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Characterization of Arp2/3 Complex in Chicken Tissues

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ABSTRACT. **Arp2/3 protein complex consists of seven subunits (Arp2, Arp3, p41-Arc, p34-Arc, p21-Arc, p20-Arc and p16-Arc) in apparent 1:1 stoichiometry. This complex has been shown to promote the formation of Y-branch structures of F-actin in cultured cells. We generated specific antibodies against chicken Arp2, Arp3, and p34-Arc to analyze the distribution of these subunits in chicken tissues.In whole samples of brain and gizzard, antibodies against each recombinant protein reacted with single bands of predicted molecular mass based on their cDNA sequences of the antigens. Anti-p34-Arc antibody detected at least two neighboring spots in 2D-PAGE, which might suggest the existence of isoforms or modified forms. Arp2/3 complex bound to an F-actin affinity column from gizzard extract. However, Arp2/3 complex did not tightly bind major actin cytoskeleton because the complex was extracted easily when gizzard smooth muscle was homogenized in PBS. Immunoblot analysis of various tissues revealed that the amounts of Arp2/3 subunits were lower in striated muscle than in non-muscle and smooth muscle tissues. Amounts and ratio of the three subunits varied in tissues, as estimated by quantitative immunoblotting. With immunofluorescence microscopy, we also observed localization of Arp3 and p34-Arc in frozen sections of gizzard with different staining patterns around blood vessels. These results suggest that the Arp2/3 complex exists also in places where rapid actin polymerization does not occur, and that a part of the subunits may exist in different forms from the complex containing the seven subunits in some tissues.**

Key words: Arp2/3 complex/subunits/chicken/F-actin column/localization/tissue

Actin plays important roles in morphogenesis and motility in eukaryotic cells. In muscle tissue, actin is a major constituent of the contractile structure. In non-muscle tissue, actin forms such structures as stress fibers, adherence junctions, lamellipodia, microvilli, and contractile rings. Actin polymerization also drives cell locomotion (Mitchison and Cramer, 1996) and the intracellular movement of pathogens such as Listeria monocytogenes (Cossart *et al*., 2001).

Arp2/3 complex is a protein complex composed of seven subunits including the actin-related proteins Arp2 and Arp3, and five other proteins that in humans are called p41-Arc, p34-Arc, p21-Arc, p20-Arc, and p16-Arc (Welch *et al*., 1997a). All of the Arp2/3 complex subunits exhibit a significant similarity in amino acid sequences among eukaryotes (Machesky *et al*., 1997), suggesting that the structure and function of the complex have been conserved during evolution. The functional importance of the Arp2/3 complex is also underscored by the observation that deleting the genes encoding individual subunits in yeast causes lethality (Machesky and Gould, 1999).

The Arp2/3 complex has been shown to play a key role in force production by actin polymerization (Welch, 1999). This complex has nucleation and side-binding activities on actin, so actin filaments that are polymerized in its presence form Y-shaped branches. These branches are also observed in lamellipodia (Svitkina and Borisy, 1999) and Listeria monocytogenes comet tails (Cameron *et al*., 2001). Its weak basal activity is synergistically stimulated by activating proteins that include ActA (Welch *et al*., 1998), WASp family

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Abbreviations: BSA, bovine serum albumin; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CBB, Coomassie brilliant blue R-250; DTT, dithiothreitol; EGTA, ethylene glycol bis (1-aminoethyl ether) -N,N'-tetraacetic acid; FITC, fluorescein isothiocyanate; F-actin, filamentous actin; G-actin, globular actin; IEF, isoelectric focusing; LSA, low salt alkaline solution; 2-ME, 2-mercaptoethanol; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; NBT, nitroblue tetrazolium; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylydene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TAME, N-p-tosyl-L-arginine methyl ester; TCA, trichloroacetic acid; TBS, Tris-buffered saline; Tween 20, polyoxyethylene sorbitan monolaurate.

proteins (Machesky *et al*., 1999), cortactin (Weaver *et al*., 2001), and ABP1p (Goode *et al*., 2001). Together, these proteins and their regulating factors are thought to control the spatial and temporal distribution of Arp2/3 complex in cells.

The functions of each subunit have been discussed in relation to genetic approaches using yeast mutants (Balasubramanian *et al*., 1996; McCollum *et al*., 1996; Morrell *et al*., 1999; Winter *et al*., 1999). Recently, Gournier *et al*. (2001) reconstituted recombinant human Arp2/3 complex using the baculovirus expression system to define function of the subunits using biochemical approaches. Analysis of subcomplexes lacking one or more subunits revealed that a heterodimer of the p34-Arc and p20-Arc subunits constitutes a critical structural core of the complex and also binds to actin. Arp3 is crucial for filament nucleation, and p41-Arc, p21-Arc, and p16-Arc differently contribute to the nucleation and stimulation by ActA and WASp. Their study revealed that the nucleating and organizing functions of Arp2/3 complex subunits are separable. It also leads to the question of whether all of the subunits always exist as the complete complex with seven subunits to form Y-shaped branches or whether part of them exist in different subcomplexes to work in other ways *in vivo*.

The Arp2/3 complex is localized in lamellipodia (Welch *et al*., 1997a), pseudopodia (Weiner *et al*., 1999), phagocytic protrusions (May *et al*., 2000), and comet tails caused by the intracellular pathogens (Welch *et al*., 1997b; Gouin *et al*., 1999) of cultured cells in which Y-shaped branches have been observed. However, the localization of Arp2/3 complex in tissues that contain various cytoskeletal structures has not been thoroughly discussed.

