

# Newly identified type of $\beta$ actin reduces invasiveness of mouse B16-melanoma

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Low metastatic parent B16 melanoma and isolated B16-F1 cell lines have a third actin designated as  $\beta m$  ( $A^X$ ; previously).  $\beta m$  actin is scantily or not at all detected in highly metastatic cell lines, such as B16-F10 and BL6. To directly assess the physiological role of  $\beta m$  in phenotypic changes of B16 melanoma, we transfected expression plasmids of  $\beta m$  into B16-F10 cells. The actin expressed in the transfectants is located largely in cytoskeletal fractions. The transfectants exhibited a larger number of stress fibers and a lower invasiveness than did the recipient cells. Thus,  $\beta m$  actin plays an important role in the organization of actin stress fibers, the result being a decrease in invasiveness of B16 melanoma.

Actin; DNA transfer; Stress fiber; Cytoskeleton

## 1. INTRODUCTION

A third actin ( $\beta m$ ) detected in mouse B16 melanoma is a newly identified type of cytoskeletal actin [1,2]. The amino acid sequence of this actin, determined by the nucleotide sequence of the cDNA, differed from  $\beta$  actin at the 28th amino acid ( $\beta$ , arginine;  $\beta m$ , leucine) (Fig. 1A), and at five amino acids from  $\gamma$  actin. In the nucleotide sequence of  $\beta m$  cDNA, there were two sites with one base change, one site of one base insertion and four regions of deletion compared with hitherto known mouse  $\beta$  actin [3]. Therefore,  $\beta m$  actin is closely related to  $\beta$  actin, which consists of a multiple gene family containing active and pseudogenes [4,5].  $\beta m$  was co-expressed with  $\beta$  and  $\gamma$  actins in parent B16 melanoma and the B16-F1 cell lines, both of which are low metastatic, yet little or not at all in the highly metastatic B16-F10 cell line, indicating inverse correlation of  $\beta m$  actin expression and metastatic ability of B16-melanoma [1,6]. Previously, mutant  $\beta$  actin which has one amino acid exchange at the 244th amino acid (mutant  $\beta$ , aspartic acid;  $\beta$ , glycine) was reported in reference of tumor progression and disorganization of cytoskeleton [7–9]. Leavitt et al. [10,11] showed that the expression of mutant  $\beta$  actin in human fibroblast KD cells caused tumor progression, by gene transfer experiments of mutant  $\beta$  actin DNA. The site of amino

acid exchange is different between  $\beta m$  and mutant  $\beta$  actin, which may correspond to inverse function in the organization of cytoskeleton and tumor phenotypes. To investigate the physiological role of  $\beta m$  actin, we transfected  $\beta m$  actin cDNA into highly metastatic B16-F10 cells expressing no  $\beta m$  actin and analyzed the nature of the transfectants.

## 2. MATERIALS AND METHODS

### 2.1. Cells and DNA transfection

B16-F1 and F10, the origin and properties of which were described by I.J. Fidler [12], were grown in Eagle's minimum essential medium (MEM, Nissui) supplemented with 10% fetal bovine serum (FBS, Gibco), bicarbonate-buffered (3.7 g/liter) in a moist atmosphere of 10% CO<sub>2</sub> and 90% air. An expression vector was constructed from  $\beta m$  actin cDNA and 5' UTR region containing human  $\beta$  actin promoter [13] and pUC18 (pH $\beta$ A). Another one was constructed from pBR322 plasmid containing mouse mammary tumor virus LTR [14], inducible by dexamethasone,  $\beta m$  actin cDNA and Eco gpt gene (pMMA). As the Eco gpt gene was not effective as a genetic marker in B16-F10 cells, pSV2-neo (1  $\mu$ g) was co-transfected with the plasmid (10  $\mu$ g) into B16-melanoma F10 cells (10<sup>5</sup> cells/dish) by the calcium phosphate precipitation method. G-418 (600  $\mu$ g/ml) selection was performed for 7 days and independent clonal G418<sup>r</sup> cells were isolated. Total proteins and Triton X-100 insoluble cytoskeletal fractions were obtained from 10<sup>7</sup> transfectants, and then two dimensional gel electrophoresis and Western blot analysis, and rhodamine-phalloidin staining was performed as described previously [6].

### 2.2. In vitro invasion analysis

In vitro invasion assay was carried out by the method of Albini et al. [15] using polycarbonate filters, with 8  $\mu$ m pore size (Nuclepore, CA), which were coated with 50  $\mu$ g of the basement membrane matrigel.

