Jpn. J. Cancer Res. 85, 735–743, July 1994

Intracellular Localization and Biochemical Function of Variant β-Actin, which Inhibits Metastasis of B16 Melanoma

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We analyzed the biochemical nature of βm-actin protein found in mouse B16 melanoma. When we carried out immunostaining with the antibody specific to βm-actin, filamentous immunofluorescence was observed in B16-F1, a low-metastatic cell line expressing βm-actin, but not in highly metastatic B16-F10 that did not express βm-actin. When a purified actin fraction containing βm-actin was polymerized and immunoprecipitated with anti-βm-actin antibody, the immunoprecipitate contained βm-, β- and γ-actin. This indicated that the βm-actin was incorporated into an actin filament together with β- and γ-actin *in vitro*, and this phenomenon was consistently suggested by cellular double immunostaining with anti-βm-actin and common anti-actin antibody. When the actin fraction containing βm-actin under a regular depolymerizing condition was subjected to immuno-adsorption assay using anti-βm antibody and protein-A Sepharose, the immunoadsorbed aggregates contained βm-, β- and γ-actin. This indicates that the actin fraction was not completely depolymerized and contained βm-actin-containing oligomers, which were too small to be precipitated with anti-βm-actin antibody alone. The incomplete depolymerization of the βm-actin-containing fraction was also suggested by the much lower DNase 1 inhibition activity of the βm-actin-containing fraction than that of β- and γ-actin fraction. Furthermore, a DNase 1 binding assay showed that cytoplasmic supernatant prepared from B16-F1 under a low-ionic condition contained less monomeric actin than the cytoplasmic preparation from B16-F10. These results suggested that βm-actin protein in B16 melanoma probably inhibits the dynamic conversion between the monomeric and polymerized forms of actin, leading to a decrease in cell motility and consequently the suppression of invasiveness and metastasis.

Key words: Actin — Antibody — DNase 1 — Melanoma — Metastasis

Many cellular events, such as change in cell morphology, the reorganization of intracellular structures, mitosis, and cell motility, require dynamic cytoskeletal systems. In eukaryotic cells, the microfilament system, containing mainly actin and actin-related proteins, appears to be involved in mechanochemical processes for dynamic cellular activity.[1,2] Actin isoforms have a highly conserved structure throughout evolution. In mammalian tissues, at least six isoforms have been identified, which have only 0.8–5.9% differences in their amino acid sequences.[3] The biochemical function of the actin molecule is characterized by reaction with various actin-associated proteins, such as DNase 1, myosin and so on, in addition to self-polymerization.

We found a variant actin in mouse B16 melanoma, and showed that it has the same molecular weight (43 kD) and a more acidic isoelectric point than other actin isoforms.[4] The expression of the acidic actin was inversely correlated with the disorganization of actin stress fibers and with invasiveness.[5] We cloned cDNA corresponding to the actin, which proved to be similar to that of β-actin, but with various exchanges, deletions and insertions in the nucleotide sequence.[6] The amino acid sequence deduced from the nucleotide sequence showed one difference at the 28th amino acid between this actin and β-actin, which explains the change of isoelectric point (Arg of β-actin to Leu). We named this new actin βm-actin (m; mouse, melanoma, metastasis, mutant, etc.). A transfection study showed that the expression of exogenous βm-actin in highly metastatic B16-F10 and BL6 increased the organization of actin stress fibers concomitantly with the suppression of motility, invasiveness and metastasis.[7,8] The biochemical role of βm-actin protein still remains to be clarified in order to explain those phenotypic alterations seemingly attributable to βm-actin.

Variant actins can provide us with useful information on the structure-function relationship of actin isoforms. Several variant actins having amino acid exchanges in various sites have been reported in mammalian cell lines.[9–11] Those actins have some functional deficiencies in comparison with normal actin.[12]

We prepared a specific antibody against the oligopeptide including the 28th amino acid, which differs between βm- and β-actin as already noted. Using this antibody as a tool for the biochemical analysis of βm-actin, we examined several biochemical aspects of the

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βm-actin protein to understand better the mechanism of the biological effects of βm-actin.

MATERIALS AND METHODS

Antiserum preparation The peptide H_3N-LysAlaGly-PheAlaGlyAspAspAlaProLeuAlaValPheProSerIleVal-GlyArg-COOH, containing the leucine by which βm-actin differs from β-actin, was kindly provided by Dr. H. Okazaki (Hoechst, Tokyo). The peptide (1 mg) was conjugated with bovine serum albumin (2 mg) in 0.5 ml of 50 m*M* carbonate buffer (pH 8), using bis(sulfosuccinimidyl)suberate (final 10 m*M*) as a cross linker of the amino residue (30°C for 2 h). The reaction mixture was applied to Sephadex G-25 to remove free cross-linker molecules. The conjugation as checked by SDS-PAGE.[2] The prepared conjugate (0.5 mg) was mixed with TDM emulsion (RIBI ImmunoChem Research, Inc., Hamilton, USA), and was then subcutaneously injected two times into white New Zealand rabbits for immunization at an interval of 1 week. One month after the first injection, 0.2 mg of the peptide conjugate was intravenously injected. The collection of blood was started one week after the intravenous injection and the activity was examined at 5-day intervals for one month. Reactivity of the rabbit serum with the peptide oligomer used for the immunization was assessed using an ELISA system. The peptide (0.1 μg/well) was used as the antigen to titrate the antibody, and β-galactosidase-conjugated 2nd antibody and *ortho*-nitrophenyl-β-D-galactopyranoside were used for color development. The antibody used for the experiments was affinity-purified from rabbit antiserum, and the reactivity was neutralized with the βm peptide but not with the peptide corresponding to β peptide by ELISA.