In this study, we generated specific antibodies against chicken Arp2, Arp3, and p34-Arc to determine distributions of these subunits in various tissues. We confirmed F-actin binding activity of Arp2/3 complex with an F-actin affinity column, but the complex did not seem to tightly bind actin cytoskeleton *in vivo*. Quantitative immunoblotting revealed the molar ratio of three subunits seemed to vary in tissues. We also observed the localization of Arp3 and p34-Arc were different around blood vessels.

These results will give a new point of view for the studies of distribution of Arp2/3 complex and its complex formation *in vivo*.

Materials and Methods

Antibodies against recombinant Arp2, Arp3 and p34-Arc

Antibodies against recombinant chicken Arp2, Arp3, and p34-Arc were affinity-purified from rabbit serum. Arp2 clone (GenBank Accession. No. X73971) was a kind gift from Dr. Jean-Jacques Michaille (Michaille *et al*., 1995) and Arp3 and p34-Arc (AW239874 and AI981024) were obtained from Chick EST project. Full cDNA sequence of chicken p34-Arc has not been obtained but AI981024 showed significant homology to amino acids of 110-300 of human p34-Arc. C-terminal of Arp2 (amino acids 318-394), Arp3 (amino acids 214-418) and p34-Arc (full sequence of AI981024) were subcloned into pRSET vectors (Invitrogen Japan K.K., Tokyo, Japan) to allow production of His-tag fusion proteins. As recombinant proteins were mostly contained in inclusion bodies, they were solubilized with 4 M or 6 M urea and purified with Ni-NTA beads (QIAGEN K.K. Tokyo, Japan). The gel bands were excised and immunized to rabbits separately. The same part of each clone was subcloned into pGEX vectors (Amersham Biosciences Corp., Piscataway, NJ, USA). GST-tagged proteins were solubilized with PBS containing 4 M or 6 M urea, dialyzed against 2 M urea and purified with glutathione-sepharose 4B (Amersham Biosciences Corp). Purified proteins were stabilized on the resin by washing with PBS. Antisera against His-tagged proteins were affinity-purified with GST-fusion proteins via the GST sepharose columns to avoid contamination of antibodies against tag sequences.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed according to the method of Laemmli (1970), using 12.5% acrylamide or 3–15% acrylamide gradient gel. The gels were stained with CBB or Silver Stain Plus kit (BIO-RAD, Hercules, CA, USA).

Immunoblotting analysis was performed according to the procedure developed by Towbin *et al*. (1979) with modifications. Electrophoresed proteins were transferred onto PVDF membranes, and the membranes were blocked with 5% skimmed milk in TBS (0.5 M NaCl, 20 mM Tris-HCl, pH 7.6). They were reacted with the primary antibodies in TBS containing 0.05% Tween 20 (TTBS) for 2 h and incubated with alkaline phosphatase-labeled secondary antibody (Cappel Research Product, Durham, NC, USA) for 1 h. Developement was carried out with BCIP and NBT.

2D-PAGE

Brain and gizzard tissue were suspended in sample buffer consisting 40 mM Tris, 8 M urea, 4% CHAPS, 20 mM DTT, and centrifuged at 11,000×g. Supernatants were applied onto 7 cm pH 3–10 Immobiline IEF gel strips (Amersham Bioscience) preswelled according to the manufacturer's protocol. In the first PAGE, the gel strips were electrophoresed with Multiphor II, and then placed on the 12.5% gel for the second PAGE.

F-actin affinity column chromatography

Columns for affinity chromatography were prepared according to the method of Miller and Alberts (1989). Rabbit back muscle actin was prepared by the method of Spudich and Watt (1971) and further purified through a Sephadex G-100 column (Amersham Biosciences). F-actin polymerized in 50 mM Hepes-KOH (pH 7.5), 0.1 M KCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.2 mM ATP was stabilized with 10 μg/ml phalloidin, and then coupled to Affi-Gel 10 (BIO-RAD) mixed with Sepharose CL-6B (Amersham Biosciences).

Chicken tissue extract for F-actin affinity chromatography was prepared as described by Miller and Alberts (1989). Fresh gizzard

or brain was homogenized in 4 volumes of E-buffer (5 mM Hepes-KOH (pH 7.5), 0.05 % NP-40, 0.5 mM EGTA/EDTA, 2 mM TAME, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin) with a kitchen mixer. The homogenate was centrifuged at 12,000×g for 30 min, and to the supernatant was added 1 M Hepes-KOH (pH 7.5) to 50 mM and 0.5 M DTT to 2 mM. This was centrifuged again at 100,000×g for 2 h. The supernatant from this step was applied to F-actin affinity columns.

F-actin columns were equilibrated with A-buffer (50 mM Hepes-KOH (pH 7.5), 2 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, 10% glycerol, 0.05% NP-40, 2 mM TAME, and 0.1 mM PMSF,).

The extracts were applied to the column at a flow rate of one column volume/h. The columns were rinsed with A-buffer at a flow rate of three column volumes/h. The columns were then eluted successively with A-buffer containing 0.1 M KCl, A-buffer containing 0.5 M KCl, and A-buffer containing 1.0 M KCl plus 1.0 mM ATP and 3 mM $MgCl_2$.

