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fosB Gene Products Trigger Cell Proliferation and Morphological Alteration with an Increased Expression of a Novel Processed Form of Galectin-1 in the Rat 3Y1 Embryo Cell Line

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In this study, we established rat 3Y1 embryo cell lines expressing FosB and ΔFosB as fusion proteins (ER-FosB, ER-ΔFosB) with the ligand-binding domain of human estrogen receptor (ER). The binding of estrogen to the fusion proteins resulted in their nuclear translocation. After estrogen administration, exponentially growing cells expressing ER-ΔFosB, and to a lesser extent ER-FosB, underwent morphological alteration from the flat fibroblastic shape to an extended bipolar shape, and ceased proliferating. Such morphological alteration was also induced in quiescent cells expressing ER-ΔFosB and ER-FosB after one round of cell division triggered by estrogen administration. The cells expressing ER-ΔFosB changed shape frequently, and the content of F-actin in the cytoplasm detected by binding of Alexa 488-phalloidin significantly decreased after the morphological alteration. By two-dimensional gel electrophoresis analysis of cellular proteins from the cells expressing ER-ΔFosB, we identified several proteins whose expression either increased or decreased after estrogen administration. Two of these proteins were identified from their amino acid sequences as novel processed form of galectin-1.

Key words: cell fate, differentiation, *fosB*, galectin-1, rat3Y1.

Jun and Fos family proteins are major components of AP-1 (activator protein–1) transcription factor (*1, 2*), which is known to regulate the expressions of various genes that determine different cellular fates, such as cell proliferation, differentiation and programmed cell death (*3, 4*). Of the four *fos* family genes (*c-fos*, *fosB*, *fra-1*, *fra-2*), only *fosB* forms two mature mRNAs, *fosB* and *ΔfosB*, by alternative splicing (*5*), and each of these encodes three polypeptides by alternative initiation of translation, FosB, Δ1FosB, Δ2FosB, and ΔFosB, Δ1ΔFosB, Δ2ΔFosB, respectively (*6, 7*). The proteins encoded by *ΔfosB* mRNA lack the C-terminal 101 amino acid region of the proteins encoded by *fosB* mRNA, the region that contains the motifs responsible for the interaction with TATA-box binding protein (TBP) and TFIID complex, and also for the repression of *c-fos* and *fosB* promoters (*5, 8*).

Like other Fos family proteins, *fosB* gene products form heterodimers with each of the Jun (c-Jun, JunB, JunD) proteins, thereby stimulating the latter's DNA-binding activities. The proteins encoded by *ΔfosB* mRNA, such as ΔFosB, suppress the Jun transcription-activating ability acting on AP-1 dependent promoters, while FosB dramatically enhances such activity. Furthermore, ΔFosB counteracts other Fos proteins that repress the serum-activated *c-fos* and *fosB* promoters. It is thus likely that the *fosB* gene products play an important role in the modulation of the gene expression regulated by AP-1 (*5*).

In most types of rodent tissue, *fosB* expression is either absent or barely detectable, while a basal expression of *fosB* is detected in some neurons scattered throughout the brain cortex and in the dorsal portion of the dentate gyrus in the hippocampus (*9*). The expression of the *fosB* gene, as well as *c-fos* or *c-jun*, is rapidly and transiently induced in brains in response to electrical stimulation, physiological perturbations, stress, and psychotropic drugs (*9–12*). The *fosB* gene products, especially ΔFosB, have been reported to have a potential to regulate the neuronal function as well as cell growth and differentiation (*7, 13–15*), but the downstream events and the target molecules of FosB or ΔFosB remain unknown.

We previously developed a conditional regulatory system of nuclear proteins, in which each protein is expressed as a fusion to the ligand-binding domain of human estrogen receptor (ER) (*13, 16*). Using this system, we also demonstrated that the fusion proteins (ER-FosB and ER-ΔFosB) translocate to the nucleus in the presence of estrogen, and

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Abbreviations: CBB, Coomassie Brilliant Blue R250; DMEM, Dulbecco's modified Eagle's medium; ER, estrogen receptor; FBS, fetal bovine serum; MGMT, O^6-methylguanine DNA methyltransferase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; nano-ESI, nanoflow electrospray ionization; PBS, phosphate buffered saline; 2-D, two-dimensional.

thus trigger quiescent rat1A cells, an embryonic rat fibroblast cell line, to transit G1, initiate DNA replication, and ultimately undergo cell division at least once (*13, 17*). Recently, we found that persistent expression of ER-ΔFosB but not ER-FosB in rat1A cells results in cell death (K. Tahara and Y. Nakabeppu, in preparation), indicating that ΔFosB and FosB may have a potential to regulate cell fate such as cell death and cell proliferation.

To examine whether the potential of *fosB* gene products regulating the cell fate is limited to certain types of cells, we applied the ER-fusion system to rat 3Y1 embryo cell line, in the present study. We found that expression of ER-ΔFosB and ER-FosB in the rat 3Y1 cells induced an alteration of cell morphology after one round of cell division and identified a novel processed form of galectin-1 as a potential downstream target of ΔFosB.

MATERIALS AND METHODS

Materials—Estrogen (β-estradiol) and Coomassie Brilliant Blue R250 (CBB) were obtained from Sigma. Sequencing grade trypsin was obtained from Roche (Mannheim, Germany). Prestained protein molecular weight standards, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies. Most other chemicals were obtained from Wako (Osaka). The sources of all other materials are indicated at the appropriate places in the text.

Plasmids—The expression vector pcDEBΔ carrying the SR-α promoter and hygromycin phosphotransferase (*hph*) gene, plasmids pcDEBΔ:FosB, pcDEBΔ:ΔFosB and pcDEBΔ:c-Myc, pcDEBΔ:ER-FosB, pcDEBΔ:ER-ΔFosB and pcDEBΔ:ER-MGMT have been described previously (*13, 16*).