Cells Mouse B16 melanoma cell lines, B16-F1 and B16-F10, were used in this study; their properties have been described by Fidler.[13] They were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, bicarbonate-buffered (3.7 g per liter) in a moist atmosphere of 10% CO_2 and 90% air.

Western blot analysis and cellular immunostaining The cells were harvested to prepare total protein fraction and cytoskeletal fraction, which were subjected to Western blot analysis, as previously described.[14] In the Western blot analysis, we used the prepared rabbit anti-βm antibody as already noted, or mouse monoclonal anti-actin antibody[15] (Amersham Japan, Tokyo), which reacts with all isoforms of actin, as the 1st antibody, and peroxidase-conjugated goat anti-rabbit Ig antibody (Medical & Biological Labs. Co., Ltd., Nagoya) or peroxidase-conjugated goat anti-mouse IgM antibody (Medical & Biological Labs. Co., Ltd.) as the 2nd antibody, respectively.

The cells were cultured overnight on cover slips (1 cm × 1 cm) coated with 10 μg of Matrigel under the culture conditions described above. The cells were washed twice with NaCl solution [0.13 *M* NaCl, 2.7 m*M* KCl, 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4 (pH 7.5)] and permeated with cold 1% Triton X-100 in the NaCl solution for 5 min at room temperature. The permeated cells were washed with the NaCl solution containing 0.1% Triton X-100, fixed with cold methanol for 10 min and then dried to remove methanol. The antiserum to βm peptide was diluted to 1:200 with NaCl solution containing a 0.1% blocking reagent (Boehringer Mannheim, Yamanouchi Company, Tokyo), and it was then poured onto the cells and incubation was continued for 1 h at room temperature. Double staining was performed by further adding the monoclonal anti-actin antibody diluted 1:100 with the NaCl solution for 1 h at room temperature. After three washings of the cells with the NaCl solution, the cells were treated with rhodamine-conjugated goat anti-rabbit Ig (Organon Teknika Corp., Durham, USA), and for double staining, FITC-conjugated goat anti mouse Ig2a (Organon Teknika Corp.), diluted 1:1000 with the NaCl solution containing 0.1% blocking reagent for 1 h at room temperature. The cells on the cover slips were rinsed with the NaCl solution and were then mounted with a 1:9 mixture of glycerol and the NaCl solution containing 1% 2-mercaptoethanol. The fluorescence of the cells was examined with a Zeiss Axio Scope and photographs were taken on Kodak Tri-X 100 film.

Actin preparation B16-F1 cells (5×10^5) were inoculated into the thigh of 10 mice (C57BL/6). When tumors had grown to about 1 cm^3, they were excised and homogenized with a homogenizer equipped with a Teflon pestle in 100 ml of 2 m*M* Tris-HCl (pH 8.0) and 0.2 m*M* Na_2ATP (G-buffer), containing protease inhibitors, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml trypsin inhibitor, and 1 m*M* p-APMSF. The homogenate was centrifuged at 100,000*g* for 90 min. The precipitate was reextracted with G-buffer and centrifuged. The combined supernatant was applied to a DE-52 (Whatman International, Maidstone Kent, UK) column (1.6×30 cm) equilibrated with 10 m*M* imidazole-HCl (pH 8.0), 0.5 m*M* ATP, 0.05 m*M* $MgCl_2$, 0.2 m*M* DTT and the protease inhibitors, and then washed with the buffer containing 0.1 *M* KCl. Elution was carried out with the buffer containing a gradient from 0.1 *M* to 0.4 *M* KCl.

Eluates of actin fractions were examined by ELISA using anti-βm serum and anti-actin antibody. A fraction containing β- and γ-actin alone was first eluted at about

[2] The abbreviations used are: SDS-PAGE; polyacrylamide gel electrophoresis, ELISA; enzyme-linked immunosorbent assay, DTT; dithiothreitol, p-APMSF; (*p*-amidinophenyl)methanesulfonyl fluoride, FITC; fluorescein isothiocyanate.

0.23 *M* KCl, and a fraction containing βm-actin was eluted with β- and γ-actin at about 0.25 *M* KCl. The two fractions were dialyzed against G-buffer overnight and concentrated with a Centriprep-10 concentrator (Amicon Grace Company). The actin fractions were applied to a Sephadex G-200 column. The eluted actin fractions were held under a polymerizing condition of 0.15 *M* KCl, 1 m*M* $MgCl_2$ at 30°C for 30 min and then at 4°C overnight. The polymerized actin obtained by ultracentrifugation (100,000*g* for 1.5 h) was dialyzed against G-buffer for 48 h and depolymerized. The average yields of βm-actin fraction and β- and γ-actin fraction were about 0.5 mg and 0.25 mg from 10 g of tumors, respectively.