Extraction of chicken gizzard extracted with various buffers

The extraction procedure was performed according to the protocol of Feramisco and Burridge (1980) with some modifications. Fresh chicken gizzard smooth muscle was homogenized in PBS containing 10 mM EDTA and 0.1 mM PMSF and then centrifuged at 7000×g for 10 min. The pellet was resuspended with the same buffer and washed eight times in the same manner. The myofibril-rich pellet was then washed with distilled water and extracted stepwise with LSA (2 mM Tris, 1 mM EDTA, and 0.1 mM PMSF, pH 9.2) at 4°C and Hasselbach-Schneider's solution (0.1 M K-PO_4, pH 6.5, 0.6 M KCl, 1 mM $MgCl_2$, and 10 mM $Na_4P_2O_7$). The pellet was washed again with distilled water and extracted with LSA at 37°C.

Quantitative immunoblotting

Whole samples of chicken brain and known amounts of recombinant His-Arp3 were electrophoresed in the same gel, blotted on the same membrane, and reacted with the anti-Arp3 antibodies under the same conditions. The intensity of bands on immunoblots was quantified using an imaging densitometer (BIO-RAD GS-700) and Quantity One software (BIO-RAD). Amounts of native Arp2 and p34-Arc were estimated in the same manner.

Immunofluorescence microscopy

Chicken gizzard was fixed with PBS containing 10% formalin for 5 min and frozen in liquid nitrogen. Sequential sections (5 μm) on slide glasses were fixed again with PBS containing 10% formalin for 5 min and washed with PBS. The sections were incubated at room temperature for 30 min with PBS containing 1% BSA and reacted with the primary antibodies for 2 h. After washed well with PBS, Alexa488-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., Junction City, OR, USA) for 1 h.

Results

Reactivity of antibodies

In whole samples of brain and gizzard, affinity-purified antibodies against each recombinant protein reacted with single bands of predicted molecular mass (chicken Arp2 : 44.7 kDa, chicken Arp3 : 47.4 kDa, human p34-Arc : 34.3 kDa) from their cDNA sequences (Fig. 1). In addition, all antibodies clearly reacted with human melanoma cell line M2 (Cunninghum 1992), and anti-Arp3 also reacted against sea urchin oocytes (data not shown).

In 2D-PAGE, the amount of Arp2/3 complex was so low that it was difficult to determine immunoreactive spots even though the membranes contained more than 1 mg of protein.

With anti-Arp3 antibody, a clear spot in gizzard (Fig. 2A) and a clear spot with a faint spot next to the main spot in brain (Fig. 2B) were detected. The pIs of the main spots were estimated to be 5.6 in gizzard and 5.8 in brain, which were close to pI 5.5 predicted from amino acid sequence of chicken Arp3. Anti-p34-Arc antibody detected two isolated spots in gizzard (Fig. 2C) and at least two spots that were not clearly separated in brain (Fig. 2D). The pIs of two spots were estimated to be 7.0 and 7.4 in gizzard, 6.8 and 7.2 in brain, which were close to pI 7.0 predicted from amino acid sequence of human p34-Arc. These results seemed to indicate the existence of isoforms or some modified forms of Arp3 and p34-Arc. We have not been able to detect distinct spots with the anti-Arp2 antibody either in gizzard or brain.

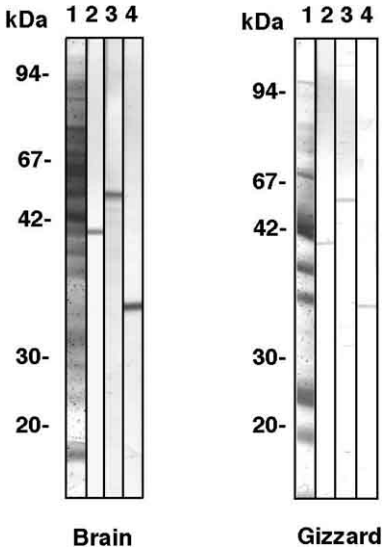

Fig. 1. Reactivity of affinity-purified antibodies against Arp2, Arp3, and p34-Arc to chicken brain and gizzard (1D-PAGE). Whole protein of each tissue was run in 12.5% gels. 1, CBB staining pattern; 2, immunoblot of anti-Arp2 antibody; 3. immunoblot of anti-Arp3 antibody; 4, immunoblot of anti-p34-Arc antibody.

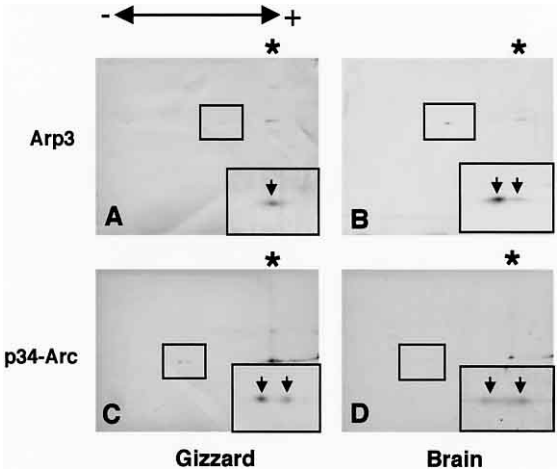

Fig. 2 Reactivitiy of affinity-purified antibodies against Arp3 and p34-Arc to chicken brain and gizzard (2D-PAGE). Whole proteins of chicken gizzard and brain were separated in pH 3-10 Immobiline gels and then analyzed in the second SDS-PAGE of 12.5% gels. Spots were transferred to PVDF membrane and reacted with anti-Arp3 or anti-p34-Arc antibodies. Magnified images of spots (boxed) are added to blots. Arrows indicate detectable spots. Asterisks indicate sample-adding positions in the first PAGE. (A) Blot of gizzard reacted with anti-Arp3 antibody. (B) Blot of brain reacted with anti-Arp3 antibody. (C) Blot of gizzard reacted with anti-p34-Arc antibody. (D) Blot of brain reacted with anti-Arp3 antibody.