Cell Cultures—Rat 3Y1 embryo cell line (*18*) was transfected with pcDEBΔ, pcDEBΔ:ER-FosB, pcDEBΔ:ER-ΔFosB and pcDEBΔ:ER-MGMT, and cell lines established were designated 3Y1H, 3Y1(ER-FosB), 3Y1(ER-ΔFosB), and 3Y1(ER-MGMT), respectively. The transfection of cells with plasmid DNA was done according to the method of Chen and Okayama (*19*). The cells were maintained in phenol red-free DMEM supplemented with 10% charcoal-dextran-stripped FBS, 100 μg of hygromycin B, 100 units of penicillin and 100 μg of streptomycin per ml. The number of viable cells in culture was counted using a hemocytometer after staining with trypan blue.

Western Blotting—Cell extracts were separated on SDS-PAGE and transferred onto Immobilon-P membrane (Millipore), and the membranes were subjected to Western blotting, according to a procedure described previously (*20*). Primary antibodies, anti-FosB(N) for detecting ER-FosB (*5, 13*) and anti-rhGAL-1 (*21*), were used in combination with horseradish peroxidase-labeled protein A (Amersham Pharmacia Biotech), and the antibodies bound to each blot were detected by the chemiluminescence method with ECL-Plus Kit (Amersham Pharmacia Biotech). Digitized images were obtained by LAS1000 Plus (Fuji Film, Tokyo) and processed for publication using the Adobe Photoshop 5.5J software package (Adobe Systems).

Laser-Scanning Fluorescence Microscopy—Cells cultured in collagen-coated chamber slide glass (Beckton-Dickinson) were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 10 min, followed by incubation in 0.1% Triton X-100 in PBS for 5 min, at room temperature. Cells on the slides were incubated with Alexa-488 labeled phalloidin (Molecular Probe) for 20 min at room temperature, and bound phalloidin was observed under an Axiovert 135M microscope (Carl Zeiss) equipped with a Radiance 2000 laser-scanning fluorescence microscope system (Bio-Rad). Digitized images were obtained and processed for publication using Adobe Photoshop 5.5J.

Two-Dimensional Gel Electrophoresis—Cells harvested from a 14-cm dish were lysed in 1 ml of 8 M urea, 2% Triton X-100, 10 mM DTT, 0.5% carrier ampholite (pH 3.5–9.5) (Amersham Pharmacia Biotech), and protein concentration was determined with a protein assay kit (Bio-Rad). Samples were applied overnight to Immobiline Drystrips (pH 4–7, 11 cm; Amersham Pharmacia Biotech), and isoelectric focussing was performed for a total of 60 kVh using a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech). Second-dimension SDS-PAGE was performed in 15% acrylamide gels (ExcelGel SDS Homogeneous 15; Amersham Pharmacia Biotech). The two-dimensional (2-D) gels were stained with CBB. Protein spots on the 2-D gels were detected and analyzed by use of an Image Scanner combined with Image Master 2D Elite (Amersham Pharmacia Biotech). For 2-D Western blotting analysis of galectin-1, the second-dimension SDS-PAGE was performed in 8–18% gradient acrylamide gels (ExcelGel SDS Gradient 8-18; Amersham Pharmacia Biotech), according to Lutomski *et al.* (*22*).

Amino Acid Sequencing—Proteins separated on 2-D gels were transferred onto Immobilon-P membrane and detected by CBB staining. Each protein spot was excised and subjected to Edman degradation using Procise 491cLC-1 (PE Biosystems) to determine the N-terminal amino acid sequence. For nanoflow electrospray ionization (nanoESI) mass spectrometry (MS) and tandem mass spectrometry (MS/MS), excised gel spots were destained, reduced, alkylated and digested with trypsin, and peptides were extracted with 5% formic acid in 50% acetonitrile, according to the protocol developed by Shevchenko and Shevchenko (*23*). The samples were then subjected to nanoESI-MS and MS/MS analysis using a Q-TOF2 mass spectrometer (MicroMass, Manchester, UK). The instrument operation, data acquisition, and analyses were performed using the MassLynx/BioLynx 3.2 software package (Micromass), according to the manufacturer's instructions.

RESULTS

Ectopic Expression of FosB and ΔFosB Alters Cell Fate of Rat 3Y1 Embryo Cell Line—When FosB and ΔFosB expression plasmids, pcDEBΔ:FosB and pcDEBΔ:ΔFosB, which also carry an *hph* gene for hygromycine B resistance, were introduced into the rat 3Y1 embryo cell line (*18*), few hygromycin B–resistant colonies were obtained, while many colonies were obtained from the cultures which received either pcDEBΔ itself or pcDEBΔ:c-Myc (data not shown). These results strongly suggest that the ectopic expression of FosB and ΔFosB alters the fate of 3Y1 cells, resulting in either the suppression of cell growth or the induction of cell differentiation or cell death.

To elucidate this hypothesis, we constructed pcDEBΔ:ER-FosB and pcDEBΔ:ER-ΔFosB, which encode fusion proteins (ER-FosB, ER-ΔFosB) with a ligand-binding domain

of the human estrogen receptor (ER), as shown in Fig. 1A, and introduced them into the 3Y1 cells. We obtained many hygromycin B-resistant colonies, in which most of these cells expressed fusion proteins in their cytoplasm. The functions of the proteins, however, tended to be suppressed in the absence of estrogen (*13*). Estrogen administration induced an accumulation of the fusion proteins in nuclei as well as in cytoplasm, and the level of fusion proteins in the nuclei was maintained for at least 3 days in the presence of estrogen (Fig. 1B). Using the established cell lines derived from 3Y1 in which the functions of FosB and ΔFosB are regulated by estrogen, we examined whether the expression of functional FosB and ΔFosB indeed alters the cell fate of the rat 3Y1 embryo cell line.