Assay of polymerization and depolymerization of actin The finally dialyzed actin fractions showed only actin proteins on two-dimensional gel electrophoresis. Protein staining was carried out with Coomassie Brilliant Blue R-250 as described previously.[5] The dialyzed fraction was used for the following experiments. The purified actin fractions (0.5 mg/ml) were subjected to polymerization in 0.15 *M* KCl, 10 m*M* Tris-HCl (pH 8.0), 10 m*M* $MgCl_2$ at 4°C overnight. After ultracentrifugation (100,000*g* for 1.5 h) of the polymerized actin fractions, the precipitates and supernatants were analyzed by two-dimensional gel electrophoresis. Aliquots of the precipitates were dialyzed against G-buffer for depolymerization and were centrifuged under the same conditions. The precipitates and supernatants were also analyzed using two-dimensional gel electrophoresis.

Each actin fraction (0.5 mg/ml) under the polymerizing or depolymerizing condition was mixed with anti-βm antibody alone and incubated for 1 h at 30°C, then centrifuged at 2,500*g* for 10 min. The pellets and supernatants were again subjected to two-dimensional gel electrophoresis.

To examine the existence of oligomers, not precipitated with anti-βm-actin antibody alone, the purified actin fractions (23 μg) dialyzed against G-buffer for 48 h were reacted with 3 μl of anti-βm antibody at 37°C for 30 min, and then reaction mixtures were mixed with 5 μl of protein-A Sepharose 4B and further incubated for 1 h for immunoadsorption. The preparations were centrifuged at 3,000*g* for 15 min to precipitate protein A Sepharose 4B, which was washed four times with 10 m*M* Tris-HCl (pH 9.0) containing 0.9% NaCl. The pellet was dissolved in O'Farrell's lysis buffer and boiled for 15 min, and then subjected to two-dimensional gel electrophoresis and Western blot analysis.

Interaction of actin with DNase 1 A DNase 1 inhibition assay was carried out according to the method of Bilkstad[16] with slight modifications as follows. Two μl of DNase 1 solution, 0.2 mg/ml DNase 1 in 5 m*M* imidazole-HCl (pH 7.0) containing 0.2 m*M* $CaCl_2$, was mixed with 2–10 μl of actin solution (2–160 μg) in G-buffer. Then 400 μl of DNA solution, 40 μg/ml salmon sperm DNA (Sigma Type I) in 5 m*M* imidazole-HCl (pH 7.0) containing 2 m*M* $MgCl_2$ and 1 m*M* $CaCl_2$, was added to the reaction solution and quickly mixed, and the change of absorbance at 260 nm was monitored with a photometer. All reagents and biochemicals used were of analytical grade.

A DNase 1 column was prepared by conjugating 20 mg of DNase 1 (Boehringer Mannheim, Yamanouchi) to 1 ml of Affigel-10 (BioRad Laboratories, Richmond, USA).[17] B16-F1 and B16-F10 cells (3×10^5 cells) were harvested and homogenized with 1 ml of G-buffer, containing protease inhibitors, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml trypsin inhibitor, and 1 m*M* p-APMSF. The homogenates were centrifuged at 100,000*g* for 1.5 h. One ml of the supernatant was applied to a DNase 1 column, which was washed with 1 volume of G-buffer. The column was washed with 0.75 *M* guanidine-HCl (pH 8.0) and eluted with 3 *M* guanidine-HCl (pH 8.0) in G-buffer. The unbound fraction and 3 *M* guanidine-HCl-eluted fraction were dialyzed against G-

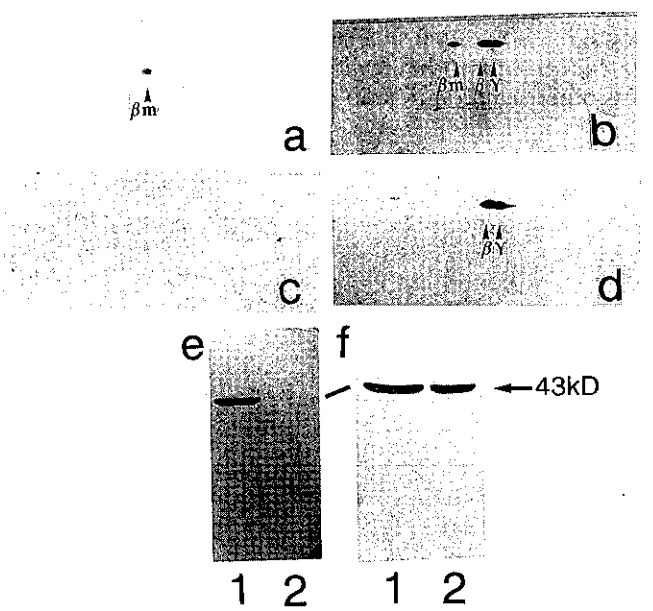

Fig. 1. Western blot analysis with the antibody (anti-βm-actin antibody) against oligopeptide of βm-actin. Total cellular protein (a, b, c, d) and cytoskeletal protein (e, f), prepared from B16-F1 (a, b, e-lane 1, f-lane 1) and B16-F10 (c, d, e-lane 2, f-lane 2), were separated by two-dimensional gel electrophoresis or SDS-PAGE, and electroblotted. Western blots were stained with anti-βm-actin antibody (a, c, e) and a common anti-actin antibody (b, d, f) as described in "Materials and Methods." An arrow at 43 kD indicates the molecular weight of actin. Prestained SDS-PAGE standards (BIO-RAD) as molecular size markers were used to assess the quality of an electrophoretic transfer.