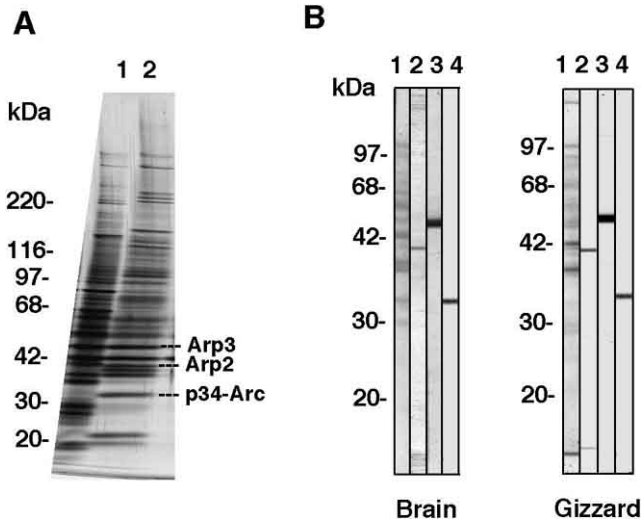

Fig. 3 Chicken Arp2/3 complex bind specifically to the F-actin column. Starting material and 0.1 M eluent was run in 3-15% gel and silver-stained. Bars indicate Arp2, Arp3, and p34-Arc bands that were confirmed with immunoblot. (A) Typical elution pattern from chicken brain extracts. 1, brain extract; 2, 0.1 M eluent of F-actin column. (B) Reactivity of antibodies to 0.1 M KCl eluent from F-actin column of gizzard and brain extract. The eluent was concentrated with TCA and run in 12.5% gels. 1, CBB staining pattern; 2, immunoblot of anti-Arp2 antibody; 3, immunoblot of anti-Arp3 antibody; 4, immunoblot of anti-p34-Arc antibody.

Analysis of F-actin binding activity of Arp2/3 complex with F-actin column

The extracts of chicken brain and gizzard were loaded in an amount exceeding the capacity of the F-actin column in order to detect minor proteins that have high affinity to F-actin. Lane 2 of Figure 3A shows SDS-PAGE of the 0.1 M KCl eluent from the brain extract. When extracts containing 300 mg protein were applied to 20 ml F-actin columns (0.5–0.8 mg F-actin/ml resin), more than 20 major proteins containing previously identified actin-binding proteins were found to bind specifically to the columns (Terasaki *et al.*, unpublished data). Arp2, Arp3, and p34-Arc were concentrated as clear bands in 0.1 M eluent (Fig. 3A). Reactivity of anti-Arp2, Arp3, and p34-Arc antibodies against the column eluent also suggested that Arp2/3 complexes were collected with the F-actin column (Fig. 3B). Additionally, the peptide sequence derived from the protein band reacted with anti-p34-Arc antibody was similar to the predicted amino acid sequence of human p34-Arc (MILLEVNNRIIEETL).

Extraction of Arp2/3 complex from gizzard

Gizzard smooth muscle was extracted stepwise with various buffers as shown in Figure 4A. Obtained extracts and residues (Fig. 4B) were reacted with antibodies against Arp2, Arp3, and p34-Arc to analyze their solubilities. All three antibodies reacted with whole samples and PBS-soluble fractions, but not with myofibrillar fraction. Terasaki *et al.* (1995) reported that actin, tropomyosin, and α-actinin were extracted with LSA at 4°C, myosin and filamin were extracted with Hasselbach-Schneider's solution, and filamin, vinculin, and α-actinin were extracted with LSA at 37°C. But neither anti-Arp2 antibody nor anti-p34-Arc antibody reacted with these extracts and residue containing cytoskeletal proteins (upper bands with asterisks stained with anti-p34-antibody in lanes 3, 5, 7 of Figure 4C seemed to be non-specific when blots developed excessively). Anti-Arp3 antibody weakly reacted the extract with the Hasselbach-Schneider's solution but anti-Arp2 and anti-p34-Arc antibodies did not (Fig. 4C).

Relative amounts of subunits of Arp2/3 complex in brain

Whole samples of chicken brain and known amounts of recombinant His-Arp3 on the same membrane were reacted with anti-Arp3 antibody to determine the amount of native Arp3 in brain (Fig. 5A). Immunoblot density increased in proportion to amount of the recombinant protein (Fig. 5B). With this approach, native Arp3 in 30 μg whole brain samples showed the same density as 0.0082 μg (mean value, n=5) of recombinant Arp3, which was calculated as 0.289 pmol from its molecular mass. Amounts of Arp2 and p34-Arc were estimated with the same method (n=4 each) and molar ratios of Arp2, Arp3, and p34-Arc in brain were estimated at 1.00±0.37 : 0.71±0.17 : 1.12±0.28 (Fig. 5C).