FosB and ΔFosB Induced Growth Arrest of the Exponentially Growing 3Y1 Cells Followed by Their Morphological Alteration—To evaluate the effects of FosB and ΔFosB expression in 3Y1 cells, exponentially growing cells of 3Y1-(ER-FosB), 3Y1(ER-ΔFosB), and 3Y1(ER-MGMT) in which a ER-fusion protein with O^6-methylguanine DNA methyltransferase (MGMT) was expressed, were treated with 1 μM estrogen. As shown in Fig. 2, all three cell lines grew normally in the absence of estrogen, but 3Y1(ER-ΔFosB) cells, and to a lesser extent 3Y1(ER-FosB) cells, altered their morphology from fibroblast-like shape to bipolar shape, 2 days after estrogen administration. 3Y1(ER-ΔFosB) and 3Y1(ER-FosB) cells increased in number by only twofold after estrogen administration, and thereafter ceased proliferating (Fig. 3, A and B). No such morphological alteration or suppression of cell growth was observed in the parental 3Y1 (data not shown) or 3Y1(ER-MGMT) cells (Figs. 2C and 3C). The morphological alterations observed in cells expressing ER-ΔFosB and ER-FosB were partly reversed 2 days after the medium was replaced by one without estrogen, as shown in Fig. 2, A and B (8 days), but these cells did not appear to proliferate again.

FosB and ΔFosB Induces Cell Proliferation of Quiescent 3Y1 Cells Followed by Their Morphological Alteration—We previously reported that ER-ΔFosB, and to a lesser extent ER-FosB, triggers the quiescent rat1A cells to transit G1, initiate DNA replication, and ultimately undergo cell division at least once (*13*). To examine whether ER-ΔFosB or ER-FosB triggers a proliferation of quiescent 3Y1 cells, fully confluent cultures of 3Y1(ER-ΔFosB), 3Y1(ER-FosB),

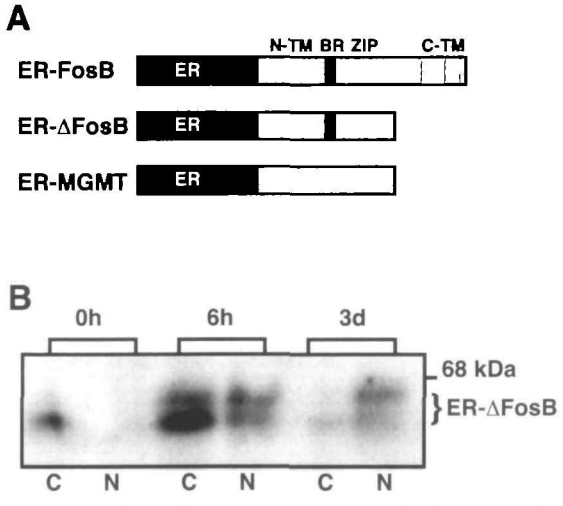

Fig. 1. **Expression of estrogen-receptor fusion proteins of FosB and ΔFosB in a rat 3Y1 embryo cell line.** A. Structures of ER-fusion proteins. ER-FosB, fusion protein of FosB with the hormone-binding domain of the human estrogen receptor (ER); ER-ΔFosB, fusion protein of ΔFosB with ER; ER-MGMT, fusion protein with human O^6-methylguanine DNA methyltransferase (MGMT) with ER. Structural motifs in FosB or ΔFosB are also shown. N-TM, N-terminal transactivation motif; BR, basic region; ZIP, leucine zipper; C-TM, C-terminal transactivation motif. B. Subcellular localization of ER-ΔFosB in 3Y1 cells grown with estrogen. 3Y1(ER-ΔFosB) cells were grown with 1 μM estrogen for the time noted, then harvested. Nuclear (N) and cytoplasmic lysates (C) were prepared and subjected to a Western blotting analysis. The blots were probed with the anti-FosB (N). A single band corresponding to a 65-kDa unmodified form of ER-ΔFosB was detected in the cytoplasmic lysate prepared from 3Y1 (ER-ΔFosB) cells cultured in the absence of estrogen, while an additional modified form of ER-ΔFosB (67-kDa) was also detected both in nuclear and cytoplasmic lysates prepared from the cells cultured in the presence of estrogen, as reported previously (*13*).