buffer and 10 μl of the sample was subjected to SDS-PAGE and Western blot analysis, using anti-actin antibody which reacts with all actin isoforms.

RESULTS

Preparation of antibody against βm-actin The synthesized oligopeptide specific to βm-actin was reacted with bovine serum albumin and the preparation was electrophoresed in SDS-polyacrylamide gel. A smear band was observed at a higher position than that of untreated serum albumin, indicating that the peptide was conjugated with most of the molecules of albumin (data not shown). Production of antibody to the oligopeptide in an immunized rabbit was confirmed with an ELISA system using the rabbit serum and the oligopeptide as the antigen (data not shown).

To examine the reactivity of the antiserum to actin molecules, we carried out Western blot analysis in comparison with a common anti-actin antibody, which cross-reacts with all six isoforms of actin. As shown in Fig. 1, the antiserum against the βm peptide stained a single spot at the position of βm-actin monomer on the blot of the two-dimensional gel electrophoresis in a B16-F1 cell extract, but no protein spot reacted with this antibody in an extract from B16-F10 cells not expressing βm-actin. On the other hand, anti-actin antibody stained all the actin protein spots corresponding to βm-, β- and γ-actin in B16-F1 as well as, β- and γ-actin in B16-F10, as previously described. Fig. 1e shows that the antiserum reacted with βm-actin protein in cytoskeletal fractions from B16-F1 cells.

Immunostaining To examine the reactivity of the antiserum (anti-βm antibody) and the localization of βm-actin *in vivo*, we carried out immunostaining of B16 melanoma cell lines (Fig. 2). Soluble fractions were removed from the B16-F1 and F10 cells by extraction with 1% Triton X-100. The remaining cytoskeletal fractions of the cells were subjected to the immunostaining. Fibrous structures like actin stress fibers in B16-F1 were clearly stained with anti-βm antibody. The structures stained with this antibody were also stained with the common anti-actin antibody as described later (Fig. 3a, b), establishing those structures as microfilaments and actin stress fibers. On the other hand, no significant immunofluorescence was observed in B16-F10 cells with the anti-βm antibody. In the B16-F1 cells, the staining was mainly seen on actin stress fibers, and some thin filaments which parallel the leading edges were also observed crossing actin stress fibers.

To examine the interrelation of βm-actin with other actin isoforms, β- and γ-actin, double staining with anti-βm-actin antibody and common anti-actin antibody, which cross-reacts with all actin isoforms, was carried

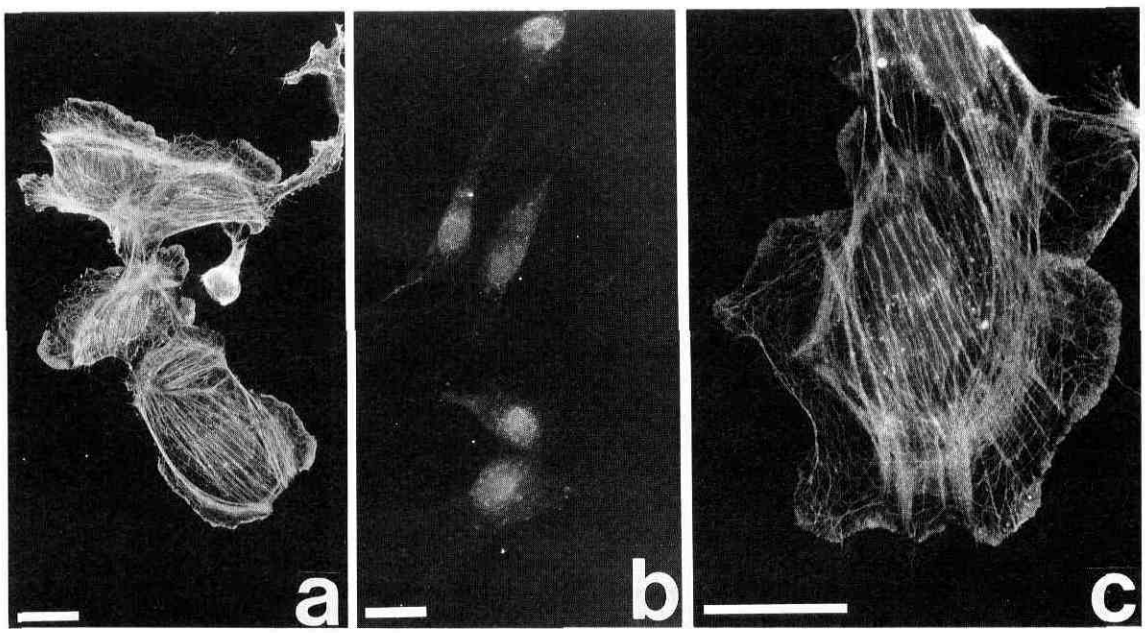

Fig. 2. Immunofluorescence staining of B16 melanoma. B16-F1 (a, c) and B16-F10 (b) were cultured on Matrigel-coated cover slips for 3 days. The cells were permeated with 1% Triton X-100 and fixed with methanol. All cells were stained with anti-βm-actin antibody and rhodamine-conjugated second antibody as described in "Materials and Methods." Bar: 10 μm.