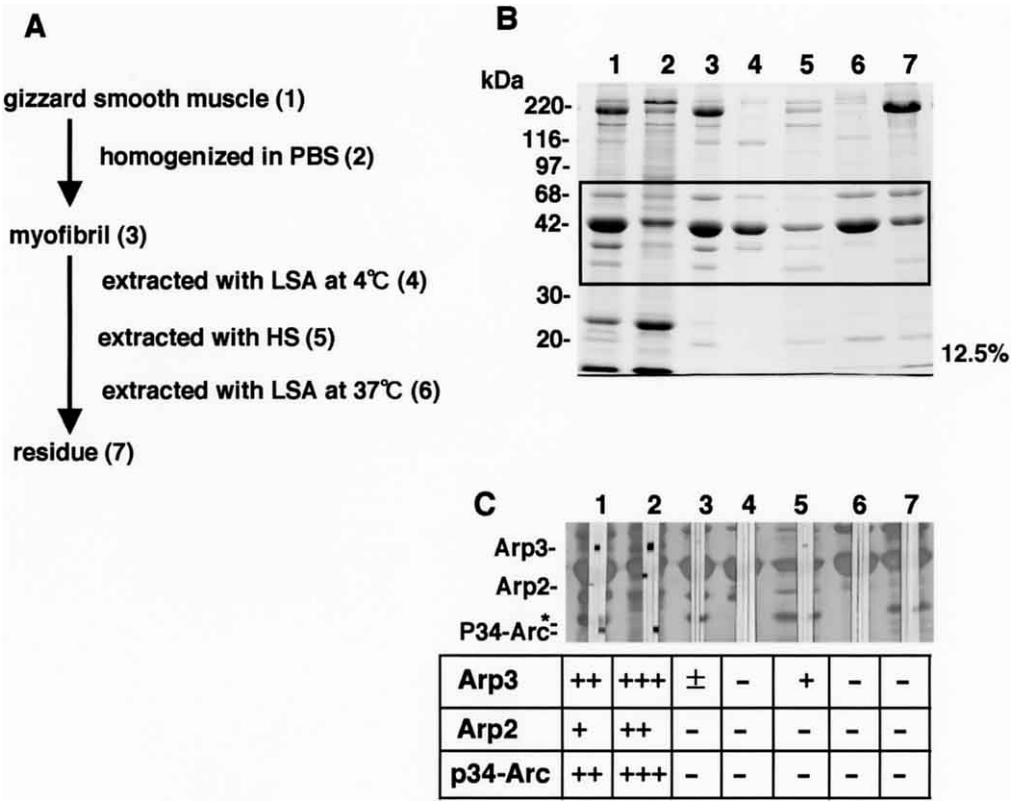

Fig. 4 Detection of chicken Arp2, Arp3, and p34-Arc in chicken gizzard extracted with various buffers. Chicken gizzard smooth muscle was extracted stepwise with PBS, LSA, and HS solution. Each fraction were run in 12.5% gels and reacted with anti-Arp3, anti-Arp2, and p34-Arc antibodies in the same condition. Asterisks indicate bands stained with anti-p34-Arc antibody that seemed to be non-specific when blots developed excessively. (A) Extraction procedure. (B) SDS-PAGE patterns of fractions obtained in A. (C) Blots of samples in B. Membranes were excised to 4 strips to be stained with CBB, reacted with three antibodies. 1, whole sample; 2, PBS extract; 3, myofibril; 4, LSA extract (4°C); 5, HS extract; 6, LSA extract (37°C); 7, residue.

Amounts of subunits in various tissues

Whole extracts of brain, liver, kidney, gizzard, breast muscle, and heart were run in the same gels and reacted separately with the anti-Arp2, Arp3, and p34-Arc antibodies (Fig. 6). Densities of immunoreactive bands, which were shown to correlate to amounts of native subunits in Figure 5B, varied among tissues. All three subunits of the Arp2/3 complex were less abundant in striated muscle than in non-muscle and smooth muscle tissues. In many tissues, reactivity of three antibodies did not vary in the same manner. In kidney, for example, the density of immunoreactive band for anti-Arp3 antibody was 124% of the value for brain, but the density of the bands for anti-Arp2 antibody and for anti-p34-Arc antibody were 49% and 58%.

Localization of Arp3 and p34-Arc in chicken gizzard

Sequential sections of chicken gizzard were stained with the antibodies against Arp3 and p34-Arc in order to compare their localization. In smooth muscle tissue, both of them showed weak filamentous staining pattern throughout the muscle layer and did not show the clear dotted patterns often observed with antibodies against components of dense bodies and dense plaques (Fig. 7A, 7C). The staining patterns of anti-Arp3 and anti-p34-Arc antibodies around blood vessels appeared to follow a pattern based on various cells in the region (Fig. 7B, 7D). Anti-p34-Arc strongly stained the inner layer of the blood vessels, which may indicate the presence of epithelial cells compared with phase contrast image (Fig. 7F), but anti-Arp3 antibody stained the layer much less.

Discussion

Distribution of Arp2/3 complex was well described in cultured cells and monocellular organisms. Localization of the complex in lamellipodia, comet tails, and other structures in which actin polymerization occur supported the Y-branch model (Svitkina and Borisy, 1999), but their localization in tissues is not well documented.