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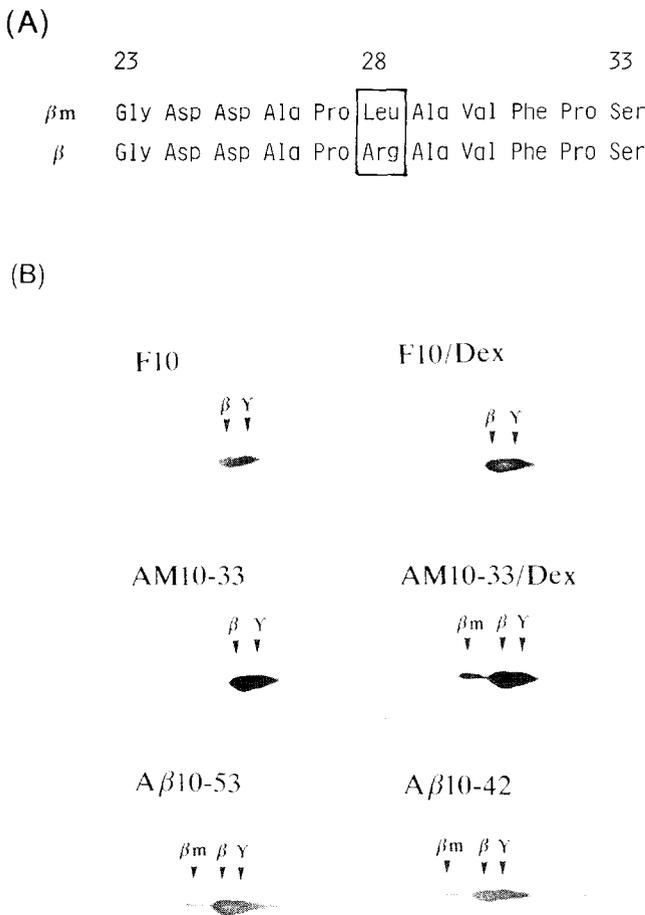


Fig. 1. (A) The amino terminal side sequences of mouse  $\beta m$  actin containing a different amino acid from  $\beta$  actin, whereas the carboxyl side sequences are the same as  $\beta$  actin. (B) Expression of  $\beta m$  actin in the transfectants (B16-F10) carrying  $\beta m$  cDNA grown with or without  $5 \mu M$  dexamethasone for 3 days. Cells ( $10^7$ ) were harvested and treated with 9 vols of cold acetone. The acetone powder was dissolved in O'Farrell's lysis buffer, and the sample corresponding to  $10^6$  cells was analyzed with two dimensional gel electrophoresis and Western blot immunostaining, using anti-actin antibody and peroxidase conjugated second antibody. The AM10-33 clone is a clonal transfectant with pMMA, the A $\beta$ 10-53 and 42 were independent clones transfected with pH $\beta$ A.

### 3. RESULTS

The transfectants were cloned and the actin expression was examined by Western blot analysis, using anti-actin antibody. Independent clones, A $\beta$ 10-42 and A $\beta$ 10-53 carrying pH $\beta$ A plasmid expressed  $\beta m$  actin (Fig. 1B). In clone AM10-33 carrying pMMA,  $\beta m$  actin was induced only when cultured with  $5 \mu M$  dexamethasone, but not without dexamethasone (Fig. 1B). Exogenous plasmid DNA containing  $\beta m$  cDNA in these three clones was detected as extra DNA bands by Southern blot analysis (data not shown). Since B16-F10

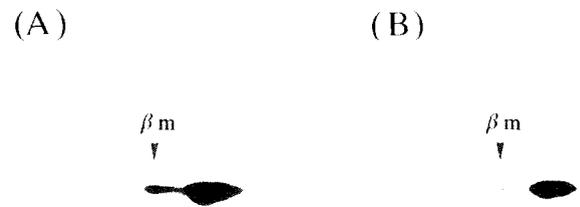


Fig. 2. Distribution of  $\beta m$  actin in the transfectants.  $10^7$  cells were harvested and extracted with PBS-buffer containing 0.5% Triton X-100 at  $4^\circ C$  for 10 min, and Triton X-100 insoluble cytoskeletal fractions (A) and soluble fractions (B) were prepared and dissolved in equal volumes of lysis buffer. The samples corresponding to  $10^6$  cells were used for two dimensional gel electrophoresis and Western blot analysis with anti-actin antibody.

cells did not express  $\beta m$  actin irrespective of treatment with dexamethasone (Fig. 1B), the  $\beta m$  actin expression in B16-F10 transfectants was derived from the exogenous DNA plasmid. Densitometric measurement showed that the relative proportion of  $\beta$  to  $\gamma$  actin ( $\beta/\gamma$ ) was unchanged between F10 and transfectants (data not shown).