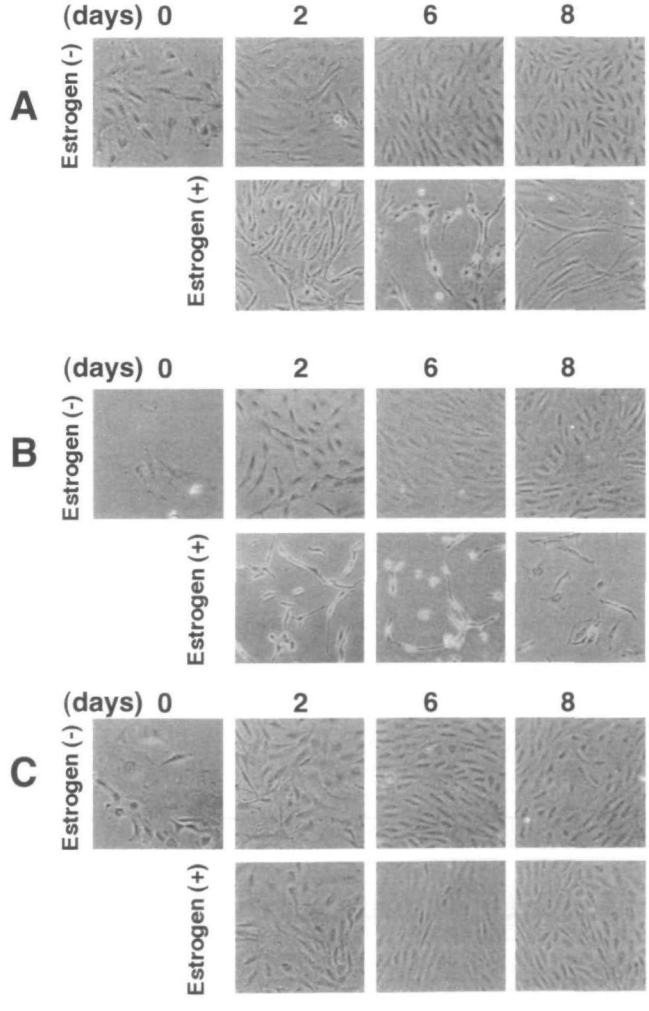

Fig. 2. **Exponentially growing 3Y1(ER-FosB) and 3Y1(ER-ΔFosB) cells alter their morphology in the presence of estrogen.** Exponentially growing cultures of 3Y1(ER-FosB) (A), 3Y1(ER-ΔFosB) (B) and 3Y1(ER-MGMT) (C) cells received 1 μM estrogen at day 0 and were removed to estrogen-free medium at day 6. At the times noted, photographs were taken using a phase contrast microscope. (+), treated with estrogen; (–), no treatment.

and 3Y1(ER-MGMT) were stimulated with estrogen. The number of 3Y1(ER-FosB) cells increased twofold within 2 days after estrogen administration and remained constant thereafter (Fig. 4A). 3Y1(ER-ΔFosB) cells also increased in number, but the increase was slightly less than twofold within 2 days after estrogen administration (Fig. 4B). 3Y1 (data not shown) and 3Y1(ER-MGMT) cells showed no change in cell number in 7 days after exposure to estrogen (Fig. 4C).

As seen in the exponential cultures (Fig. 2), the quiescent 3Y1(ER-ΔFosB) cells, and to a lesser extent 3Y1(ER-FosB) cells, altered their morphology from fibroblast-like shape to bipolar shape within 3 days after estrogen administration (data not shown). Again, no such morphological changes were observed in the parental 3Y1 or 3Y1(ER-MGMT) cells (data not shown).

Alteration of Morphology and Actin Filaments in Cells Expressing ER-ΔFosB—The morphological changes of 3Y1-(ER-ΔFosB) cells were monitored by use of a microscope equipped with a video camera (Fig. 5). Bipolar cells seen in the culture with estrogen frequently changed their morphology. One end of the extended cell body elongated along the matrix, then the other end detached from the matrix and the cell body shrank. By repeating this process, the cells expressing ER-ΔFosB appeared to move on the matrix. Such cell movement was barely seen in the culture without estrogen.

Content of actin filaments in the cells expressing ER-ΔFosB or ER-MGMT was examined by the binding of Alexa-488–labeled phalloidin detected by laser-scanning confocal fluorescence microscopy. As shown in Fig. 6, thick, straight actin filaments were apparent in 3Y1(ER-MGMT) cells in the presence or absence of estrogen, but such actin filaments disappeared in 3Y1(ER-ΔFosB) cells in the presence of estrogen, and only a weak fluorescence signal along the cell axis was observed. 3Y1(ER-FosB) cells showed an intermediate phenotype between 3Y1(ER-MGMT) and 3Y1(ER-ΔFosB) cells, suggesting that cellular content of the actin filament correlates with the extent of morphological alteration of these cells.

Altered Expression of Various Proteins in Cells Expressing ER-ΔFosB—Our data suggest that ΔFosB and FosB have a potential to trigger cell proliferation and to induce cell differentiation, but it is difficult to define the type of

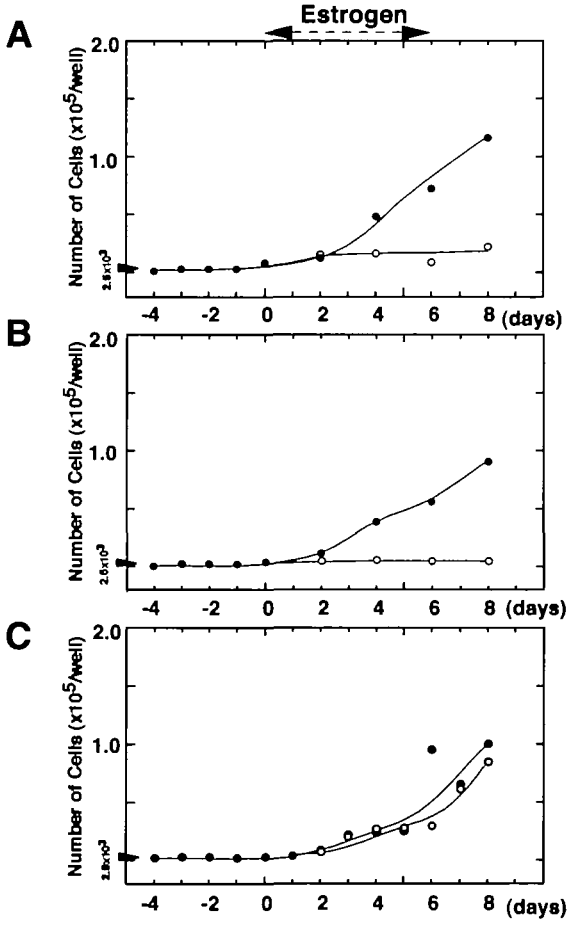

Fig. 3. **Growth arrest of exponentially growing 3Y1(ER-FosB) and 3Y1(ER-ΔFosB) cells in the presence of estrogen.** 3Y1(ER-FosB) (A), 3Y1(ER-ΔFosB) (B), and 3Y1(ER-MGMT) (C) cells were plated at 2.5×10^3 cell/well, and 1 μM estrogen was administered to some of the cultures 4 days later (0 day). On day 6, the medium was replaced with an estrogen-free medium. At the times noted, the cells were harvested and any viable cells not stained with trypan blue were counted. ●, no treatment; ○, 1 μM estrogen.