Among the subunits of Arp2/3 complex, the functions of Arp2, Arp3 and p34-Arc have been well discussed. Arp2 and Arp3 have been suggested to have nucleation activity

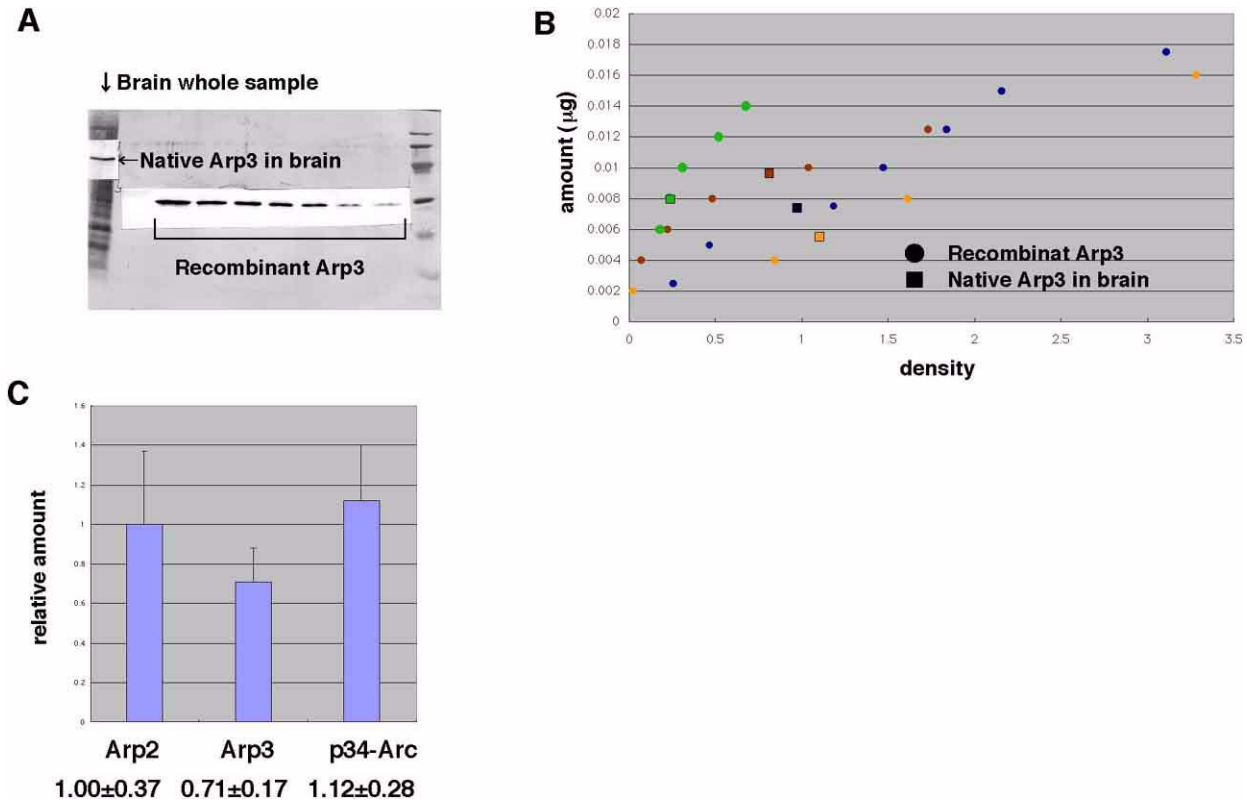

Fig. 5 Estimation of amounts of chicken Arp2, Arp3, and p34-Arc in brain by quantitative immunoblotting. A total of 30 μg protein of chicken brain sample and His-Arp3 were blotted on the same membrane and reacted with anti-Arp3 antibody.(A)Typical immunoblot patterns of recombinant Arp3 and brain native Arp3. (B) Relationship between densitometry and amount (μg) of the recombinant proteins. Green, red, blue, and yellow circles indicate densitometry and amount of recombinant Arp3 in four independent experiments. Squares indicate ones of native Arp3 in brain. (C) Molar ratio of Arp2, Arp3, and p34-Arc in brain, which were calculated with amounts and their molecular mass. Bars in C represent standard deviations (Arp2, n = 4 : Arp3, n = 5 : p34-Arc, n = 4).

from their structure similarity to actin (Kelleher *et al*., 1995). Recently, side-binding activity of p34-Arc has revealed to be essential for branching activity of the Arp2/3 complex (Bailly *et al*. 2001). Therefore, we prepared antibodies against Arp2, Arp3 and p34-Arc of chicken Arp2/3 complex for the present study.

Immunoblotting of brain, kidney, liver, gizzard, heart, and breast muscle showed that the amount of the Arp2/3 subunits was lower in striated muscle than in non-muscle and smooth muscle tissues (Fig. 6). As actin cytoskeletons in heart and breast muscle are stable, they may not need Arp2/3 complex to maintain their cytoskeletal structures. Immunoblot of the brain is more intense than liver and kidney, suggesing Arp2/3 complex might play a role in regulating the actin cytoskeleton for the growth of dendrites and intercellular attachments. It is noteworthy that gizzard contained the same amount of Arp2/3 complex as non-muscle tissue.