To investigate the distribution of  $\beta m$  actin in the transfectants, we isolated the cytoskeletal fractions and Triton X-100 soluble fractions. Fig. 2A shows that  $\beta m$  actin expressed in AM10-33 cultured with  $5 \mu M$  dexamethasone located more in cytoskeletal fractions than in the Triton X-100 soluble fractions (Fig. 2B) as compared with  $\beta$  and  $\gamma$  actin. The amounts of  $\beta$  and  $\gamma$  actins in cytoskeletal fractions were slightly larger than in soluble fractions, but the ratio of  $\beta/\gamma$  actin in both fractions was much the same. In other transfectants, A $\beta$ 10-42 and A $\beta$ 10-53, the same phenomena were found (data not shown).

We investigated the cytoskeletal organization of B16-F10 transfectants expressing  $\beta m$  actin. AM10-33 cultured with dexamethasone and A $\beta$ 10-53 showed more actin stress fibers than the non-induced cells and recipient B16-F10 cells (Fig. 3). The induction of  $\beta m$  and increase in actin stress fibers were reversible phenomena depending on dexamethasone treatment (data not shown).

To investigate the effect of  $\beta m$  actin on cell invasiveness, we carried out an in vitro analysis using matrigel-coated filters. AM10-33 cells cultured under  $\beta m$  inducible conditions showed a lower penetration than did the B16-F10 cells, irrespective of the treatment of the recipient with dexamethasone (Fig. 4A). A $\beta$ 10-53 and A $\beta$ 10-42 cells, constitutively expressing  $\beta m$ , also showed lower invasiveness than did B16-F10 cells. The invasiveness of A $\beta$ 10-53 and A $\beta$ 10-42 decreased to much the same level seen in the B16-F1 cells (Fig. 4B). On the other hand, F10 transfected pSV2-neo (Neo10) showed no significant reduction in in vitro invasiveness.