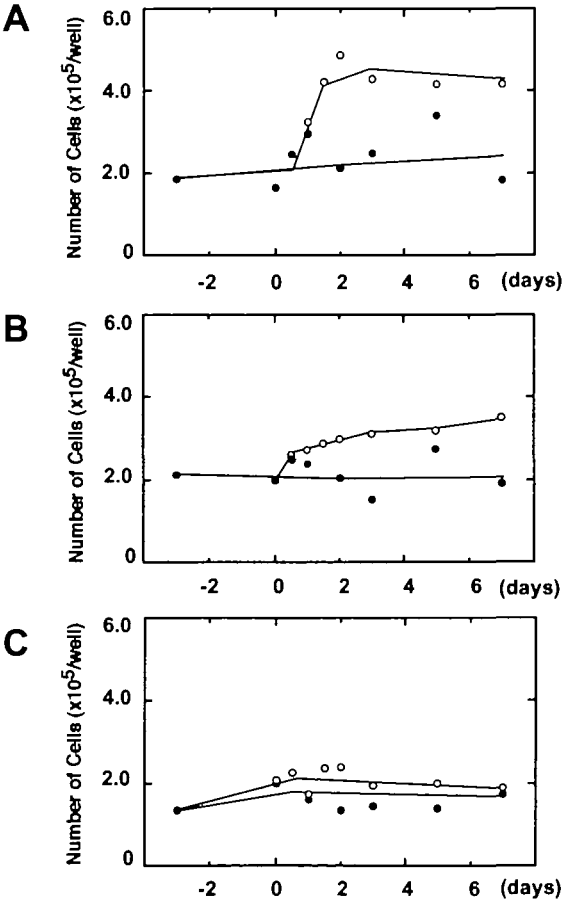

Fig. 4. **Quiescent 3Y1(ER-FosB) and 3Y1(ER-ΔFosB) cells proliferate in the presence of estrogen.** Confluent cultures of 3Y1(ER-FosB) (A), 3Y1(ER-ΔFosB) (B), and the 3Y1(ER-MGMT) (C) cells received 1 μM estrogen at day 0. The cells were harvested and any viable cells not stained with trypan blue were counted. ●, no treatment; ○, 1 μM estrogen.

differentiated cells without molecular markers. To identify proteins whose expression is changed by ER-ΔFosB activation, we subjected the cell lysates of 3Y1(ER-ΔFosB) cells to 2-D gel electrophoresis (Fig. 7). Several spots could be clearly identified (P1 to P5) that are more abundantly expressed in 3Y1(ER-ΔFosB) cells after estrogen treatment, as shown in Fig. 7. In particular, P2, a 29-kDa protein was not detected in the cell lysates from untreated 3Y1(ER-ΔFosB) cells, and 3Y1(ER-MGMT) cells with or without estrogen treatment. P3, which has a molecular mass of 14 kDa and is rather acidic compared to P2 (apparent p*I* ≅ 5),

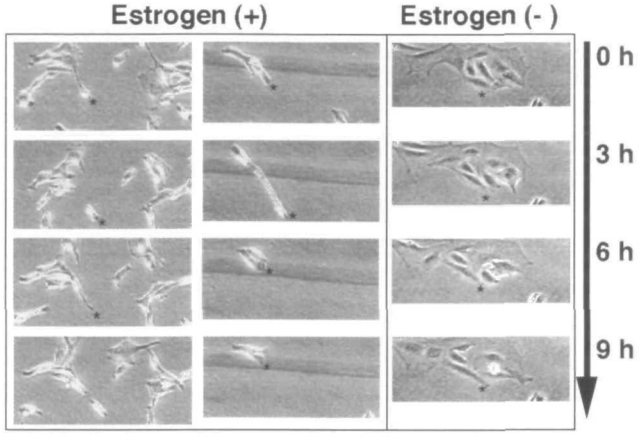

Fig. 5. **3Y1 cells expressing ER-ΔFosB frequently change their morphology.** 3Y1(ER-ΔFosB) cells were grown in the presence (+) or absence (–) of 1 μM estrogen for 2 days, and cells were monitored using a phase contrast microscope equipped with a video camera for 9 h. Two different fields in estrogen-treated culture are shown on the left. Asterisks indicate the same cells in each field.

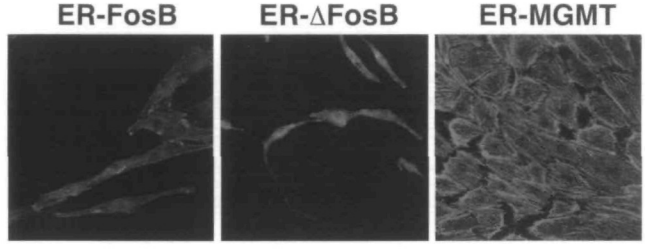

Fig. 6. **Morphological alteration of 3Y1(ER-FosB) and 3Y1(ER-ΔFosB) in the presence of estrogen accompanied by reduction in the F-actin content.** 3Y1(ER-FosB), 3Y1(ER-ΔFosB), and 3Y1(ER-MGMT) cells were grown in the presence of 1 μM estrogen for 2 days, and cellular content of polymerized actin (F-actin) were visualized by laser-scanning fluorescence microscopy using Alexa-488-phalloidin as a probe.