We observed Arp2/3 complex specifically bound to F-actin columns when the column was overloaded with extract (Fig. 3). Terasaki *et al*. (1997) detected sea urchin Arp2/3 complex with some novel actin-binding proteins using F-actin column and suggested that overcharging with extract results in the collection of actin-binding proteins which were present in low amounts but have high affinity. Arp2/3 complex in chicken tissues might have high affinity to F-actin, but the fact that Arp2/3 complex was extracted mostly with PBS from gizzard is somewhat inconsistent with the observation. Arp2/3 complex might compete with other actin-binding proteins on major actin cytoskeleton *in vivo*. It is also possible that Arp2/3 complex bound indirectly but strongly to the column through interactions with other protein(s), because some proteins have been revealed to bind Arp2/3 complex and to stimulate its activity (Higgs, 2001). These ideas explain observations that Arp2/3 complex localize in restricted cytoskeletal structure and does not localize in major actin cytoskeleton such as stress fiber in culture cells and actin cables in yeast (Welch *et al*., 1997a and Moreau *et al*., 1996).

The Arp2/3 complex was originally identified in Acanthamoeba (Machesky *et al*., 1994) and appears to be conserved in all eukaryotes. In amoebas and mammals, purified

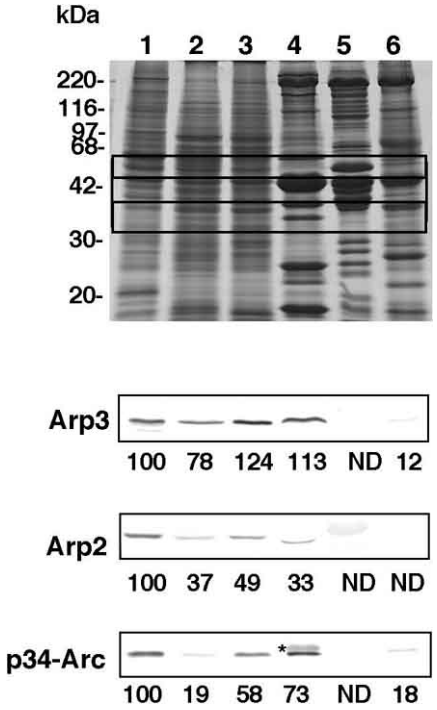

Fig. 6 Comparison of amounts of Arp2, Arp3, and p34-Arc in various tissues estimated by quantitative immunoblotting. Whole extracts of various tissues were run in the same 12.5% gels and reacted separately with anti-Arp2, Arp3, and p34-Arc antibodies. Each sample contained 30 μg protein. Numbers under blots show the relative amounts (%) of subunits in tissues compared with those of brain. Asterisks indicate non-specific band stained with anti-p34-Arc antibody as shown in Figure 4. 1, brain; 2, liver; 3, kidney; 4, gizzard; 5, breast muscle; 6, heart.

Arp2/3 complex has seven subunits, which in human are named Arp3, Arp2, p41-Arc, p34-Arc, p21-Arc, p20-Arc and p16-Arc in an apparent 1:1 stoichiometry (Machesky *et al*., 1997).

As subunits of Arp2/3 complex were usually copurified (Machesky *et al*., 1997) and the purified complex was stable, the subunits of Arp2/3 complex have been thought to exist only as a complex form and not as freely available, discrete forms. Our result on the amount of Arp2, Arp3 and p34-Arc determined with quantitative immunoblot suggests that the molar ratio of Arp2, Arp3 and p34-Arc is not 1:1:1 in some tissues (Fig. 5, 6). In case of muscle tissue, weak reactivity of anti-Arp2 antibody is possibly due to masking of antigen with actin which is abundant in muscle tissues, but uneven molar ratio between Arp3 and p34-Arc were still observed in all tissues. Immunofluoresence microscopy also showed staining patterns of anti-Arp3 and anti-p34-Arc antibodies were different around blood vessels, which suggested that epithelial cells contain different amounts of Arp3 and p34-Arc (Fig. 7B, 7D). If some subunits exist excessively *in vivo*, they would not form the complete complex of seven subunits. We expected that Arp2, Arp3, and p34-Arc would be extracted under different conditions if they were not entirely involved in the complex. Only Arp3 was detected in extract with a high concentration salt solution (Fig. 4C), but we have not been able to obtain other decisive data. Purification of putative subcomplexes would be necessary to confirm this hypothesis.

Gournier *et al*. (2001) reconstituted recombinant human Arp2/3 complex lacking one or more subunits using the baculovirus expression system. Nucleation activity and branching activity were different between the subcomplexes and one subcomplex showed only actin-binding activity in vitro. Their result suggested that the functions of subunits in Arp2/3 complex are separable. The fact that the Arp2/3 complex is stable to the loss of p41 in yeast (Winter *et al*., 1997) also suggests that a subcomplex may exist *in vivo*. If these subcomplexes have functions *in vivo*, Arp2/3 complex has other functions besides forming Y-shaped branches. This notion is also attractive because Hudson and Cooley (2002) showed that the Arp2/3 complex is required for expansion of ring canals in Drosophila where an organization of actin filaments is very different from the Y-branched network at the leading edge of moving cells. Our present study showed that smooth muscle, in which actin branches have not been reported, also contain the same content of Arp2/3 complex as non-muscle tissues (Fig. 6) and most of them were contained in the cytoplasm (Fig. 4). Subcomplexes which have different functions from Arp2/3 complex containing seven subunits may play roles in the construction and regulation of actin cytoskeleton in smooth muscle cells.

