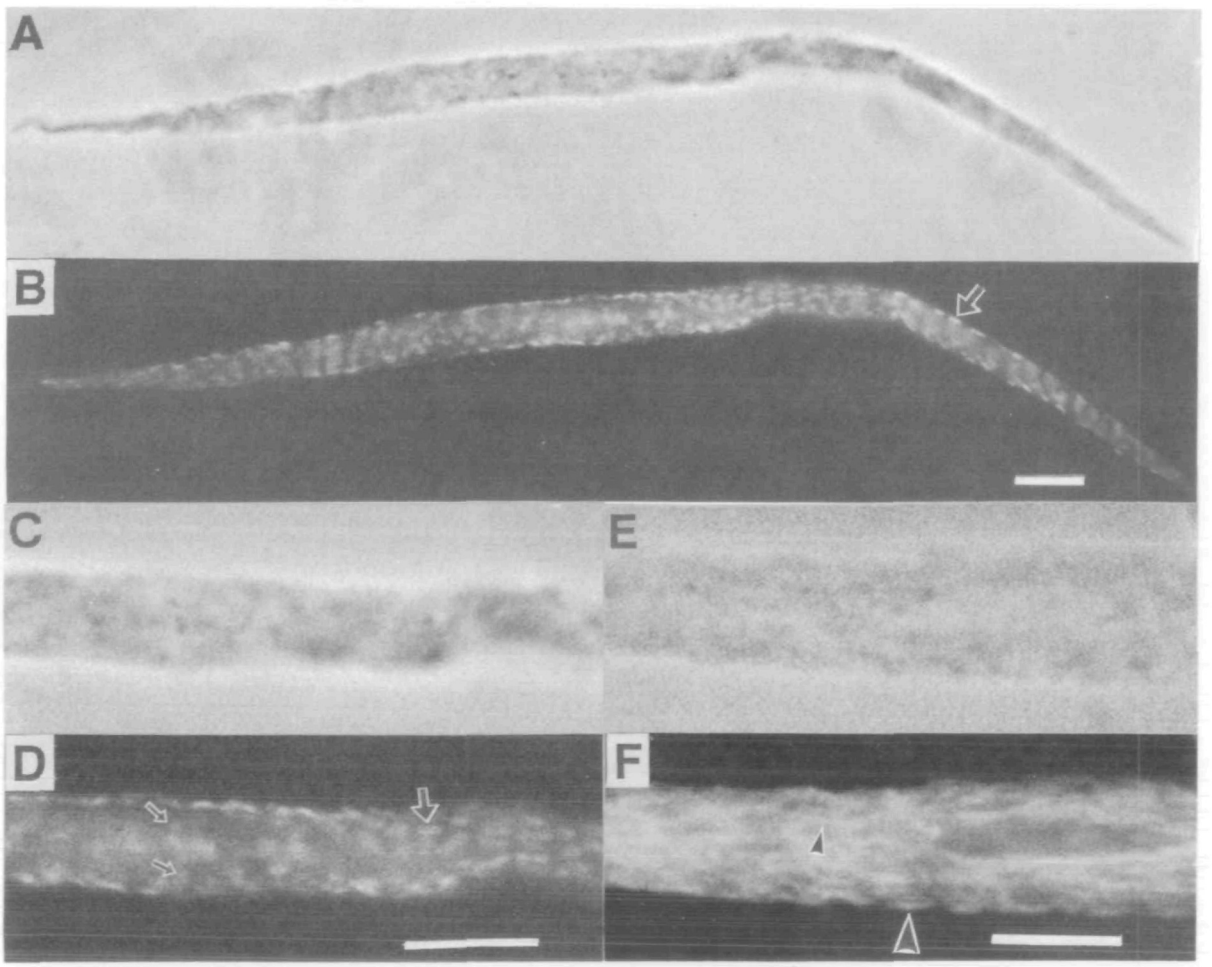

Fig. 7. **Immunofluorescence localization of cgABP260 and filamin in chicken gizzard smooth muscle cells.** (A) A phase-contrast image and (B) an immunofluorescence image with anti-cgABP260 of a single cell. (C, D) Higher-powered views of the same cell. In the thin part of the cell, the cell surface is in focus. In the central part of the cell, the inside of the cell is in focus. (E) A phase-contrast image and (F) an immunofluorescence image with anti-filamin. The middle of the cell body of an isolated smooth muscle cell is in focus. Bar, 10 μm.

α-actinin, filamin, and a 130,000-dalton protein from smooth muscle. *J. Biol. Chem.* **255**, 1194-1199
25. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
26. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
27. Ohashi, K., Mikawa, T., and Maruyama, K. (1982) Localization of Z-protein in isolated Z-disk sheets of chicken leg muscle. *J. Cell Biol.* **95**, 85-90
28. Smith, D.E. and Fisher, P.A. (1984) Identification, developmental regulation, and response to heat shock of two antigenically related form of a major nuclear envelope protein in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J. Cell Biol.* **99**, 20-28
29. Huynh, T.V., Young, R.A., and Davis, R.W. (1985) Constructing and screening cDNA libraries in λgt10 and λgt11 in *DNA Cloning 1. A Practical Approach* (Glover, D.M., ed.) pp. 49-78, IRL Press, New York
30. Sanger, F., Nickken, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
31. Gorlin, J.B., Yamin, R., Egan, S., Stewart, M., Stossel, T.P., Kwiatkowski, D.J., and Hartwig, J.H. (1990) Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J. Cell Biol.* **111**, 1089-1105
32. Burridge, K. and Connell, L. (1983) A new protein of adhesion plaques and ruffling membranes. *J. Cell Biol.* **97**, 359-367
33. Cox, D., Ridsdale, J.A., Condeelis, J., and Hartwig, J. (1995) Genetic deletion of ABP-120 alters the three-dimensional organization of actin filaments in *Dictyosterium* pseudopods. *J. Cell Biol.* **128**, 819-835