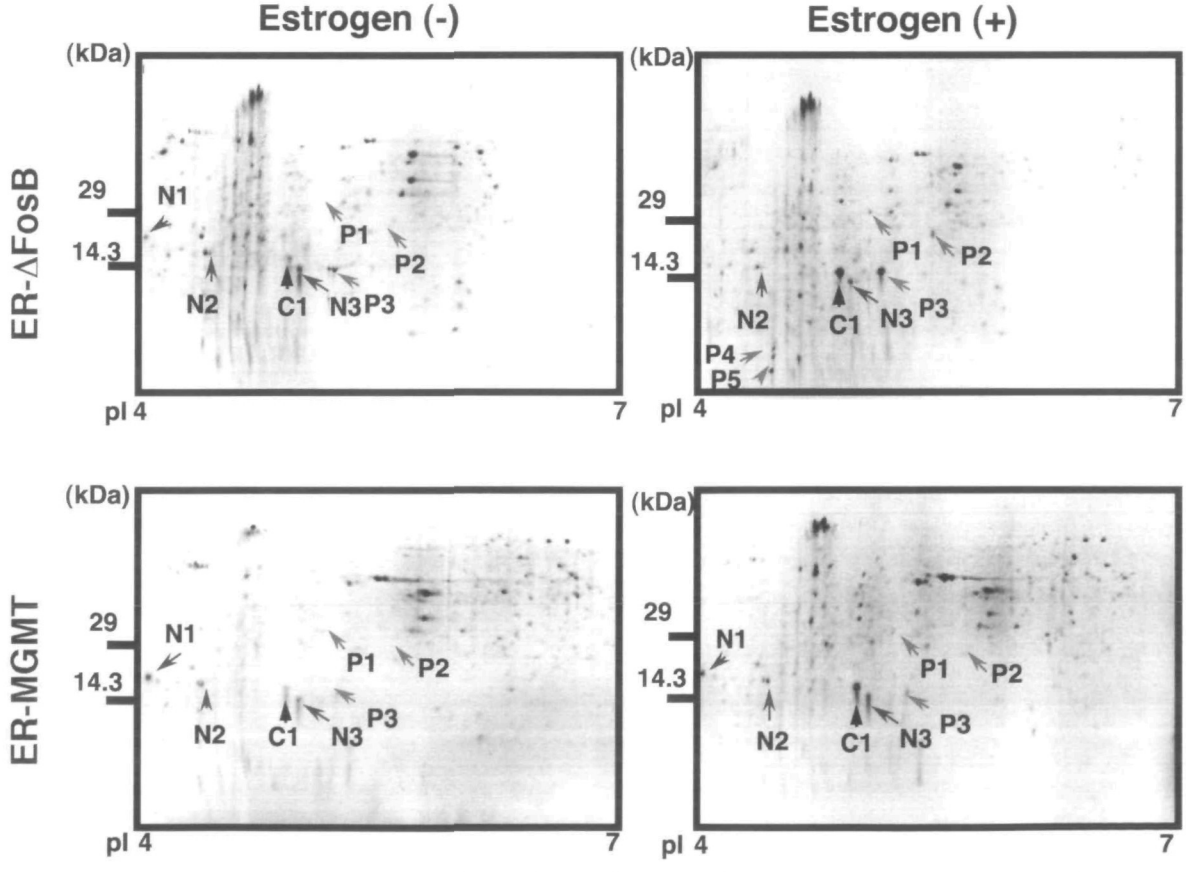

Fig. 7. **Altered expression of cellular proteins in 3Y1(ER-ΔFosB) cells with or without estrogen.** Exponentially growing 3Y1(ER-ΔFosB) and 3Y1(ER-MGMT) cells were grown in the presence (+) or absence (–) of 1 μM estrogen for 3 days, then subjected to 2-D gel electrophoresis with ExelGEL SDS Homogeneous, as described in Materials and Methods. The gels were stained with CBB and quantitatively analyzed using Image Master 2D Elite.

was detected in the cell lysates from the 3Y1(ER-ΔFosB) cells and 3Y1(ER-MGMT) cells. The intensity of P3 was increased only in the 3Y1(ER-ΔFosB) cells, more than threefold after estrogen treatment, when the intensity was normalized against that for the C1 spot, whose level in the 3Y1(ER-ΔFosB) cells was equivalent to that in 3Y1(ER-MGMT) cells after the treatment. The intensities of some spots (N1, N2 and N3) detected in the cells expressing ER-ΔFosB decreased after estrogen treatment.

The P2 and P3 spots were transferred onto a membrane and directly subjected to sequencing from their amino-terminal ends. We obtained 27-residue sequences from P2 and P3, and found that their amino-terminal sequences were identical, as shown in Table I. BlastP search revealed that the sequence matches residues 8 to 34 of rat galectin-1 (*24*). When tryptic peptides of P3 were analyzed by nanoESI MS and MS/MS on an electrospray-TOF instrument, two peptide sequences were determined. These sequences were identical to those from rat galectin-1 (aa8–29, aa30–50), thus confirming that P3 is a novel processed form of galectin-1 (Table I).

Altered Expression of Galectin-1 in Cells Expressing ER-ΔFosB—To further examine the expression of galectin-1, we performed an immunoblotting analysis after 2-D gel electrophoresis using anti–recombinant human galectin-1 antibody (anti–rhGal-1) (*21*). As shown in Fig. 8A, four spots corresponding to 14-kDa polypeptides in lysates from both 3Y1(ER-ΔFosB) and 3Y1(ER-MGMT) cells were reactive to the anti–rhGAl-1. The most acidic spot may correspond to the full-length galectin-1, since it has been shown that galectin-1 carries acetylated alanine at the N-terminal end (*25*). A spot of p*I* 5.1 most likely corresponds to P3, a processed form of galectin-1, because its signal intensity apparently increased in the lysate from estrogen-treated 3Y1(ER-ΔFosB) cells compared to 3Y1(ER-MGMT) cells treated with estrogen. In the lysate from estrogen-treated 3Y1(ER-ΔFosB) cells, several minor spots corresponding to 29-kDa polypeptides were also detected. On blots from 1-D SDS-PAGE, in addition to the 14-kDa major band(s), a 29-kDa band that was reactive to the anti-rhGal-1 was detected in the lysate from 3Y1(ER-ΔFosB) cells treated with estrogen, but was barely detectable in other cells (Fig. 8B). As a result, the 29-kDa band in 1-D SDS-PAGE and one of the 29-kDa spots in the 2-D gel may correspond to P2 identified in Fig. 7 and Table I.