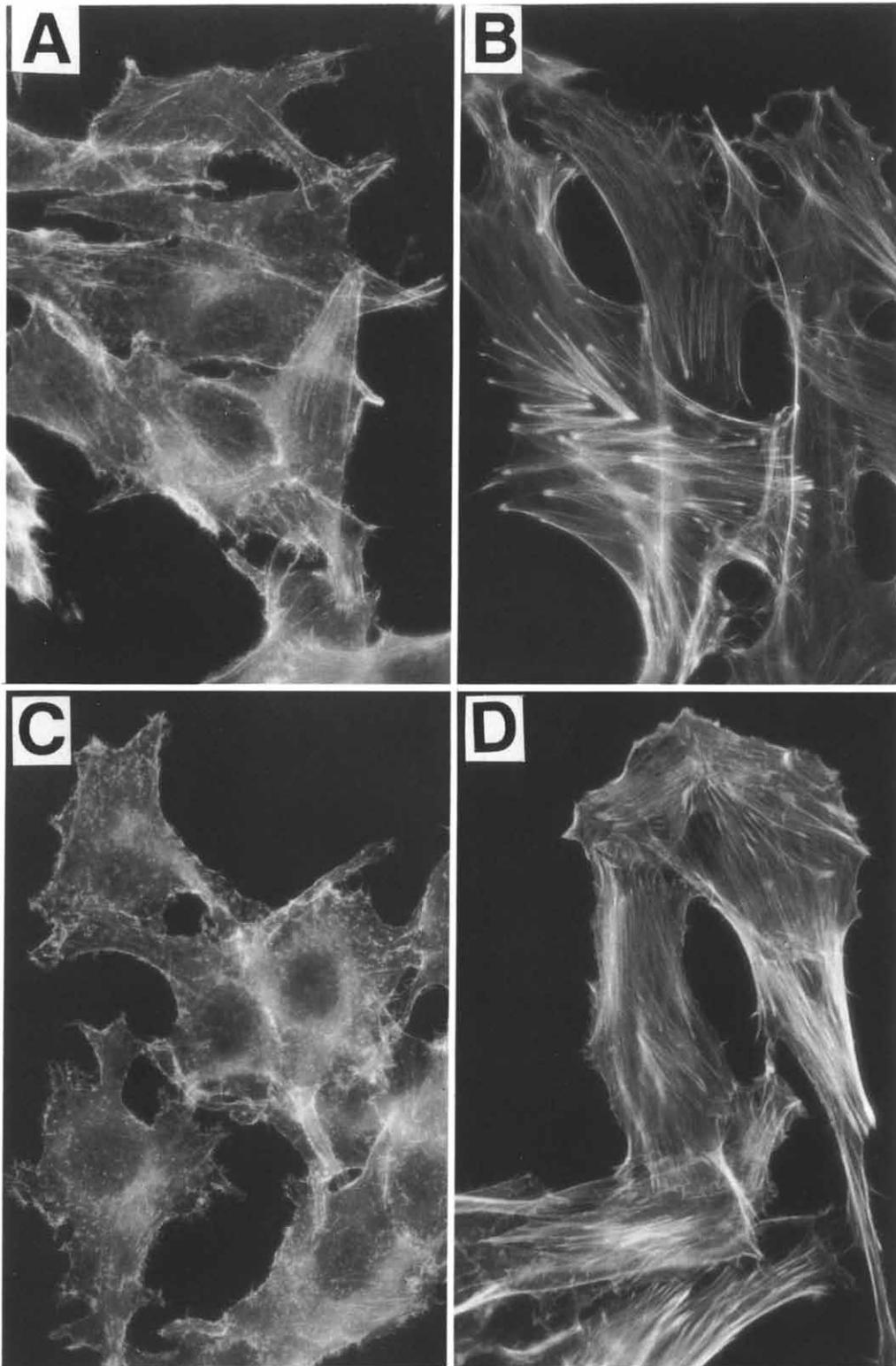


Fig. 3. Organization of actin stress fibers associated with expression of  $\beta$ m actin. The cells AM10-33 were grown without (A) or with (B) 5  $\mu$ M dexamethasone for 3 days. C, D indicated the staining of F10 and A $\beta$ 10-53, respectively. The cells were cultured on cover glasses and fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, then were made permeable with 0.1% Triton X-100 in PBS for 2 min. Staining with rhodamine-conjugated phalloidin was carried out for 30 min. After a wash with PBS, the preparations were mounted with 90% glycerol-10% PBS containing 0.1% 2 $\beta$ -mercaptoethanol and observed under a fluorescence microscope (Olympus, Tokyo).