It appears that only one gene codes for each Arp2/3 complex subunit in yeast (Machesky and Gould, 1999) but in mammals, Sop2Hs, which would be homologue of p41-Arc, has been reported (Welch *et al*., 1997a). Additionally, a single Arp3 gene generated splicing isoform Arp3 beta, and the expression of ARP3 beta in brain was shown to be restricted to neurons and epithelial cells (Jay *et al*., 2000). A minor spot detected on blots of 2D-PAGE with anti-Arp3 antibody (Fig. 2B) might be the isoform. In the present study, isoforms or some modified forms of p34-Arc were first revealed (Fig. 2C, 2D) and there might be the same case in other subunits. However, the functional differences of these isoforms or their modified forms have yet to be clarified, and they may possibly affect complex formation and activity.

Many questions still remain in the relationship of the activity, regulation and localization of Arp2/3 complex *in vivo*. Further analysis of complex formation is necessary to elucidate the functions of the subunits of Arp2/3 complex in tissues.

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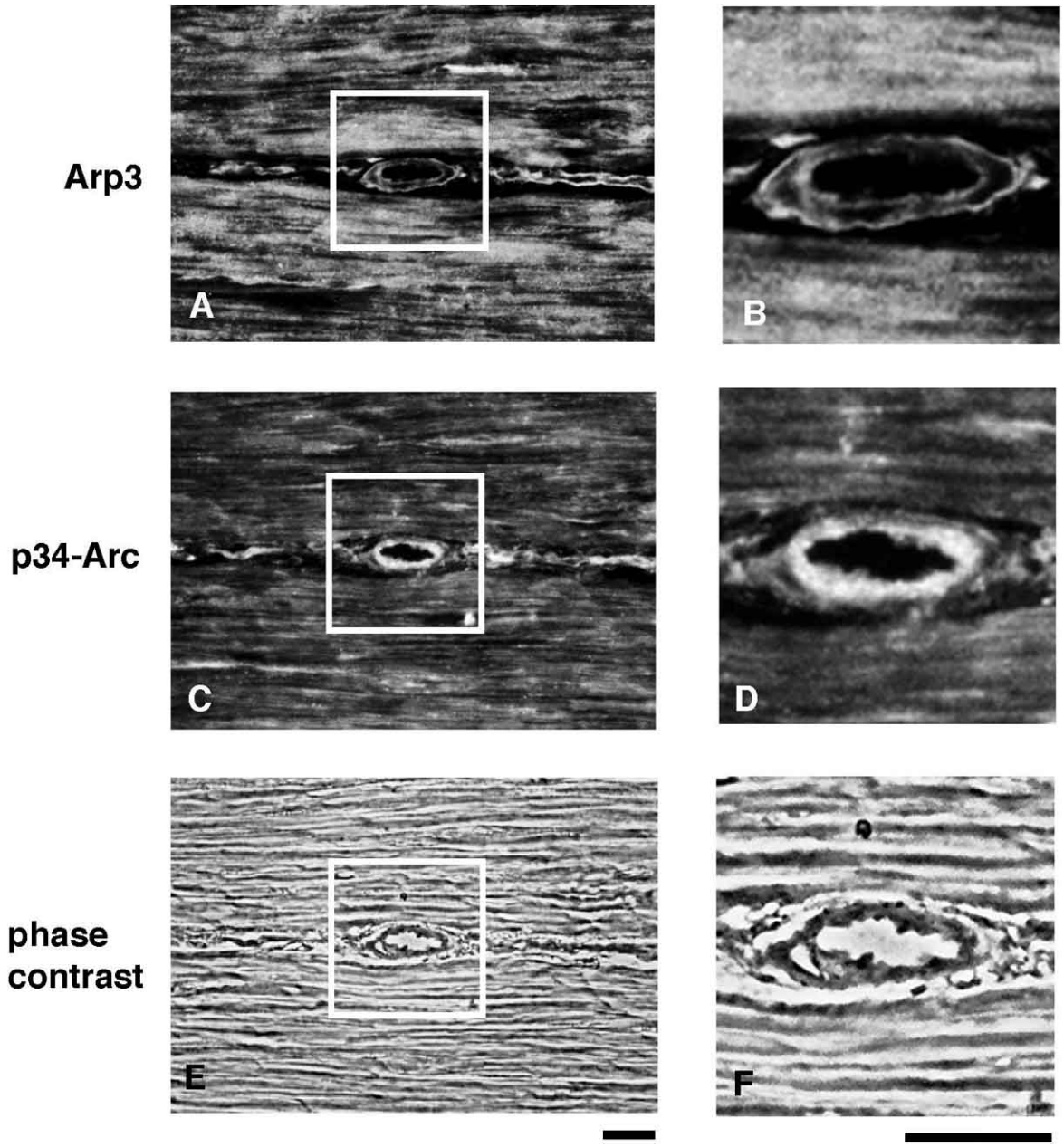

Fig. 7 Immunolocalization of Arp3 and p34-Arc in frozen sections of chicken gizzard. Chicken gizzard was fixed with 10% folmalin and frozen in liquid nitrogen. Sequential sections of 5 μm were reacted with anti-Arp3 and p34-Arc antibodies. Phase contrast image of the section reacted with anti-p34-Arc antibody was also observed. (A) Immunofluoresence image with anti-Arp3 antibody. (C) Immunofluoresence image with of anti-p34-Arc antibody. (E) Phase contrast image of C. B, D and F are magnified images of A, C, and E. Bars indicate 10 μm.

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