These results indicate that 3Y1(ER-ΔFosB) cells express different isoforms of galectin-1, and the levels of two isoforms corresponding to P2 and P3 in Fig. 7 clearly increased after exposure to estrogen.

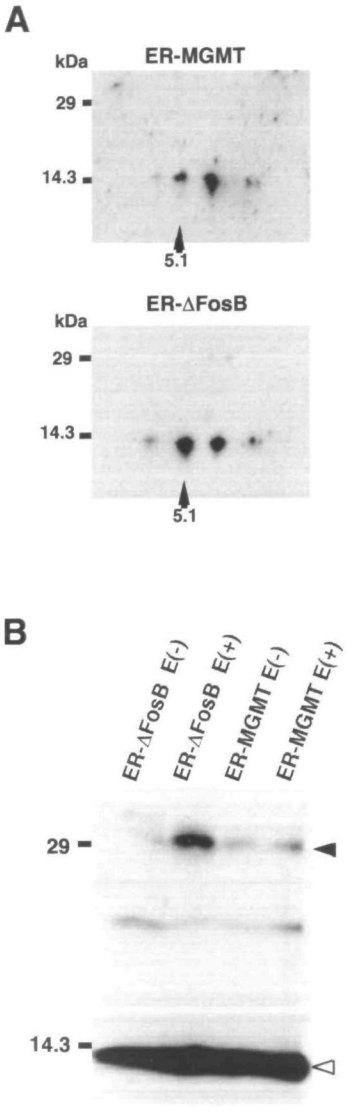

Fig. 8. **Differential expression of galectin-1 in 3Y1(ER-ΔFosB) and 3Y1(ER-MGMT) cells in the presence of estrogen.** Exponentially growing 3Y1(ER-MGMT) and 3Y1(ER-ΔFosB) cells were grown in the presence of 1 μM estrogen for 4 days, then subjected to Western blotting either after 2-D gel electrophoresis with ExelGEL SDS Gradient 8-18 (A) or after 1-D 15% SDS-PAGE (B) using anti-rhGAl-1. Estimated position of p*I* = 5.1 is shown by arrows in (A). The 14-kDa and 29-kDa proteins that reacted with anti-rhGAL-1 antibody are shown by open and closed triangles, respectively.

TABLE I. **Partial amino acid sequences of polypeptides P2 and P3.**

P2	N-terminal	SNLNLKPGEXLKVRGELAPDAKXFVLN
P3	N-terminal	SNLNLKPGEXLKVRGELAPDAKSFVLN
	tryptic peptide-1	SNLNLKPGEC* LKVRGELAPDAK
	tryptic peptide-2	SFVLNLLGKDSNNLC* LHFNPR
Rat galectin-1		MACGLVASNLNLKPGECLKVRGELAPDAKSFVLNLLGKDSNNLCLHFNPRF
		NAHGDANTIVCNSKDDGTWGTEQRETAFPFQPGSITEVCITFDQADLTIKL
		PDGHEFKFPNRLNMEAINYMAADGDFKIKCVAFE

C*, carboxyamidomethyl cysteine; —, sequence identical to N-terminal sequences of P2 and P3; ——, sequence identical to tryptic peptide-1 of P3; ——, sequence identical to tryptic peptide-2 of P3.

DISCUSSION

In the present study, we have shown that *fosB* gene products have a potential to trigger one round of proliferation of the quiescent rat 3Y1 cells followed by their morphological alteration, while, in addition, ΔFosB alters the expression of a novel processed form of galectin-1.

The 3Y1 cell line has been used for *in vitro* cell transformation experiments with various oncogenes, such as *src*, *myc*, and *fos* (*26–28*). c-Fos and v-Fos continuously promote the proliferation of 3Y1 cells in cooperation with other oncoproteins, resulting in transformation or the acquisition of a potential for metastasis (*27, 29*). Our findings show that neither FosB nor ΔFosB transforms 3Y1 cells, but both rather suppress the proliferation of exponentially growing 3Y1 cells, followed by the morphological alteration with enhanced motility reflecting a reduced content of F-actin. Because both FosB and ΔFosB are able to transform the rat1A cells as does c-Fos (*5, 30*), biological responses of 3Y1 cells to the expression of FosB and ΔFosB are completely different from those of rat1A cells, and also from those of 3Y1 cells to c-Myc, c-Fos, or v-Fos.

Based on our observation that 3Y1 cells carrying ectopic FosB or ΔFosB expression plasmid could not be established (data not shown), we introduced new plasmids into the 3Y1 cells that encode the ER-FosB or ER-ΔFosB fusion proteins with the ligand-binding domain of human estrogen receptor, which moves into the nuclei only in the presence of estrogen (*13, 17*). In the absence of estrogen, 3Y1 cells expressing ER-FosB or ER-ΔFosB were indistinguishable from those expressing ER-MGMT or the parental 3Y1 cells in terms of cell growth and cell morphology. Using this conditional expression system for the functional FosB and ΔFosB in nuclei, we were able to successfully evaluate the function of these proteins in 3Y1 cells.