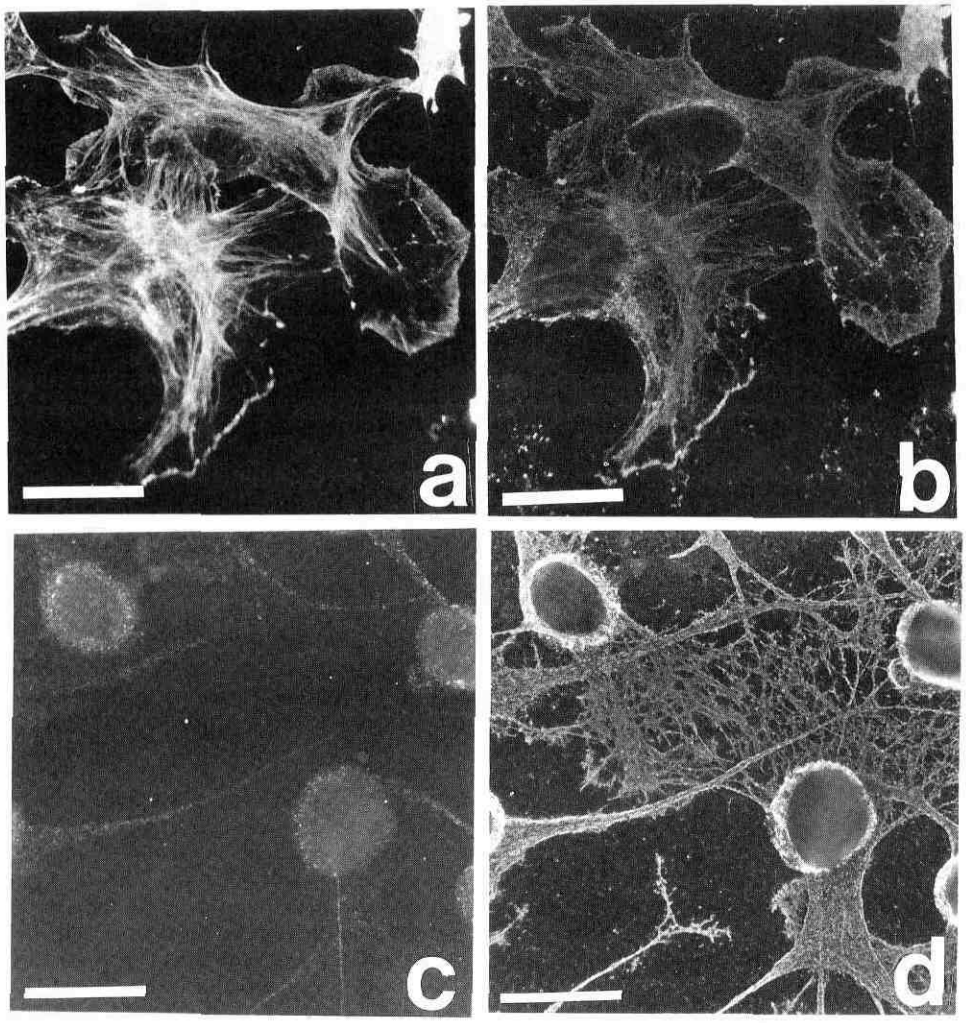

Fig. 3. Localization of βm-actin and actin fibers. B16-F1 (a, b) and B16-F10 (c, d) were cultured and fixed as described in Fig. 2. Double staining for βm-actin with anti-βm-actin antibody and rhodamine-conjugated second antibody (a, c), and for actin fibers with a common anti-actin antibody and FITC-conjugated second antibody (b, d) was carried out as described in "Materials and Methods." Bar: 10 μm.

out. Anti-βm-actin antibody and rhodamine-conjugated second antibody visualized βm-actin localized throughout a B16-F1 cell, including fibrous structures, and much the same staining pattern was observed with both common anti-actin antibody and FITC-conjugated second antibody (Fig. 3a, b). The B16-F10 cells were stained only by the common anti-actin antibody but not by the antibody specific to βm-actin (Fig. 3c, d).

These results indicated that anti-βm-actin antibody clearly reacted specifically with βm-actin in the cultured B16-F1 cells, and that βm-actin was incorporated into an actin filament together with β- and γ-actin.