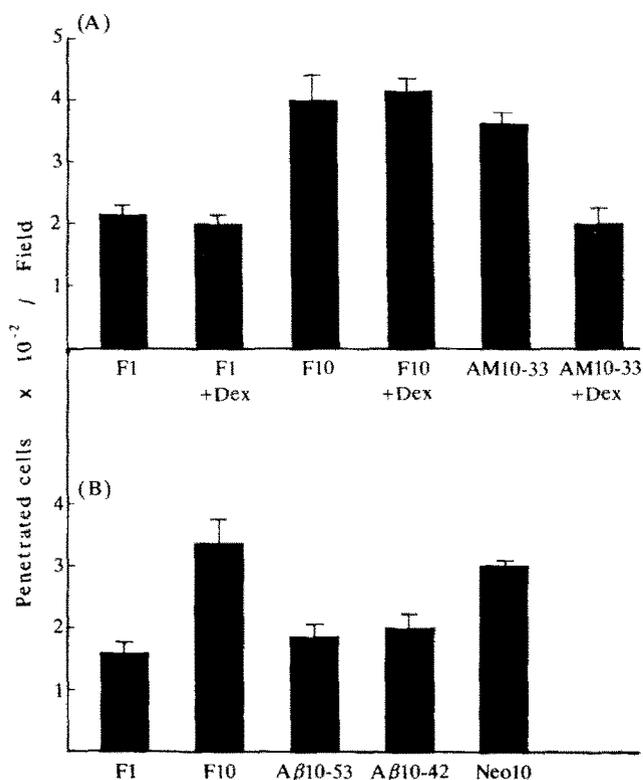


Fig. 4. In vitro invasion assay of  $\beta_m$  actin transfectants. B16-F1(F1), B16-F10(F10), and AM10-33 were cultured with (+Dex) or without 5  $\mu$ M dexamethasone for 3 days in (A). In (B), F1, F10, A $\beta$ 10-53, A $\beta$ 10-42 and pSV2-neo-transferred F10 (Neo10) were cultured and harvested. Cells ( $3 \times 10^5$ ), suspended in DMEM containing 0.1% BSA, were added to the upper compartment of Boyden chambers and incubated at 37°C in 10% CO<sub>2</sub> for 4 h. The lower compartments of the chambers were filled with conditioned medium, obtained by incubating NIH 3T3 cells for 24 h in serum-free medium containing 250 mg ascorbate per liter. The number of the cells penetrated through the matrigel coated filters was counted. Each column indicates the mean of triplicate samples; bar, SE. The number of cells per field was evaluated by Student's *t*-test. *P* value: AM10-33 + Dex versus AM10-33 and F10 + Dex, <0.01; AM10-33 versus F10 + Dex, >0.05; A $\beta$ 10-53 and A $\beta$ 10-42 versus F10, <0.01; Neo10 versus F10, >0.05.

#### 4. DISCUSSION

We reported that the B16-F1 cell line, expressing  $\beta_m$  actin at a high level, has a larger number of actin stress fibers and a lower metastatic potential than did the B16-F10 cell line not expressing  $\beta_m$  actin [6].  $\beta_m$  actin was incorporated into the cytoskeleton of B16-F1 cells as seen with  $\beta$  and  $\gamma$  actin [6], and had the DNase I binding potential seen in other native actin species [2]. Induction of the  $\beta_m$  expression derived from exogenous  $\beta_m$  cDNA was associated with increase in number of actin stress fibers and decrease in the in vitro invasion across matrigel-coated filters. Thus,  $\beta_m$  actin seems to promote construction of the stress fibers, leading to reduction of the cell motility and/or flexibility. Since there was no difference in the amount of  $\beta + \gamma$  actins between F1 and F10,  $\beta_m$  actin is an additive population in B16-F1. High efficiency of incorporation of  $\beta_m$  actin

into cytoskeletal fractions in the transfectants appears to indicate higher competence of  $\beta_m$  actin to form actin filaments than  $\beta$  and  $\gamma$  actins. We previously observed much the same efficiency in incorporation of  $\beta_m$ ,  $\beta$  and  $\gamma$  actins into cytoskeletal fraction of B16-F1. The high ratio of  $\beta_m$  actin in the cytoskeletal fraction of the transfectants might be due to intracellular conditions, which are advantageous to nuclear formations, incorporation into actin filaments and/or interaction with actin-associating proteins. A mutant  $\beta$  actin, which has one amino acid exchange (glycine,  $\beta$ ; aspartic acid, mutant  $\beta$  at amino acid 244), had less polymerizing activity in vitro and in vivo, leading to disorganization of the actin stress fibers in KD-fibroblast [7-9]. Leavitt et al. noted that transfer of the mutant  $\beta$  actin into a transformed but low tumorigenic cell line increased the malignancy and altered the organization of the microfilaments [10,11]. These inverse biochemical and biological functions of this mutant  $\beta$  actin against  $\beta_m$  actin in B16 melanoma may be attributed to the difference in the site of amino acid change. Regardless of low expression of  $\beta_m$  actin in B16-F10 transfectants, the integrity of actin stress fibers increased and the malignancy decreased, showing efficient biological functions of  $\beta_m$  actin on such cellular phenotypes. It remains to be examined whether the integrity of actin stress fibers is restored by other exogenously transferred actin isomers, such as  $\beta$ ,  $\gamma$ , or  $\alpha$  actin.

Changes of cytoskeletal proteins are associated with malignant transformation and/or progression of various cells and it is widely assumed that these changes are involved in the alteration of cell morphology, flexibility and motility [16-19]. In addition to our finding for  $\beta_m$  actin in B16 melanoma, there are reports on the depression of a third functionally normal actin associated with cell transformation. In chick embryo cells [20], and rat and mouse fibroblast cell lines [21],  $\alpha$  type actin was either decreased or disappeared in the transformants. We also found that  $\alpha$  actin identified as the smooth muscle type was expressed in a normal rat 3Y1 cell line but decreased or was absent in the malignant counterparts [22]. The  $\alpha$  actin of smooth muscle type detected in human pigment tissues tends to decrease in malignant melanoma tissues [23]. Thus, alteration in expression of cytoskeletal actin often accompanies malignant transformation and/or progression. However, the role of the  $\alpha$  type actin in non-muscle cells remains to be investigated. The functional differences among  $\beta_m$ ,  $\beta$ ,  $\gamma$  and  $\alpha$  actin are an interesting subject. Because there is no method to reproducibly separate actin isomers maintaining the native functions, a new method for isolation has to be developed.

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