Since the 3Y1 cells expressing ER-ΔFosB underwent morphological alterations and survived for longer than a week, and only the morphological phenotype was reversed after the removal of estrogen, the phenotype is not likely to be related to terminal differentiation, but instead represents a transient state to a certain differentiated phenotype. Based on the bipolar shape observed in 3Y cells expressing ER-ΔFosB, we speculated that the origin of the 3Y1 cells is one with similar shape, such as a myoblast, glia or neuron. Each cell-specific gene expression was examined by RT-PCR using the morphologically changed cell RNA samples, but we could not determine the cell origin because many kinds of RNAs were expressed in the cells (data not shown). It is likely that 3Y1 cells are derived from some embryonic progenitor cells whose maturation can be promoted by the *fosB* gene.

ΔFosB lacks the region corresponding to the C-terminal 101 amino acid residues of FosB that contains several functional motifs involved in transcriptional regulation and counteracts the transcriptional activation by FosB/Jun or c-Fos/Jun complexes (*5*). Since the C-terminal region of FosB is not required in order to initiate the response of 3Y1 cells, and the effect of FosB was less efficient, an ability to activate AP-1 transcriptional activator does not play a major role during the process.

Six polypeptides are encoded by two forms of *fosB* mRNA, namely, FosB, Δ1FosB and Δ2FosB from *fosB* mRNA, and ΔFosB, Δ1ΔFosB and Δ2ΔFosB from *ΔfosB* mRNA, all of which are translation products from the three alternative initiation codons in the *fosB* and *ΔfosB* mRNAs (*6, 7*). In the cells expressing ER-FosB or ER-ΔFosB, only the fusion proteins are estrogen-responsive, even if alternative initiation occurred from the fusion mRNA. Our data are thus considered to represent the biological functions of FosB and ΔFosB, but not of the other subforms. Transgenic mice of ΔFosB cDNA have been reported to show an enhanced differentiation of osteoblasts in which the expression of ΔFosB and Δ2ΔFosB increased (*7*). These facts together with the present findings suggest that ΔFosB has a unique potential to promote differentiation of a certain type of cells. ΔFosB, which inhibits Fos/Jun transcriptional activity (*5, 13*), may repress expression of gene(s) that negatively regulates cell differentiation in the undifferentiated condition.

2-D gel electrophoresis analyses revealed that the expression of various proteins changed in 3Y1(ER-ΔFosB) cells with or without estrogen, indicating that ΔFosB indeed induces alteration of gene expression. Among these proteins, the amino-terminal sequences of two polypeptides (P2 and P3) whose amounts clearly increased in estrogen-treated 3Y1(ER-ΔFosB) cells were determined and found to correspond to the sequence starting from the 8th residue of rat galectin-1 (*24*). Western blotting with anti–rhGal-1 and nanoESI-MS and MS/MS analysis supported the conjecture that the P3 polypeptide (14 kDa, p*I* 5.1) is a novel processed form of galectin-1. Purified human galectin-1, whose molecular mass is 14.5 kDa, is separated into three spots in 2-D gel electrophoresis; a prominent spot of p*I* 5.1, and two minor spots of p*I* 4.9 and 5.3 (*22*). It has been shown that endogenous galectin-1 is acetylated at the 2nd alanine after the removal of the 1st methionine (*25*). As a result, P3 whose N-terminal residue was determined to correspond to the 8th residue encoded by galectin-1 mRNA, is a novel processed form of galectin-1, which is herein reported for the first time. Here, we designate the novel processed form of galectin-1 (P3) as galectin-1β, and the acetylated form as galectin-1α.

The N-terminal residues missing in galectin-1β have been shown to play an important role in the formation of a homodimer of galectin-1α (*31*), thus indicating that galectin-1β may exist as a monomer. However, the position of P2, which has a molecular mass of 29 kDa and also reacts with anti–rhGal-1, probably corresponds to the dimeric size of galectin-1β, since it has shown that an incompletely resolved dimeric form of galectin-1 is positioned at 29 kDa on SDS-PAGE, and the monomer/dimer state of galectin-1 is regulated by the galectin-1 concentration (*22*). Since P2 has an identical N-terminal sequence to galectin-1β, it is likely that P2 is made by an altered dimerization mechanism.

We performed RT-PCR analysis with 5′ and 3′ RACE for galectin-1 mRNA in various cells, and obtained only sequences corresponding to the reported galectin-1 mRNA (unpublished data), indicating that there may not be alternative splicing or alternative transcription initiation for galectin-1 mRNA. Furthermore, galectin-1 mRNA was also detected in both 3Y1(ER-ΔFosB) and 3Y1(ER-MGMT) cells with or without estrogen, and the amount of P3 was increased only in estrogen-treated 3Y1(ER-ΔFosB) cells. Our results suggest that the expression of galectin-1 is regu-

lated during the process of translation or the post-translational processing, and that ΔFosB may modulate expression of genes involved in the processes rather than expression of galectin-1 itself. To unveil the function of ΔFosB in 3Y1 cells, the regulation of galectin-1 expression and post-translational processing may provide vital clues, and such efforts are now under way.

Preliminarily, we observed that the estrogen-induced morphological change of 3Y1(ER-ΔFosB) cells is delayed by one day in the presence of lactose, which binds to galectin-1, thus suggesting that the lectin activity of galectin-1 may be involved in the modulation of morphology and polymerization of actin filaments. Galectin-1 medicates cross-talk between the cell surface and extracellular matrix by binding to its receptors, resulting in modulation of cell adhesion and cell morphology and rearrangement of actin filaments (*32*). It is most likely that the enhanced motility with reduced content of F-actin observed in ER-ΔFosB-expressing 3Y1 cells was mediated by galectin-1. In many tumor cells, galectin-1 is required for their enhanced cell proliferation (*33*), and it is therefore also likely that galectin-1 mediates the proliferative activation of quiescent 3Y1 cells. Since recombinant galectin-1 has a capacity to promote axon regeneration *in vitro* (*21, 34, 35*), the introduction of expression vectors or the administration of a recombinant form of processed galectin-1 may indeed be useful as a new tool for modulating the cellular response.

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