Polymerization The β- and γ-actin fraction was separated from βm-actin fraction by DE-52 column chromatography. These actin fractions were further purified by gel filtration chromatography. The βm-actin fraction contained βm-actin and β- and γ-actin as well (Fig. 4a). On the other hand, β- and γ-actin fraction contained only β- and γ-actin (Fig. 4b). When the βm-actin fraction was held under a polymerizing condition, the precipitate obtained by ultracentrifugation (100,000*g* for 1.5 h) contained βm-, β- and γ-actin in the same ratio as observed in the original fraction (Fig. 4c). The precipitate was again placed under a depolymerizing condition in G-buffer for 48 h. The βm-actin and other actins were not precipitated by ultracentrifugation (Fig. 4d), indicating that they had been depolymerized to monomer and/or oligomer form. The same experiments using β-

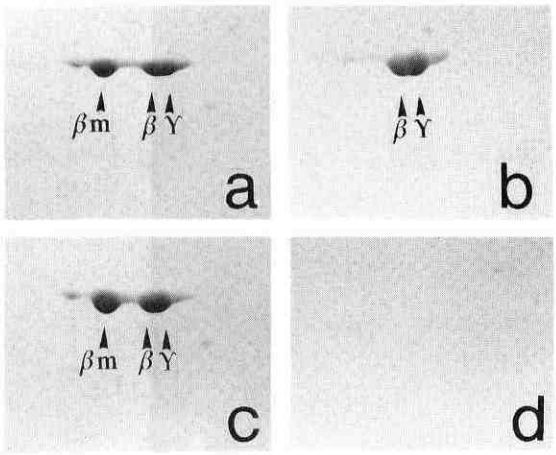

Fig. 4. Polymerization of βm-actin. Actin fractions were purified from B16-F1 tumors. The β- and γ-actin fraction (b) was separated from the βm-actin-containing fraction (a) on column chromatography. The purified βm-actin fraction was held under a polymerizing condition, and the precipitate (c) was obtained by ultracentrifugation. The precipitate was then placed under a depolymerizing condition and again ultracentrifuged to obtain precipitates (d), as described in "Materials and Methods." The precipitates were analyzed by two-dimensional gel electrophoresis and then stained with Coomassie Brilliant Blue.

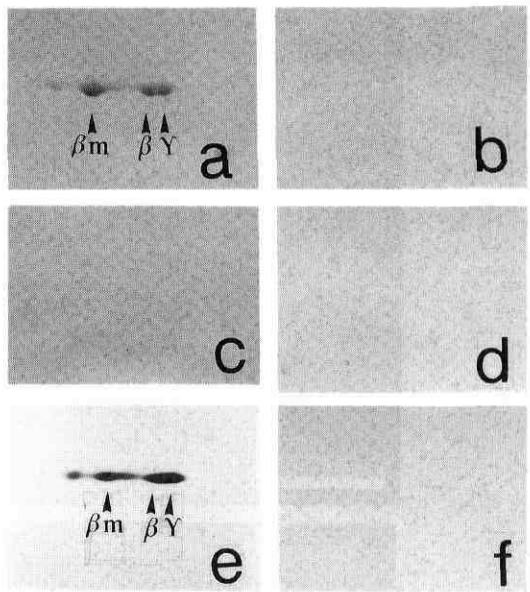

Fig. 5. Immunochemical analysis of βm-actin under various conditions. Actin fraction purified from B16-F1 tumors was also used for this experiment as shown in Fig. 4. An immunochemical analysis using anti-βm-actin antibody was performed with βm-actin-containing fractions (a, c, e) as well as β- and γ-actin fractions (b, d, f). Immunoprecipitation: the antibody was added to each actin fraction under polymerizing conditions (a, b) and depolymerizing conditions (c, d). After the immunoreaction, the immunoprecipitates were recovered by centrifugation at 2,500*g* for 10 min. Immunoadsorption: the purified actin fractions obtained in the supernatant at 100,000*g* for 1.5 h under regular depolymerizing conditions were mixed with anti-βm-actin antibody and protein A-Sepharose 4B (e, f). The immunoadsorbed materials were then recovered by centrifugation at 3,000*g* for 15 min. The immunocomplexes were analyzed by Western blot analysis using a common anti-actin antibody as described in "Materials and Methods."

and γ-actin from B16-F10 cells indicated that the actin fraction also had the ability to polymerize and depolymerize (data not shown).

To examine whether βm-actin forms a filament independently of β- and γ-actin or in a mixed form with those actins, the formed fibrous actin was precipitated with anti-βm-actin antibody as the immuno-complex (Fig. 5a, b). The immunoprecipitates contained βm-, β- and γ-actin as shown in Fig. 5a, indicating that βm-actin was polymerized together with β- and γ-actin. As a control, β- and γ-actin (Fig. 4b) were subjected to immuno-precipitation with anti-βm-actin antibody, but no actin was precipitated (Fig. 5b). Under the conditions used for immunoprecipitation, βm-actin in a regular depolymerizing solution, G-buffer, was not precipitated with anti-βm-actin antibody alone (Fig. 5c).

To examine further whether the βm-actin fraction was depolymerized to monomer or oligomer under the regular depolymerizing condition, the βm-actin-containing fraction in G-buffer was applied to an immunoadsorption assay with protein A Sepharose 4B in addition to anti-βm-actin antibody. The actin fractions were mixed with anti-βm-actin antibody and then recovered by the addition of protein-A Sepharose 4B followed by centrifugation at 3,000*g* for 10 min. The recovered preparation was analyzed by two-dimensional gel electrophoresis and Western blot analysis using the common monoclonal anti-actin antibody. From βm-actin fractions, βm-, β- and γ-actin were detected in the immunoadsorbed precipitate (Fig. 5e). From β- and γ-actin fractions, no actin was precipitated by immunoadsorption using anti-βm-actin antibody and protein A Sepharose 4B (Fig. 5f). These results indicated that in the βm-actin-containing fraction under regular depolymerizing conditions, there exist oligomer forms of βm-, β- and γ-actin, in other words, the βm-actin fraction tends not to undergo complete depolymerization.

Interaction with DNase 1 We assessed the DNase 1-inhibiting activity of actin, which is specific to the monomeric form of actin. The βm-actin fraction was about one-tenth as active as the β- and γ-actin-containing fraction under regular depolymerizing conditions (Fig. 6).

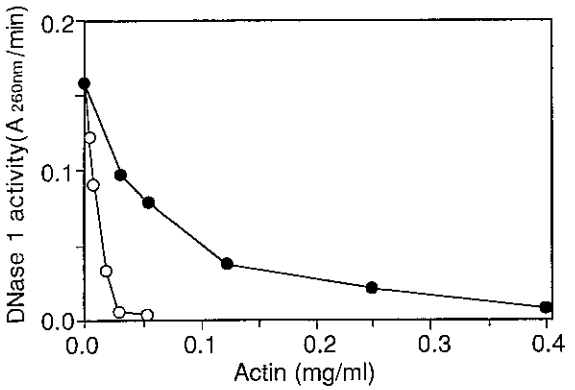

Fig. 6. DNase 1 inhibition assay with purified actin fractions. The purified actin fractions under regular depolymerizing conditions were added to the DNase 1 reaction mixture (final concentrations of actins: 5 μg/ml–0.4 mg/ml), and then deoxyribonulease activity was measured as the change of absorbance at 260 nm, as described in "Materials and Methods." Closed circles: βm-actin fraction, open circles: β- and γ-actin fraction.

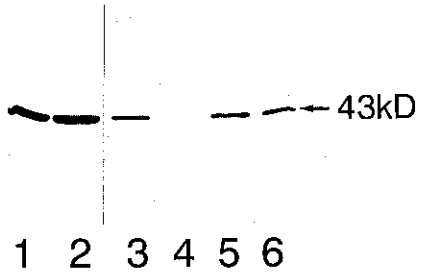

Fig. 7. Interaction of cytosolic actin with DNase 1 column. Cytosolic supernatants prepared from B16-F1 (lanes 1, 3, 5) and B16-F10 (lanes 2, 4, 6) with G-buffer were applied to a DNase 1 column. The unbound fraction (lanes 3, 4), 3 *M* guanidine-eluted fraction (lanes 5, 6), and total homogenate (lanes 1, 2) were dialyzed and subjected to both SDS-PAGE and a Western blot analysis, using anti-actin antibody which recognizes all actin isoforms.

We also carried out a DNase 1 binding assay of cytosolic actins to examine and compare the state of the cytosolic actins between B16-F1 and B16-F10 (Fig. 7). The ultracentrifugation supernatant of the cytosolic fractions in a low ionic strength G-buffer was applied to the DNase 1 column. Most of the β- and γ-actin from B16-F10 cells was bound to the DNase 1 column, indicating that β- and γ-actin exist mainly in monomeric form in the cytosolic supernatant. On the other hand, a portion of the cytosolic actin fractions containing βm-actin did not bind to the DNase 1 column under the same depolymerizing conditions.

DISCUSSION

The antibody raised against an oligopeptide containing the putative variant 28th amino acid specifically reacted with βm-actin on Western blot analysis and cellular immunostaining. This indicates that the peptide sequence deduced from the nucleotide sequence of the cDNA is actually present in the βm-actin protein. Using the polymerase chain reaction technique, we also identified the nucleotide sequence of the 28th codon corresponding to both βm- and β-actin in the genomic DNA of B16-F1 (data not shown). Whether βm-actin gene is a mutant of the β-actin allele or a duplicated gene is under investigation.

Cellular immunostaining (Fig. 3) and immunoprecipitation of *in vitro*-polymerized actin with anti-βm-actin antibody (Fig. 5) indicated that βm-actin forms a filament together with β- and γ-actin. On the other hand, the formed actin filaments containing βm-actin were resistant to complete depolymerization, as shown by immunoadsorption experiments using anti-βm-actin antibody and protein-A Sepharose (Fig. 5e). This is consistent with the results of DNase 1 inhibition activity, which is specific to depolymerized actin; the βm-actin-containing fraction under a regular depolymerizing condition showed a much lower DNase 1-inhibiting activity than β- and γ-actin fraction did. An analysis of the functional domain of the actin molecule concluded that the 28th amino acid is not involved in DNase 1 binding.[18] Our previous study showed that actin monomer synthesized *in vitro* from its cDNA could bind strongly to the DNase 1 column. Therefore, the low DNase 1 inhibition activity of the βm-actin-containing fraction can probably be attributed to the incomplete depolymerization of the actin fraction rather than to a lower DNase 1 affinity of βm-actin monomer as compared with that of β- and γ-actin monomers. The existence of actin fractions which did not bind to the DNase 1 column in the cytosolic preparation of B16-F1 under a low salt condition (Fig. 7) also suggests that βm-actin tends to form actin oligomers with other actin isoforms in the cells, though we could not exclude the possibility of association of βm-actin with other actin-related proteins.

The reluctance of βm-actin to undergo complete depolymerization remains to be explained at the molecular level. The 28th amino acid has not been shown in previous studies to be important for polymerization. However, the substitution of Arg at the 28th site to Leu would probably induce an alteration in the three-dimensional structure of the actin molecule; this seems not to affect the formation of actin filaments with β- and γ-actin but may account for the incomplete depolymerization of the formed filaments. Although the 28th amino acid of actin may be an interaction site with tropomyosin

and myosin,[18-20] our preliminary study showed that the βm-actin fraction has almost normal myosin-ATPase activation activity (data not shown).

The resistance of βm-actin fraction to complete depolymerization, suggested by the present experiments, is consistent with the higher level of organized stress fibers seen in B16-F1 than in B16-F10. In fact, the expression of exogenous βm-actin resulted in an increase of actin stress fibers, as shown by our gene transfer experiments.[7,8] In the transfectants, βm-actin was distributed more in the cytoskeletal fraction than in the cytoplasmic soluble fraction.[7] Stress fibers of B16-F1 containing βm-actin are probably more rigid or less flexible due to the resistance of the filaments to depolymerization than the actin stress fibers present in B16-F10 cells expressing only β- and γ-actin. A dynamic conversion between the monomeric and fibrous state of actin molecules is necessary for cell motility.[21] Thus, it is likely that the rigidity of actin stress fibers containing βm-actin in B16-F1 cells as well as in transfectants with βm-actin is related to the decrease in cell locomotion and/or deformability, leading to the suppression of metastatic potential.

Expression of βm actin as seen in B16-F1 has not been detected in other tumor systems by Western blot analysis with the antibody against βm-actin. However, overexpression of βm-actin in those tumor cells by transfer of βm-actin cDNA should result in a reduction of cell malignancy through similar mechanisms to those described above. Establishment of transgenic mice with βm-actin using recipients producing spontaneous cancers at high frequency is under way to examine the effects of βm-actin on tumor progression *in vivo*. There are a number of actin-associating proteins that regulate the dynamic conversion between the polymerized and depolymerized state of actin molecules. Thus, quantitative and/or qualitative alterations in such actin-associating proteins could induce similar effects to those of βm-actin in B16 melanoma. Recently, suppressive effects of such molecules on cell malignancy have been reported by several groups based on gene transfer experiments.[22-24]

We previously reported that the expression of vinculin, which is a membrane-associated protein seemingly involved in anchoring actin filaments to the membrane, was closely correlated with the expression of βm-actin in B16 melanoma, and furthermore with the expression of smooth muscle α-actin, which is also inversely correlated to malignancy in the rat 3Y1 cell system.[25] The integrity of actin stress fibers was associated with the expression of third actins (in terms of expression besides β- and γ-actins), and vinculin in both B16 melanoma and 3Y1 cells. Vinculin is known to be present at the focal contact and cell adherence junction where the actin stress fibers terminate.[26,27] The termini of the thick actin stress fibers in B16-F1 cells were certainly colocalized with broad and concentrated vinculin spots at the focal contact, when observed by double staining with anti-vinculin antibody and rhodamine-phalloidine. The small patch of vinculin at the focal contact was seen to correspond to the thin actin fibers in B16-F10.

Since the direct interaction of actin filaments with vinculin has not been reported, the interaction of actin, in particular βm-actin, with vinculin through unknown mediating molecules still remains to be examined. Recently, we reported that the elongation factor 1α, which has been identified as an actin-binding protein, ABP50 of *Dictyostelium*,[28] was more highly expressed in B16-F10 than in B16-F1.[29] We also found a higher expression of low-molecular-weight tropomyosin in B16-F10 than in B16-F1 (K. Miyado *et al.*, manuscript in preparation).

The tropomyosin isoform was reported as a molecule related to malignant phenotypic changes, and having an activity to make actin filaments unstable.[30] Study of the interaction of βm-actin with low-molecular-weight tropomyosin and elongation factor 1α as well as other actin-associating proteins may therefore improve our understanding of the role of βm-actin in the regulation of the cytoskeletal system, cell motility and the suppression of metastasis.

ACKNOWLEDGMENTS

We thank Drs. B. T. Quinn and M. Katsuki for their critical readings of and discussions regarding this manuscript. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan, by a grant from the Princess Takamatsu Cancer Research Fund and by special coordination funds for promoting science and technology from the Science and Technology Agency, Japan. We also thank Misses M. Kimura and K. Fukuda for their technical assistance.

(Received January 17, 1994/Accepted April 7, 1994)

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